Third Conference

CONTINUING EDUCATION IN TOXICOLOGIC PATHOLOGY
REPRODUCTIVE SYSTEM

ORGANIZED BY SOCIETY FOR TOXICOLOGIC PATHOLOGY IN INDIA (STPI)

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The Atria Hotel, # 1, Palace Road, Bangalore - 560 001

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Harlan Laboratories

Physiology, Histology and Lesions in the Female Reproductive System of Cynomolgus Monkeys

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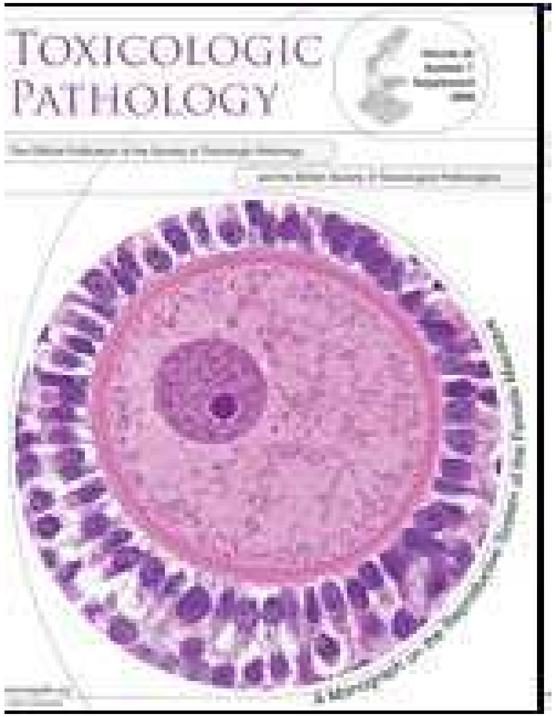
Why Monkeys?

- Excellent models for preclinical testing and safety assessment of female reproductive toxicants.
- Cynomolgus monkeys as predominant species (Marmoset and Rhesus only occasionally used)
- Well established background in-formations, summarized in:

  Buse et al., A Monograph on Female Reproductive Patho-physiology in Macaques, *Toxicol Pathol. 36, suppl. (2008)*



Cycling Monkeys

- Cynomolgus: monitoring by daily vaginal smears for menstruation
- May be combined with frequent sampling for steroid and peptide hormone analysis (not in a general design in toxicity studies).
- Marmosets: no external signs of cycle (monitoring by regular progesterone)
- Cynomolgus and marmosets do not exhibit seasonal variations in ovarian activity, but in rhesus monkeys there is a pronounced annual rhythm
- Cynomolgus: no cycle synchronization

Main Cycle Differences: Primates vs Rodents

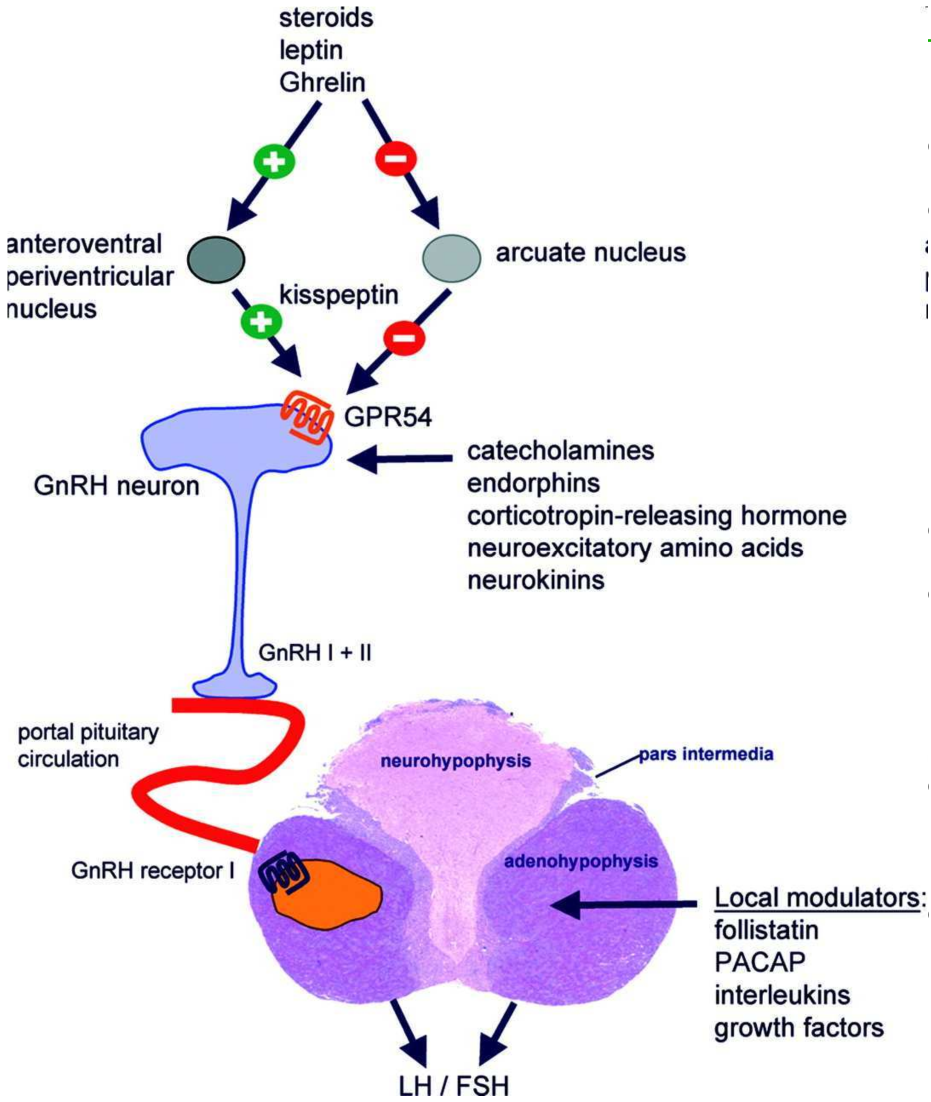
- Life span of corpus luteum in primates approx. 2 weeks
- If pregnant: c. luteum with extended duration of function and delayed luteal regression (time for implantation and luteal-placental shift)
- Unlike in rodents, prolactin does play a major role during the luteal phase
- Luteolysis in primates does not involve a uterine signal

Main Differences: Cynomolgus vs Human

- Summarized by VanEsch et al. *Toxicol Pathol. 36, suppl. (2008)*

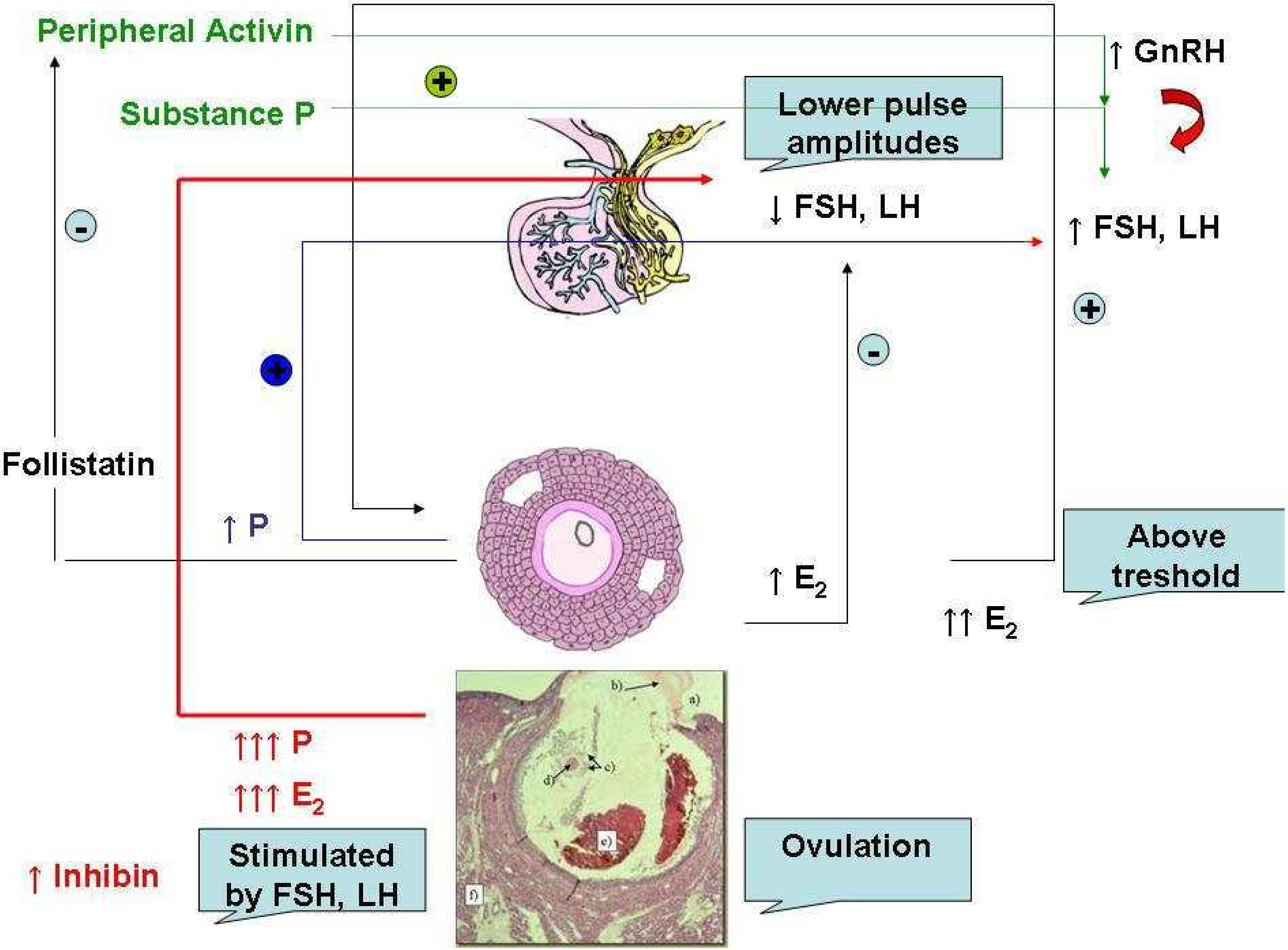
• Sexual maturity:	2.5-4 y	vs	10-18 y
• Menopause:	20-25 y	vs	50 y
• Cycle:	28-32 d	vs	28-30 d
• Implantation:	9-15 d	vs	6-13 d
• Gestation:	134-184 d	vs	259-294 d

Endocrine Regulation



- preproGnRH along axons portal blood
- GnRH is released pulsatile
- increased or decreased frequency of GnRH pulses reduces or abolishes gonadotropin secretion (disturbed cycle)
- Kisspeptin-expressing HT neurons
- is sensitive to steroid levels mediating negative feedback regulation of gona-dotropins
- no sex steroids affects GnRH secretion by direct action on GnRH neurons. Kisspeptin neurons in n. arcuate are direct targets of sex steroids in all species

Weinbauer et al, Toxicol Pathol. 36, suppl. (2008)



Peripheral Activin
Substance P
↑ GnRH
Lower pulse amplitudes
↓ FSH, LH
↑ FSH, LH
Follistatin
↑ P
↑ E_2
↑↑ E_2
Above treshold
↑↑↑ P
↑↑↑ E_2
↑ Inhibin
Stimulated by FSH, LH
Ovulation
b)
a)
c)
d)
e)
f)

Ovarian Cycle

- Day 1 of menstrual bleeding is designated as the day 1
- Entire duration is 28 to 32 days in cynomolgus monkey
- Follicular phase: 12 to 14 days
- Periovulatory interval is approximately 3 days
- Luteal phase: 14 to 16 days
- Determination by daily vaginal smears possible

Ovarian Cycle: Endocrine Profile 1

- **Follicular phase:**
  - predominant E_2 increases, LH/FSH and P low
  - rise of E_2 is by end phase along with increased FSH

- **Periovulatory phase:**
  - rise of E_2 (D12), followed by LH (D12.5), FSH (D13), and later P (permitting oocyte maturation, preventing atreasia)

Ovarian Cycle: Endocrine Profile 2

- Luteal phase:
  - gonadotropins at levels comparable to follicular phase
  - P clearly elevated during the midluteal phase (peak D22)

- Ovulation: E_2 decreases
  - contraction and release of oocyte by local prosta-glandins, plasminogen activator, leucotrienes, angio-tensins, catecholamines, vasoactive growth factors.

Ovarian Cycle Monitoring

Bleeding pattern:

- **No menstrual bleeding.**
- **Slight menstrual bleeding.**
- **Heavy menstrual bleeding.**
- **Very heavy (i.e., visible) menstrual bleeding.**

Hormone profile by blood sampling (about 2 mL):

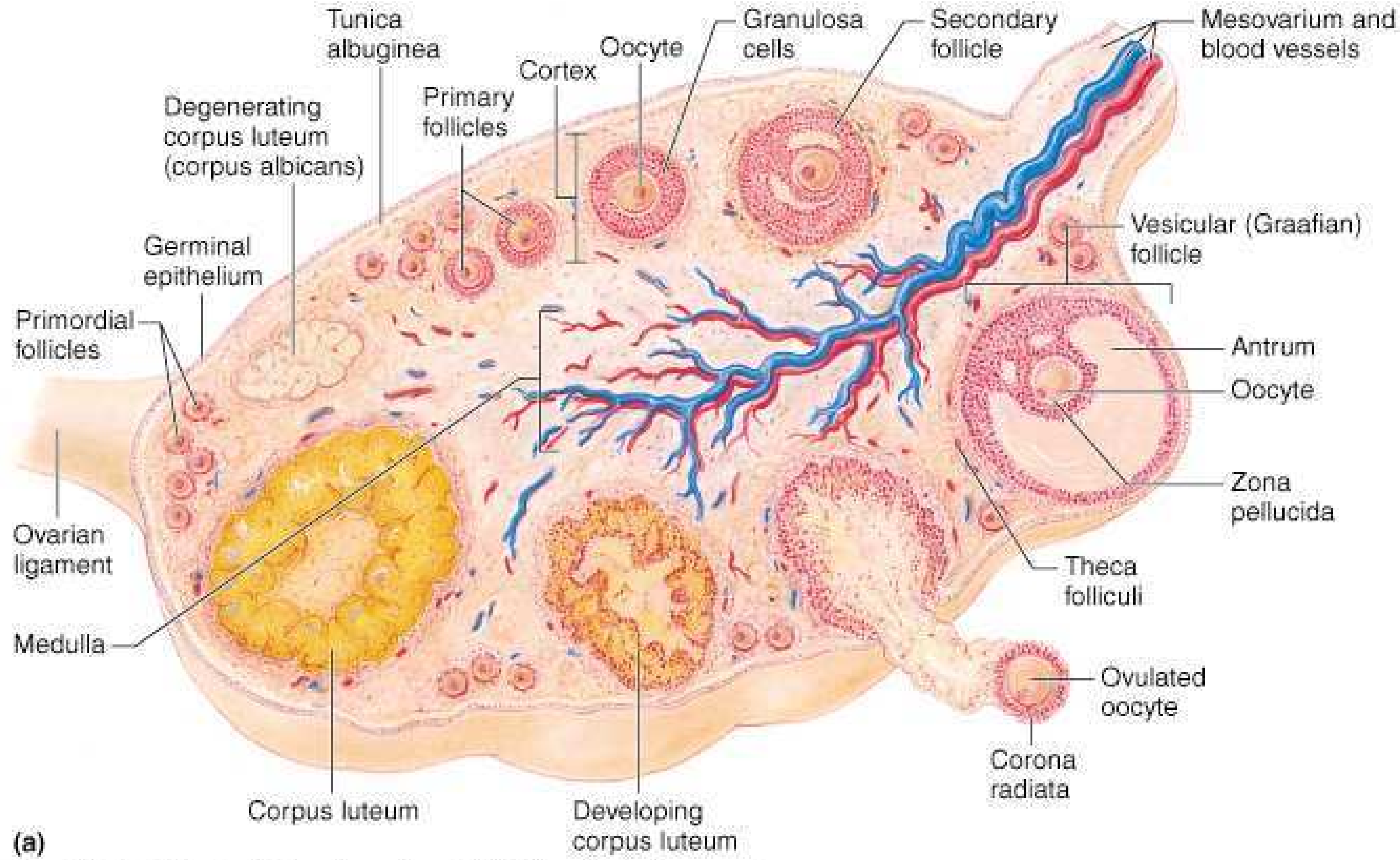
- **Days 1, 4, 7, 10, 11, 12, 13, 14, 15, 16, 18, 20, 22, 24, and 27 of menstrual cycle.**

Weinbauer et al, Toxicol Pathol. 36, suppl. (2008)

Menarche and Menopause

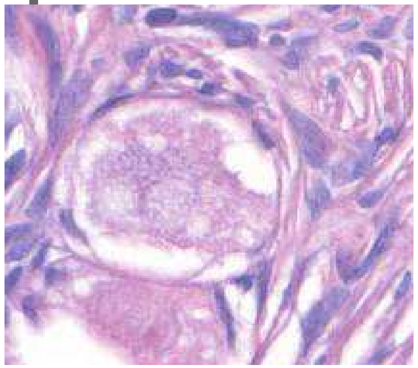
- Approx. 20 w following birth, LH/FSH levels peaks in circulation followed by a decline toward pre-pubertal levels in Cynomolgus
- Onset of puberty triggered by initiation of pulsatile GnRH release
- Juvenile/prepubertal ovary: follicle development to pre-antral and early antral follicles but no preovulatory stage
- Menarche at 2-3 y
- Menopause: increased gonadotropins, reduced E2, only slightly reduced P, increased GnRH secretion and primary follicles decreases

Normal Ovarian Structures

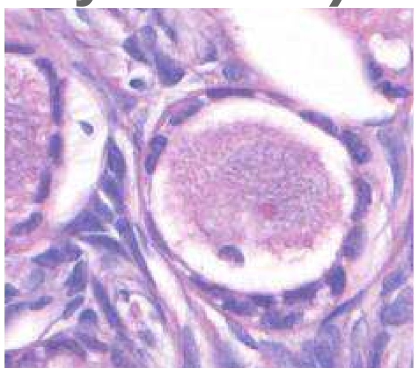


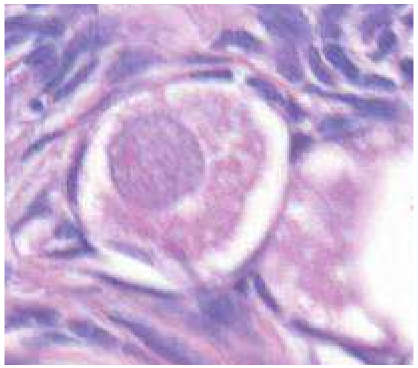
Primoridal and Primary Follicle

Category 1: Oocytes surrounded by flattened granulosa epithelial cells (classical primordial follicle).



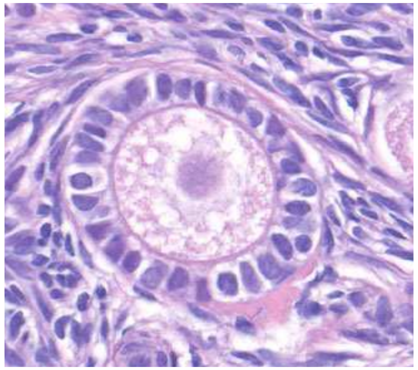
Category 2: Oocytes surrounded by both flattened and cuboidal cells (intermediate, primordial, activated primary, or early primary follicle).



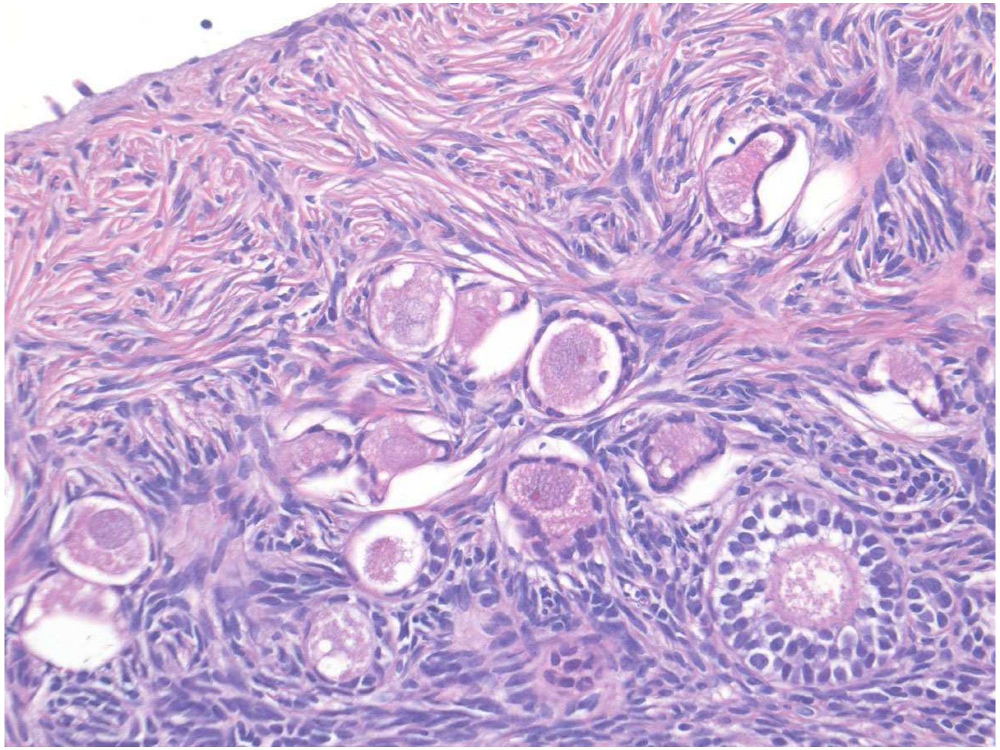


Primoridal and Primary Follicle

Category 3: Oocytes with a single layer of surrounding cuboidal cells (classical primary follicle).

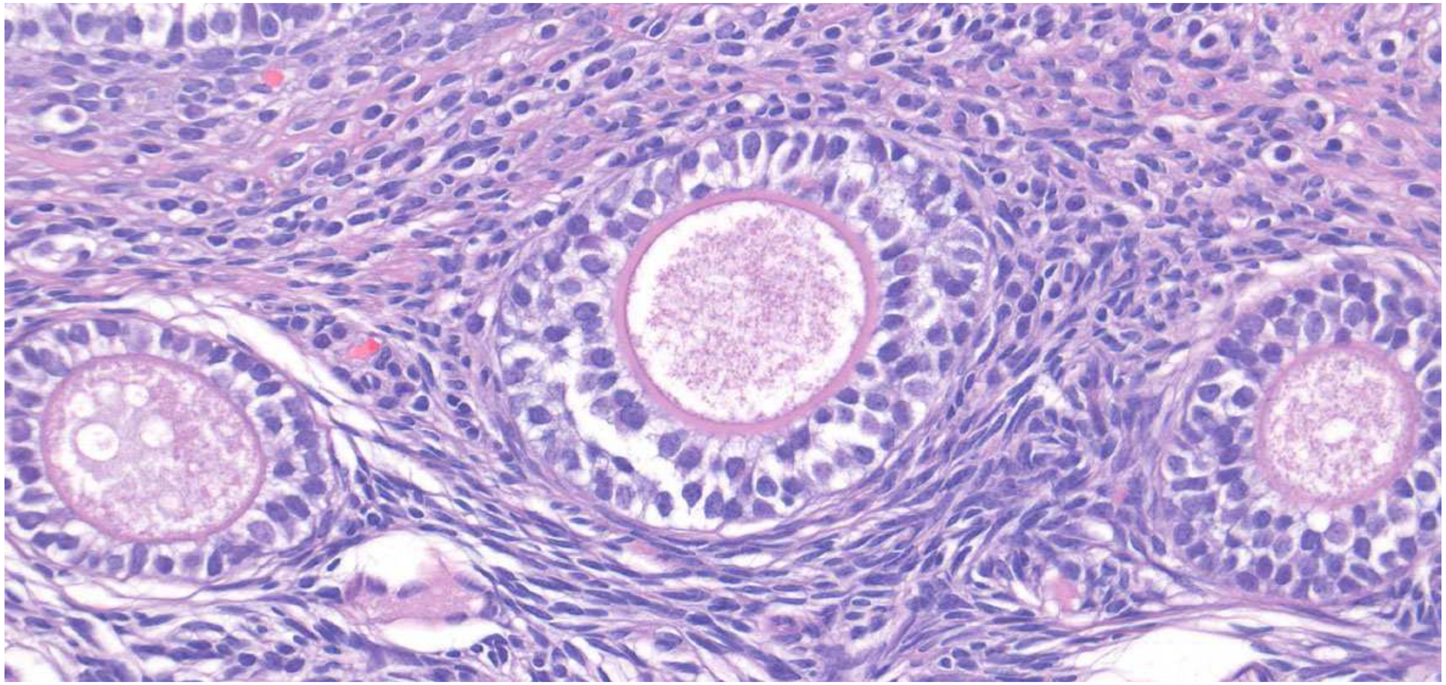


Primordial follicle re-presents dormant oocyte stage (dictyotene prophase) persisting over years



Secondary (Pre-antral) Follicle

- More than one layer of granulosa epithelial cells
- Distinct glycoprotein layer (pellucid zone or oolemma) separating it from the oocyte.



Secondary (Pre-antral) Follicle: Receptors

- Progesterone receptors expressed by almost all granulosa cells, internal and external theca cells (equally present in young and mature females)

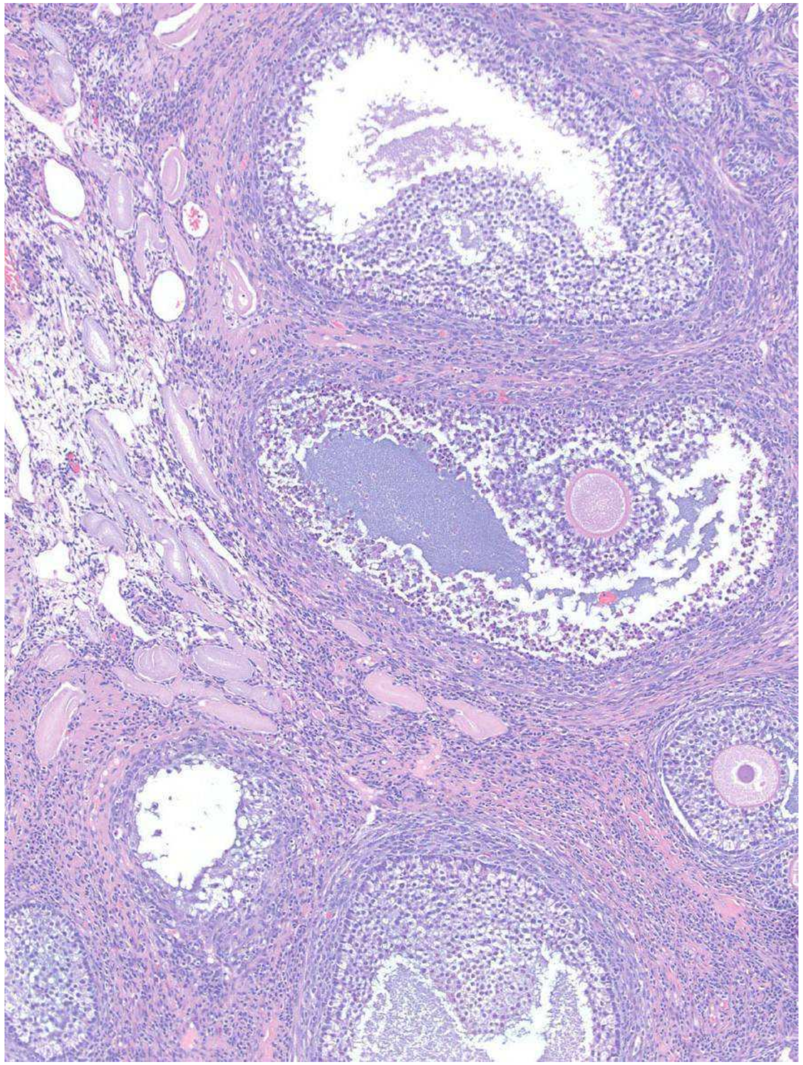
- Estrogen receptors expressed by a few granulosa cells slowly increasing in number with the size follicles and in advanced secondary follicles in 10% of granulosa cells

- Estrogen receptors expressed by some internal theca cells in preantral follicles

- Androgen receptors not expressed by secondary follicles

Tertiary Follicle

- With antrum lined by gra-nulosa cells
- Follicular liquor (mostly by granulosa cells)
- Follicle growth attributes to granulosa cell prolife-ration
- Expansion of antral cavity
- Oocyte reaches a diameter of about 120 µm



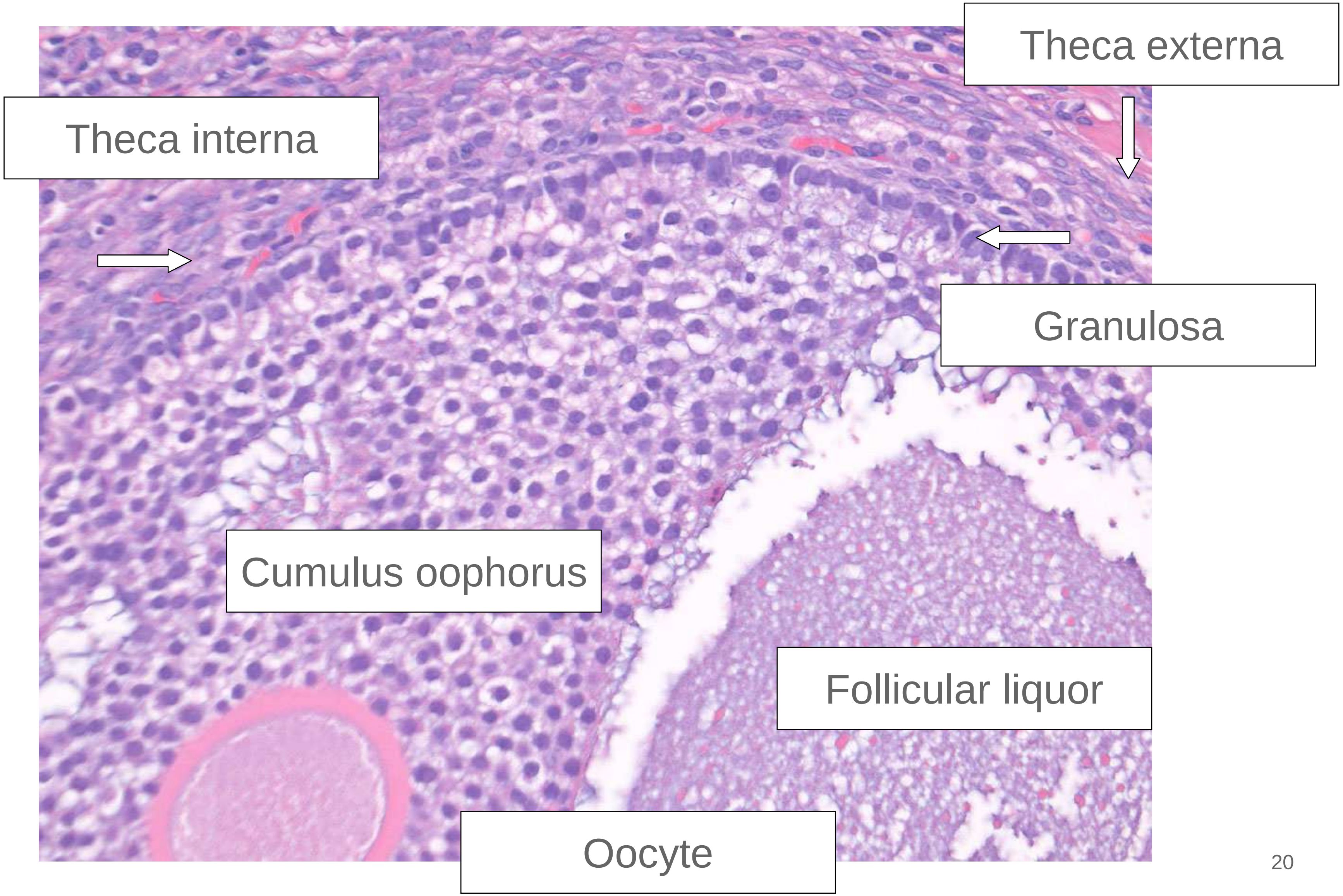
Tertiary Follicle - Receptors

- P receptors expressed by 50% of the granulosa cells (persist throughout luteal phase.
- Relatively high expressed in theca cells

- E_2 receptor expression increases with follicle growth, (almost 100% of granulosa cells in advanced tertiarys)
- Quick decline when transformationof granulosa cells into luteal epithelial cells

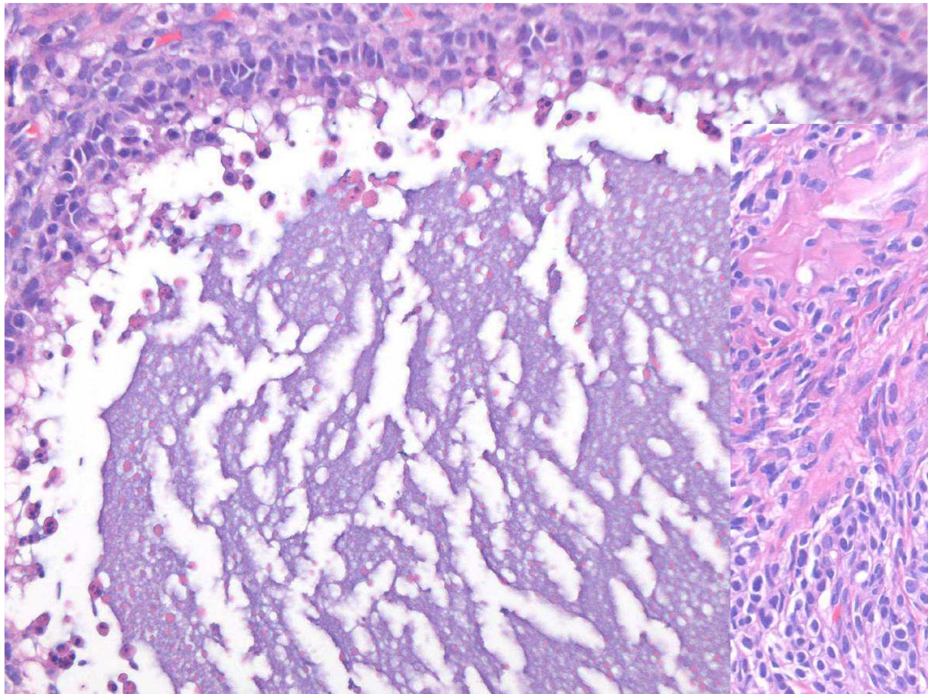
- Androgen receptor expression by over 80% of granulosa cells and by almost 100% of external theca cells.
- Absent in internal theca

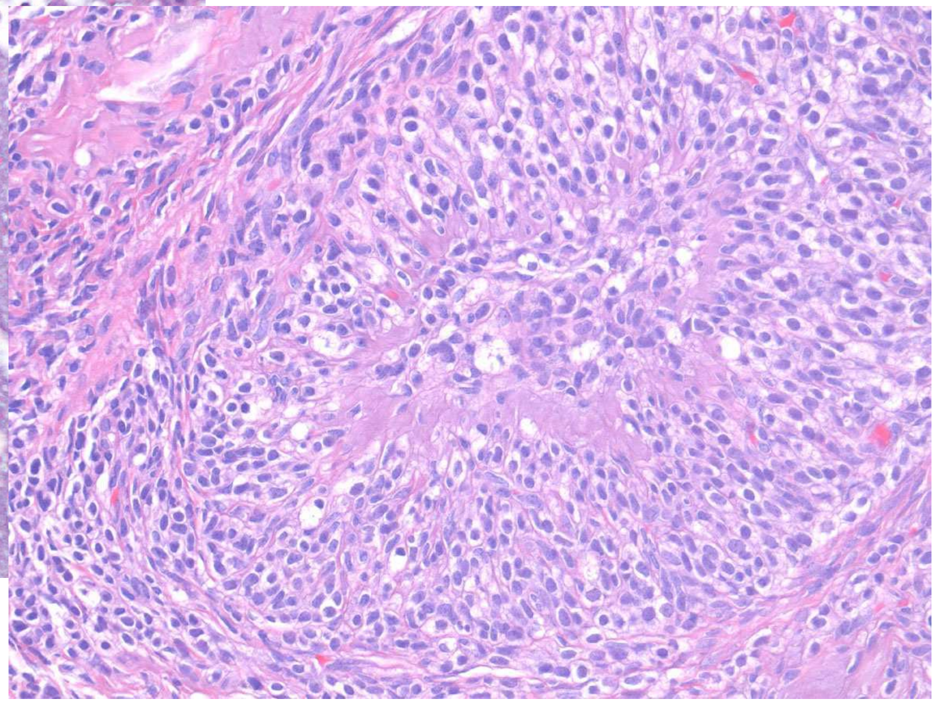
Tertiary Follicle



Tertiary Follicle - Atresia

- Majority of tertiary follicles undergo degeneration (atresia)
- Atresia may be seen also in secondary follicles





Tertiary Follicle – More than one Oocyte?

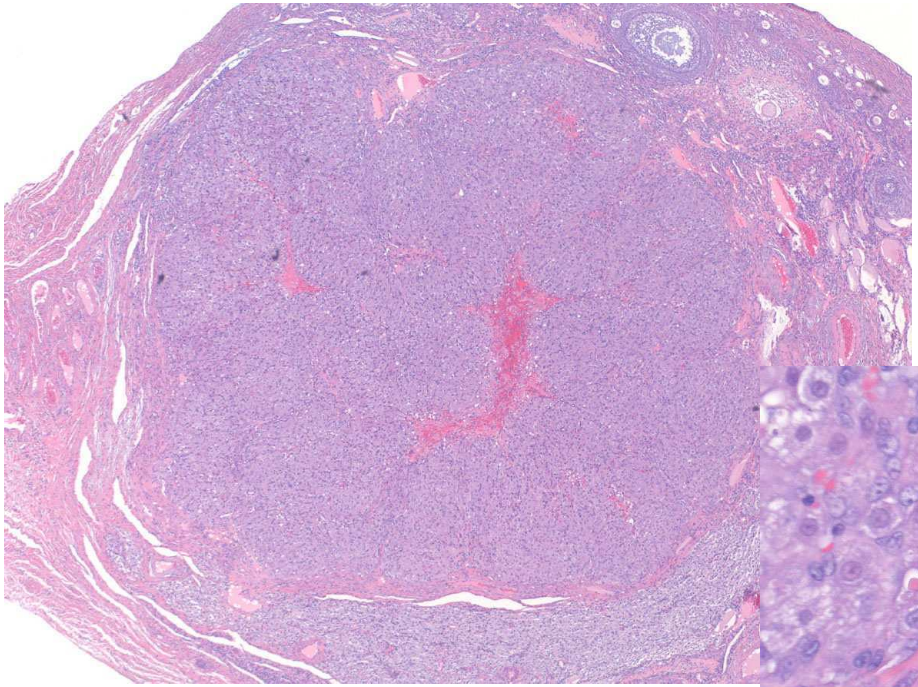
- **Very rare (Cynomolgus gives birth normally to one offspring)**

Corpus Luteum

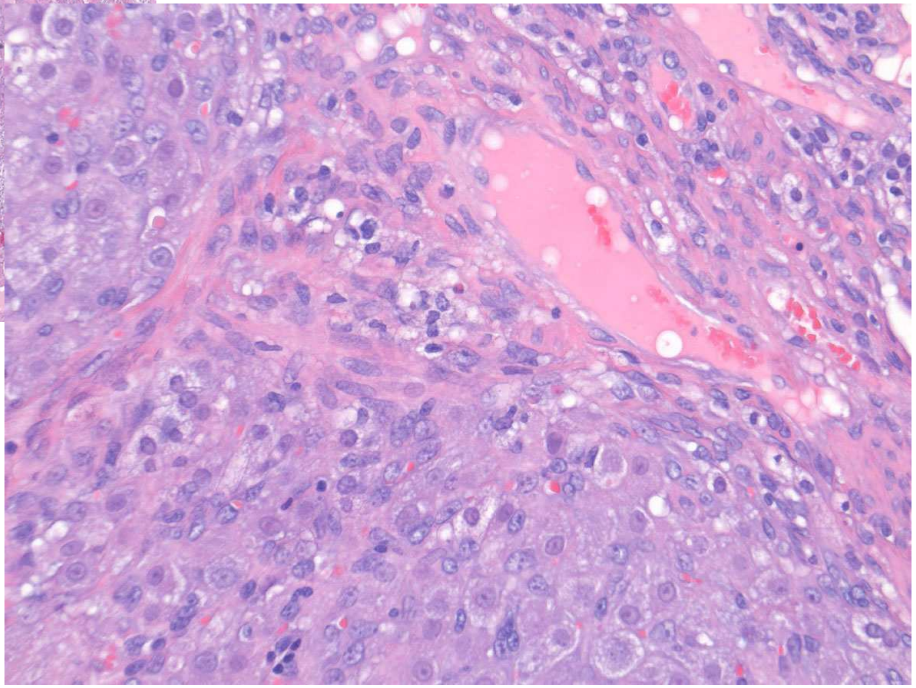
- Prior to ovulation, follicular metabolism switches
- From estrogen to progesterone synthesis
- Granulosa epithelial cells transform into large granulosa luteal cells
- Luteal cell growth by steroid genesis
- Formation of C.lutea

- If no fertilization, C. lutea involute (atrophy) and disappears within weeks

Corpus Luteum

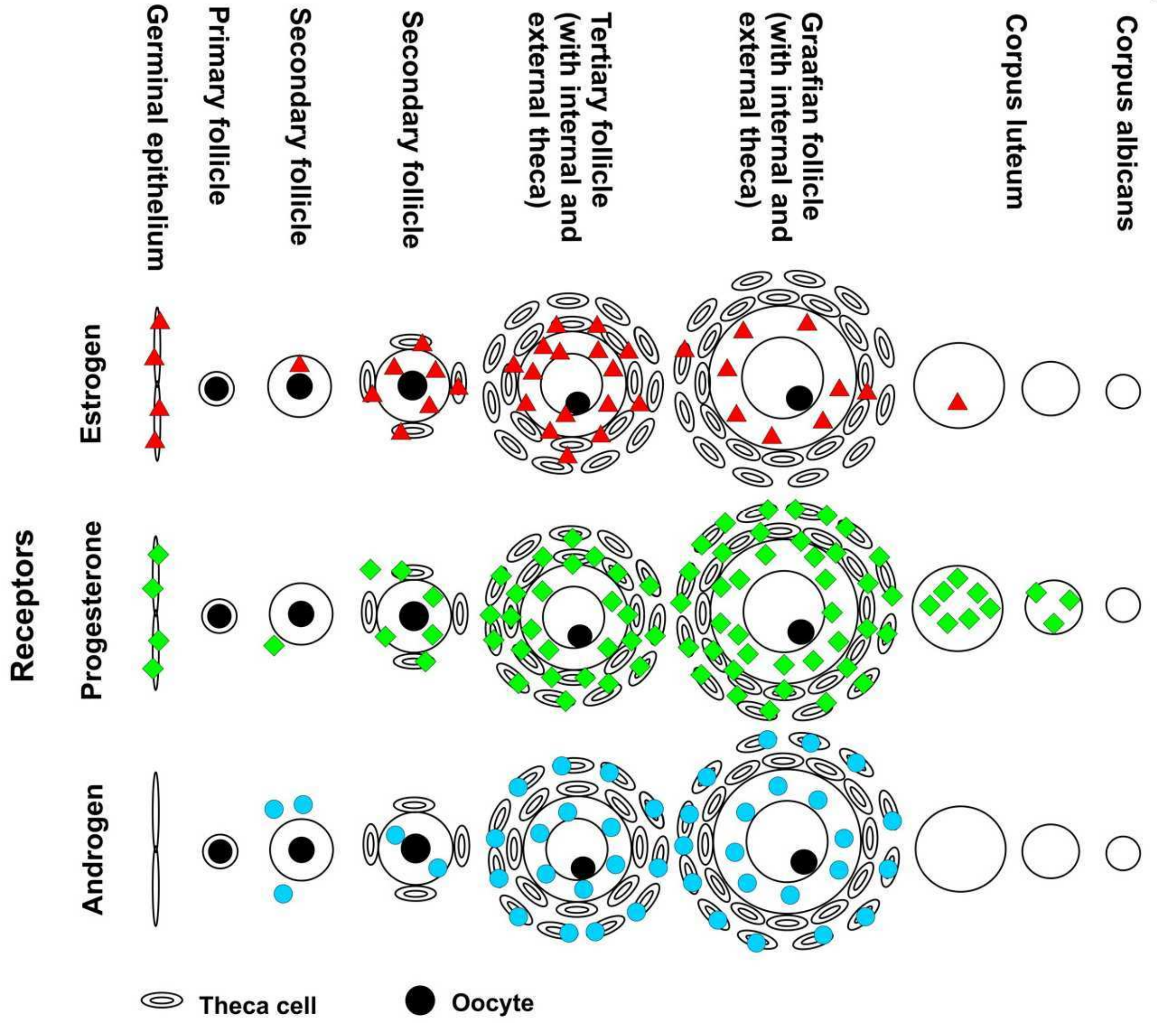


C.Lutea may be very large



Fine vacuolated luteal cells

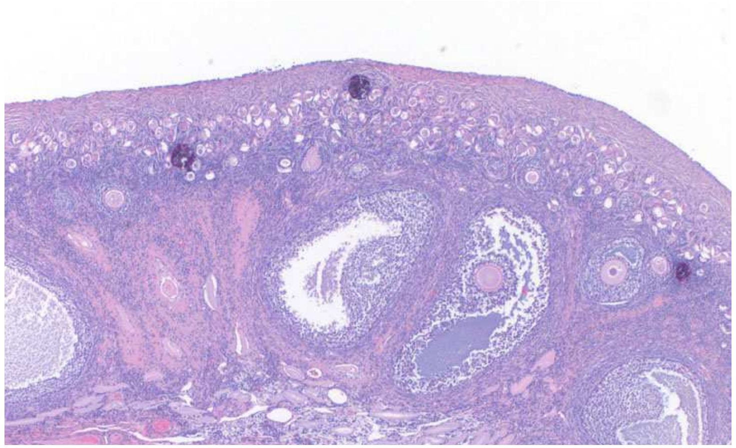
Receptor Distribution During Follicle Development (Cynomolgus)



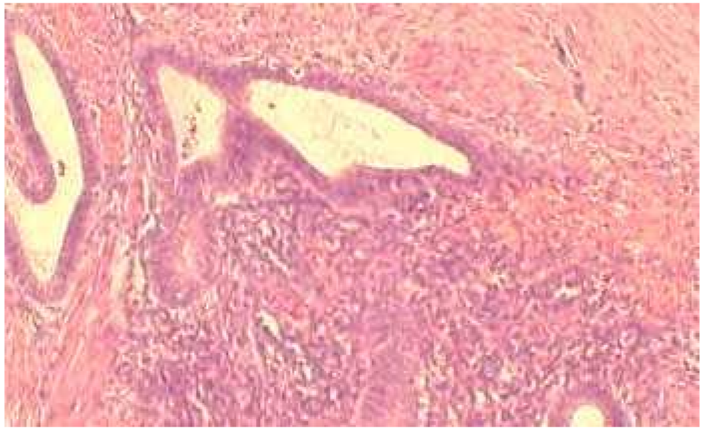
Buse et al, Toxicol Pathol. 36, suppl. (2008)

Background Lesions

- Ectopic Ovarian Tissue
- Cortical Mineralization



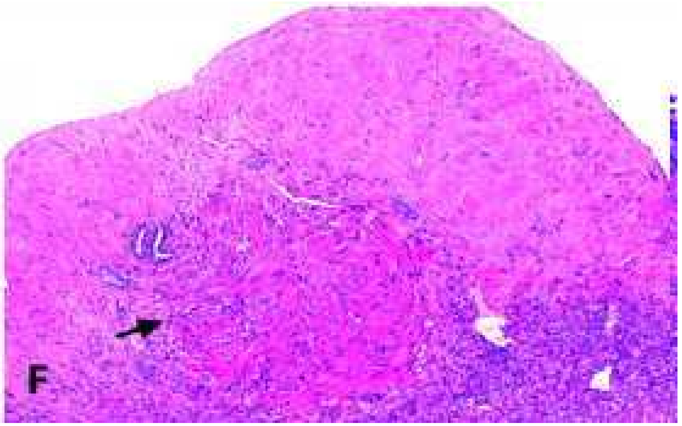
- Polyovular Follicles
- Hyperplasia of the Ovarian Surface Epithelium
- Endometriosis



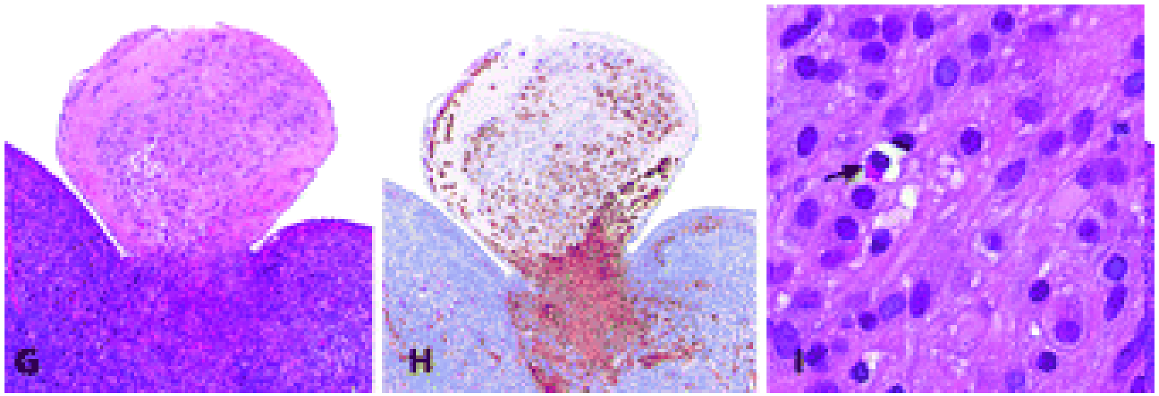
Lesions Reported

Clin et al, Toxicol Pathol. 36, suppl. (2008)

- **Ovarian smooth muscle metaplasia**



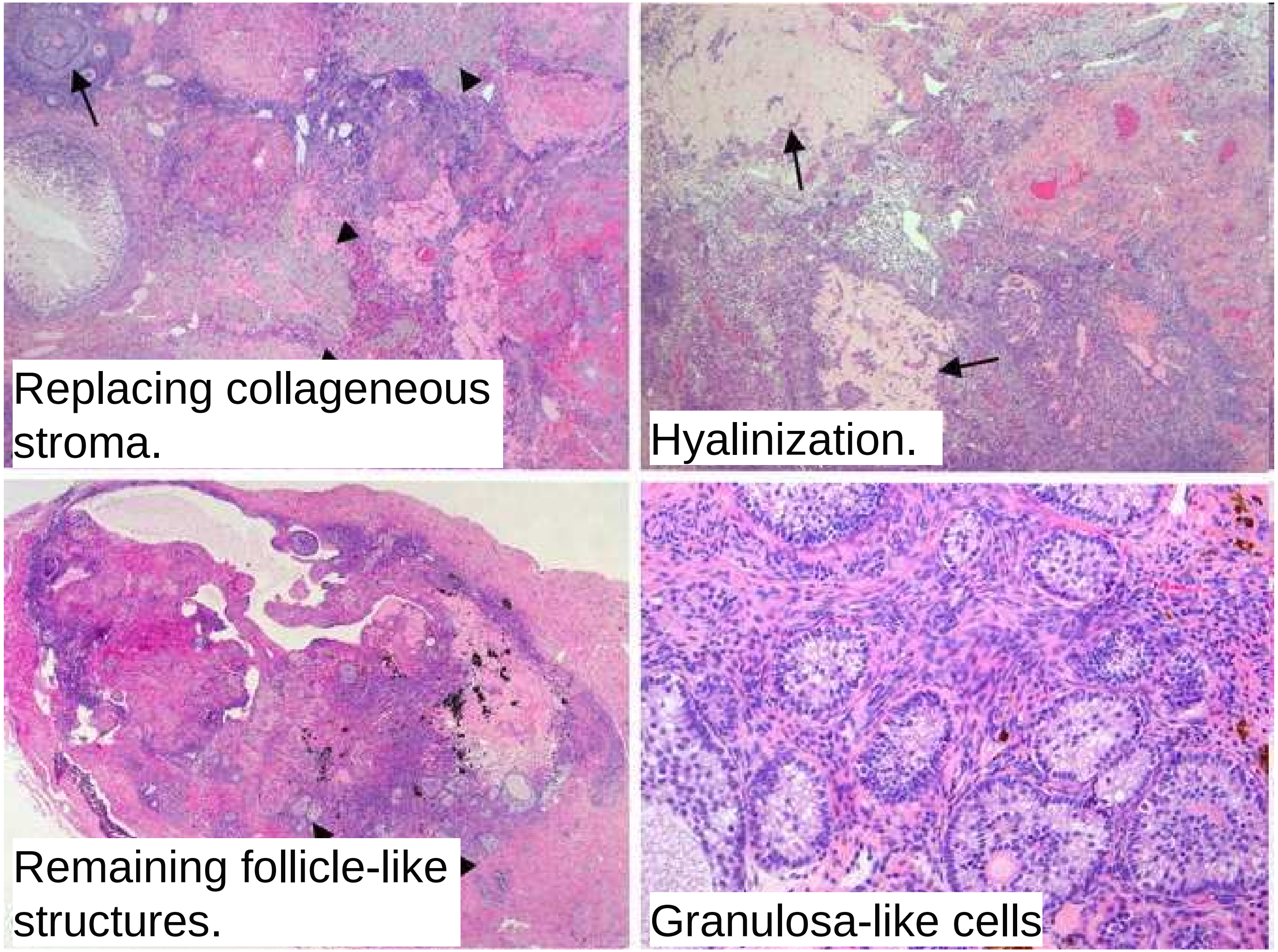
- **Ovarian "Deciduosis"**



- **Ovarian Neoplasms**

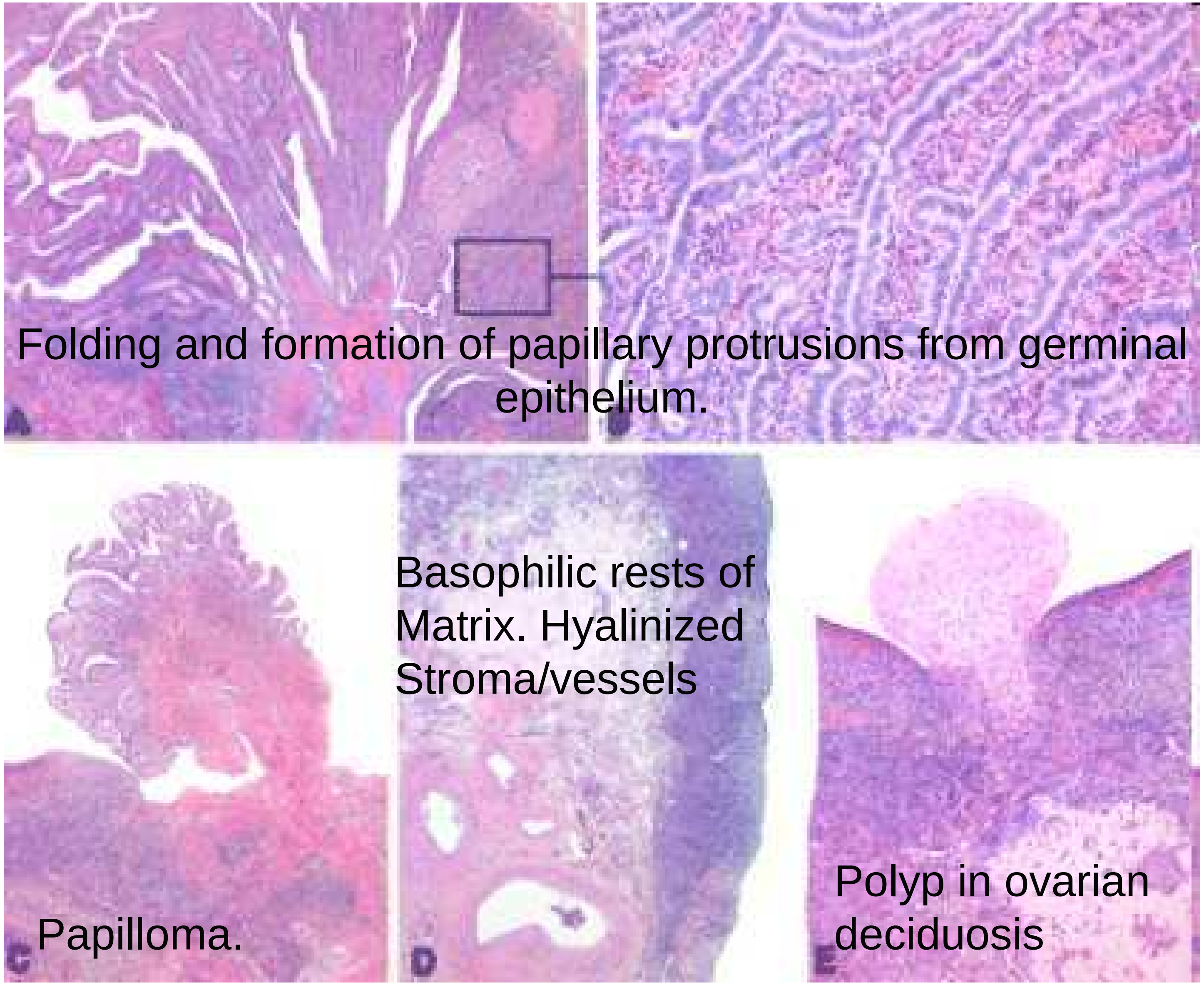
Aged Ovary

Buse et al, Toxicol Pathol. 36, suppl. (2008)



Common Findings in Aged Ovaries

Buse et al, Toxicol Pathol. 36, suppl. (2008)

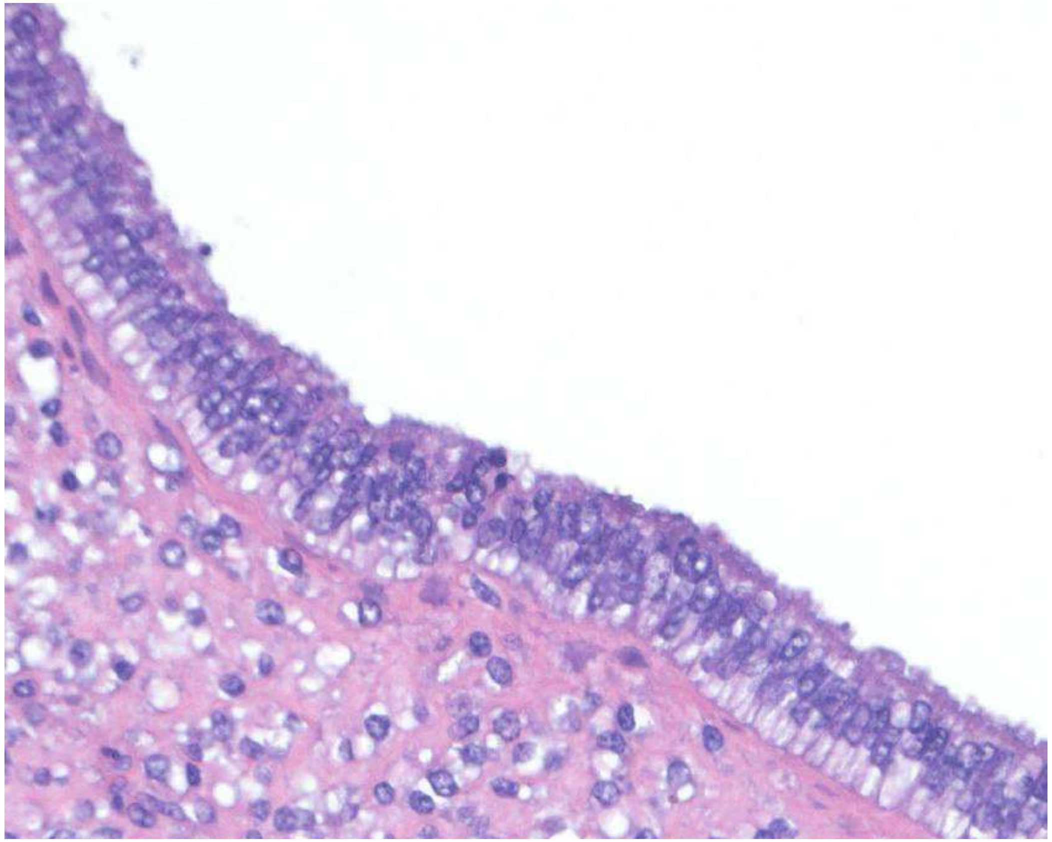


Endometrium

- Endometrial surface and glands lined by single layer secretory type epithelium
- Surface epithelium: less cyclic variation than glandular epithelium
- Glandular epithelium consists of a different cell types, varying over the length of glands and during cycle phases

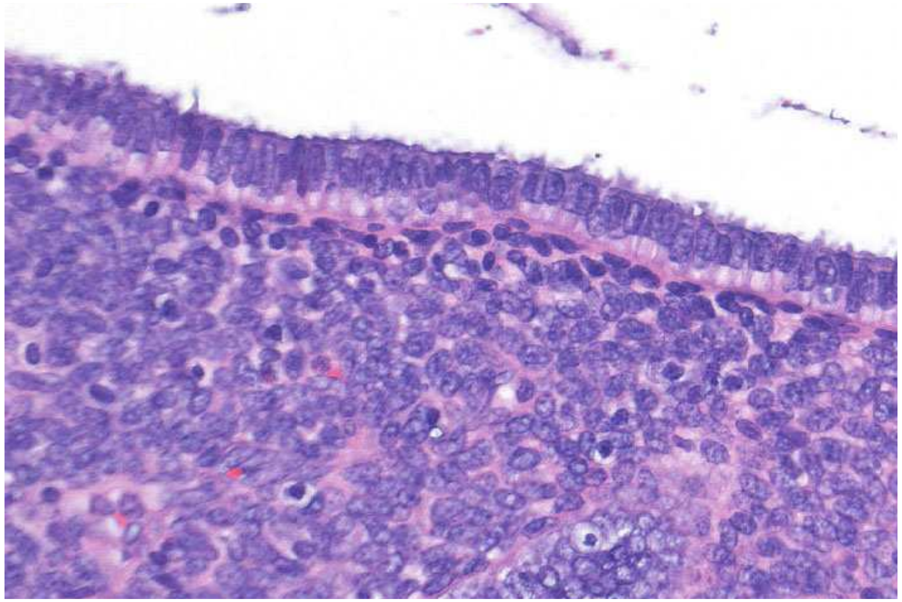
Endometrium – Secretory Cell

- **Morphology varies under influence of fluctuating estradiol and progesterone**



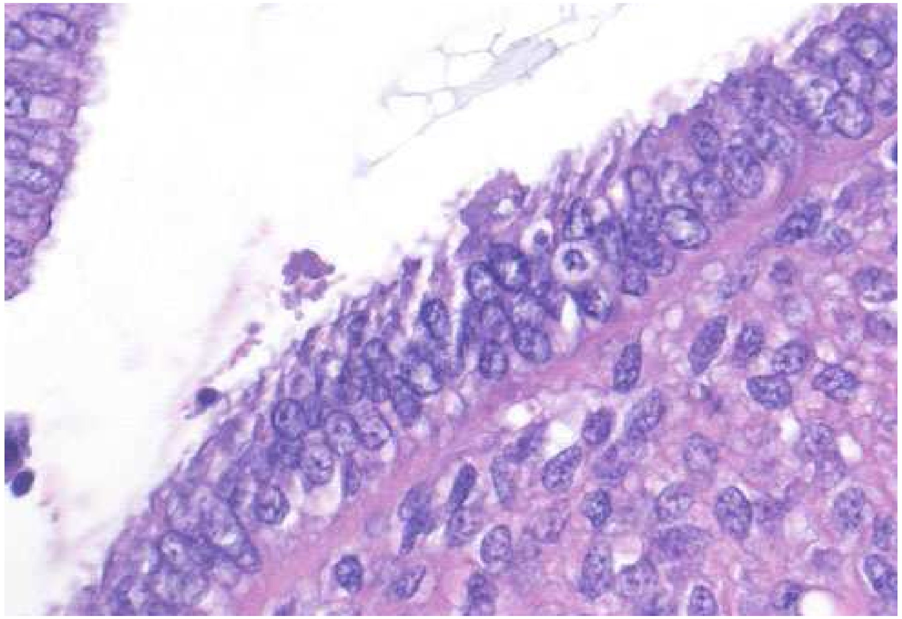
Endometrium – Ciliated Cell

- Number increase under estrogen dominance
- Not equally distributed within endometrium
- More common toward the endocervix
- Clear cytoplasm and round nuclei are often located above those of neighboring secretory cells
- Cilia difficult to recognize in routine paraffin sections



Endometrium – Clear Cell

- Less common cell type within endometrial glands
- Most frequent during follicular phase
- Probably precursor of ciliated cell that has not yet reached the luminal surface
- Clear and ciliated cells are believed to represent use-ful markers of estrogenic activity on the endometrium



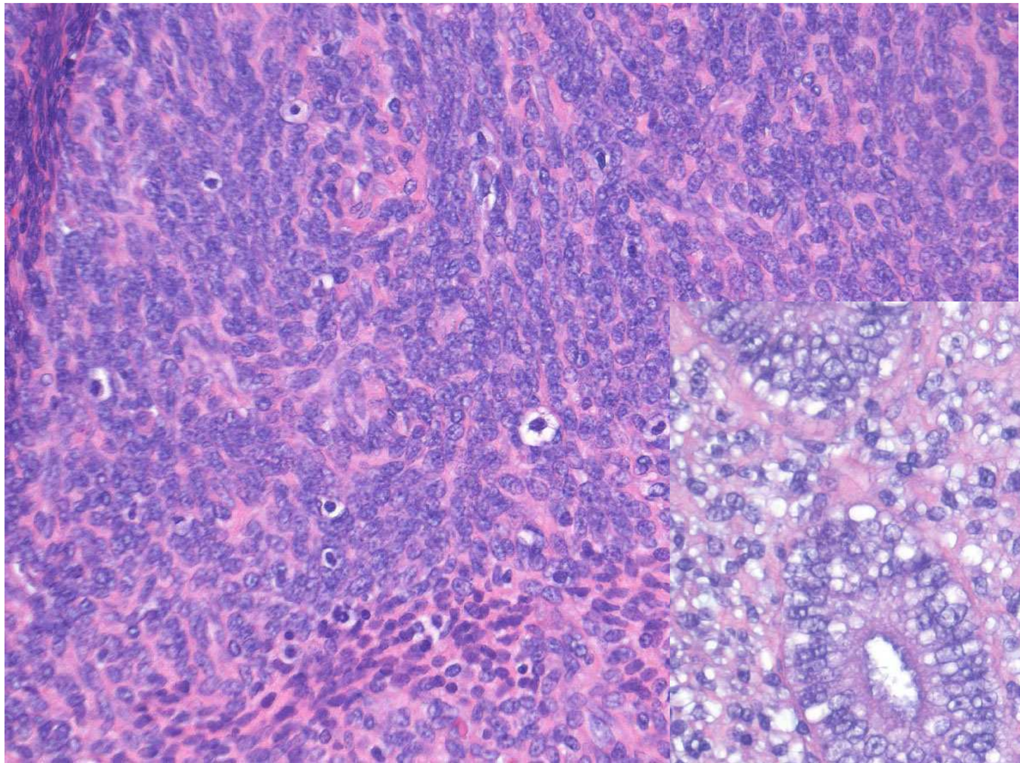
Endometrium - Stroma

- Endometrial stroma surrounds and supports glands
- Mainly formed by endometrial stromal cells and blood vessels.
- So-called “endometrial lymphocytes” are a unique type of LGL
- Endometrial lymphocytes mainly during luteal phase
- Round, often with clear cytoplasm with a centrally located round, kidney-shaped, or more segmented nucleus and eosinophilic cytoplasmic granules
- Once mistaken for infiltrating leukocytes and erroneously named ‘endometrial granulocytes’

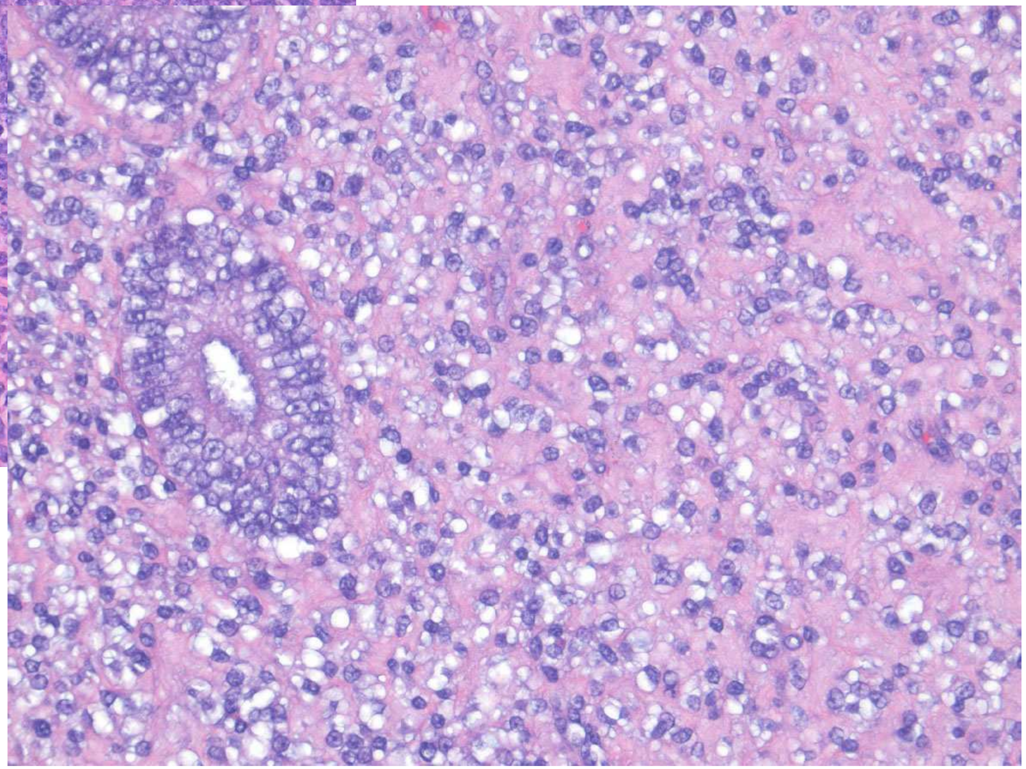
Endometrium - Decidualization

- Typical morphological change under progesterone dominance during luteal phase
- Stromal cell nlarges and becomes ‘decidualized’
- Decidualization: process in which stromal cells trans-form to large, polyhedral, cytoplasm-rich cells storing a large amount of glycogen
- Decidual changes in the stroma localized around spiral arteries and underneath superficial epithelium
- At higher progesterone levels in the whole stroma, functionalis and even part of the basalis
- Can be so prominent that glands become constricted, causing obstruction/dilation of lower parts of the glands

Endometrium - Stroma



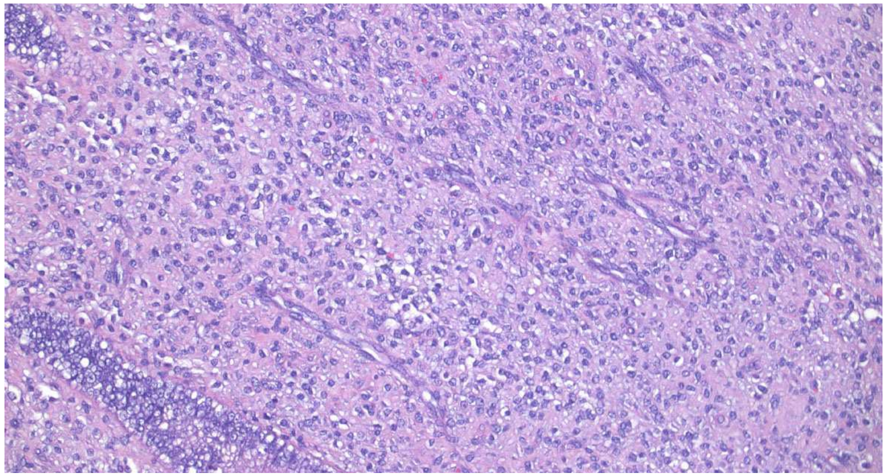
Silent stroma with endometrial lymphocyte

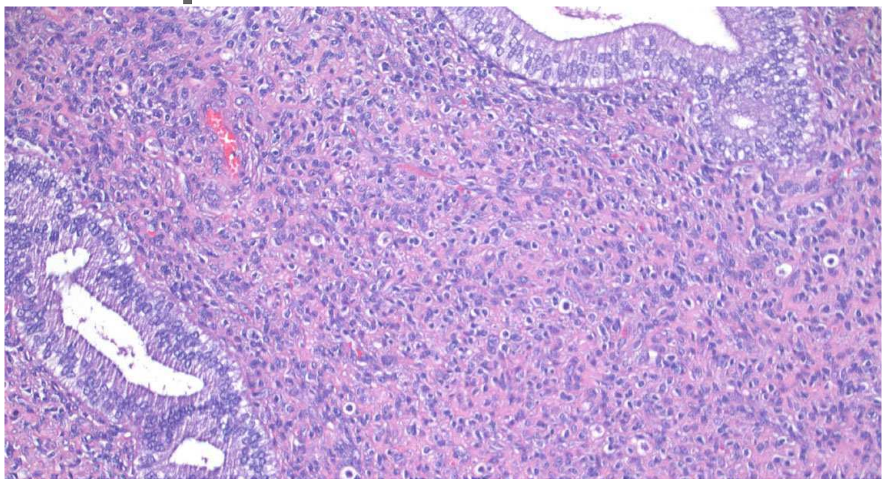


Decidualized stroma

Endometrial Vessels

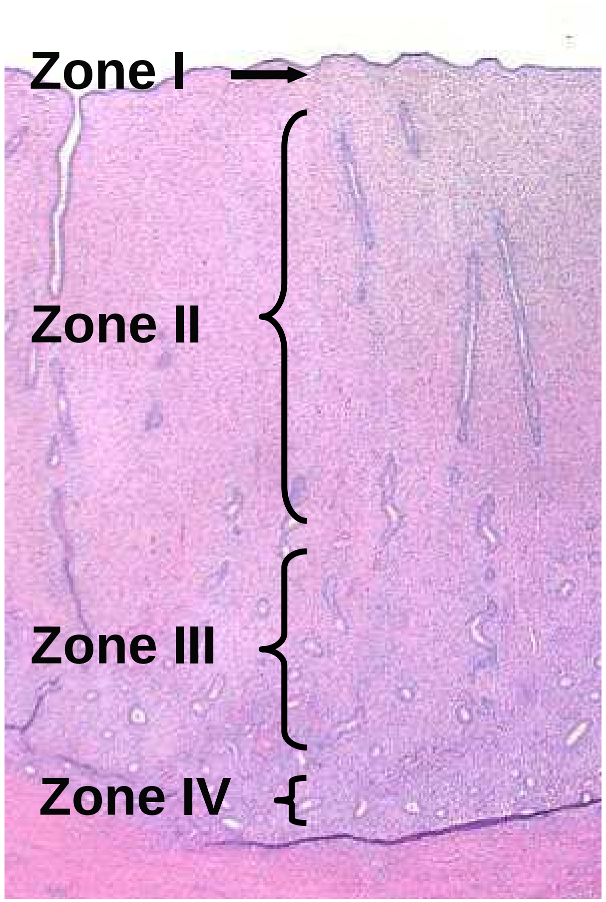
- During follicular phase, inconspicuous and located basally
- Growth under increasing progesterone
- Prominent during luteal phase
- Coiled because vessels grow rapidly and thickness of endometrium is limited
- Clusters in mid- and late luteal phase
- Capillaries branches forming network of arteriovenous anastomosis with venous counterparts





Endometrium - Zonation

- 3 functional zones: compacta, spongiosa, basalis
- Zona functionalis = compacta and upper spongiosa (shed during menstruation)
  - More affected by fluctuations in circulating ovarian hormones than zona basalis
- Zona basalis: renewal after mens-truation (escapes from shedding)



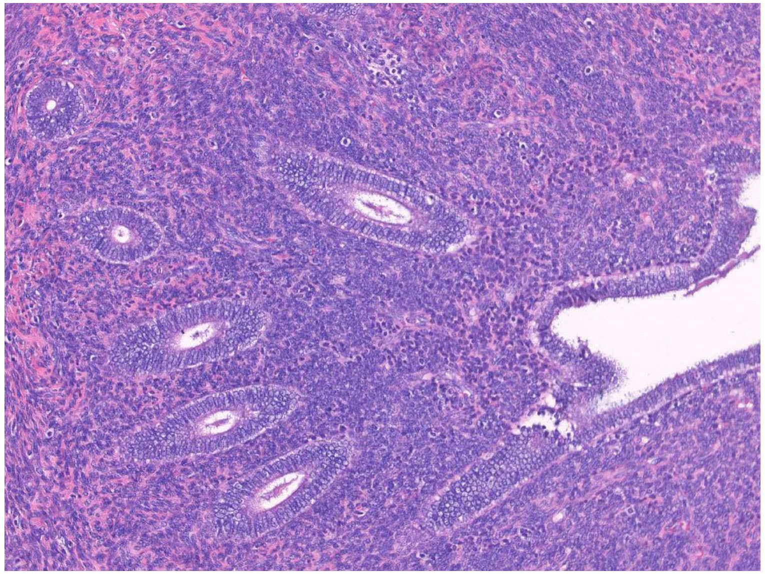
Zona I : superficial epithelium
Zone II : thick, with glands
Zone III: bodies of glands
Zone IV: basal, blind end of glands

Endometrium – Cycle: Follicular Phase

- Controlled growth of endometrium occurs as morpho-logical basis for implantation
- All tissue components (superficial and glandular epi-thelium, stroma, and endometrial vasculature are in varying degrees involved in physiological proliferation

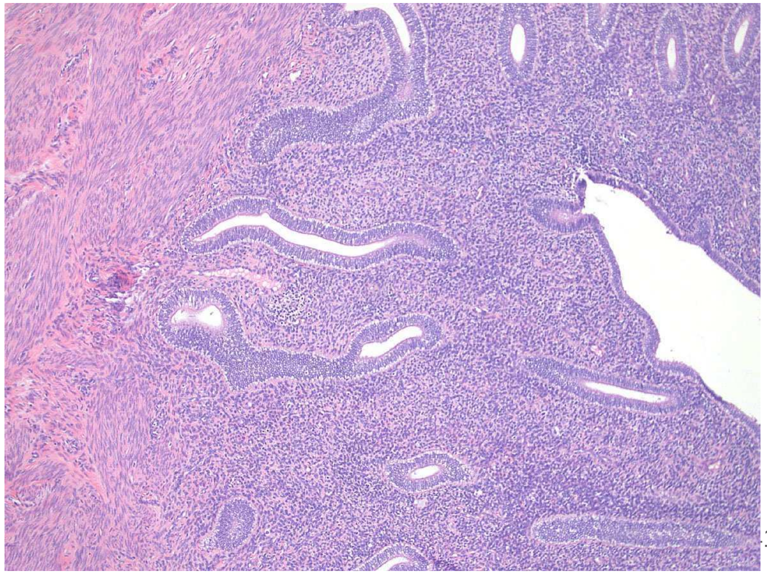
Endometrium – Cycle Morphology: Early Follicular

- Early follicular phase is characterized by:
  - low, inactive endometrium
  - sparse, narrow, and straight tubular glands within loose stroma
  - zona basalis easily recognized due to more compact stroma



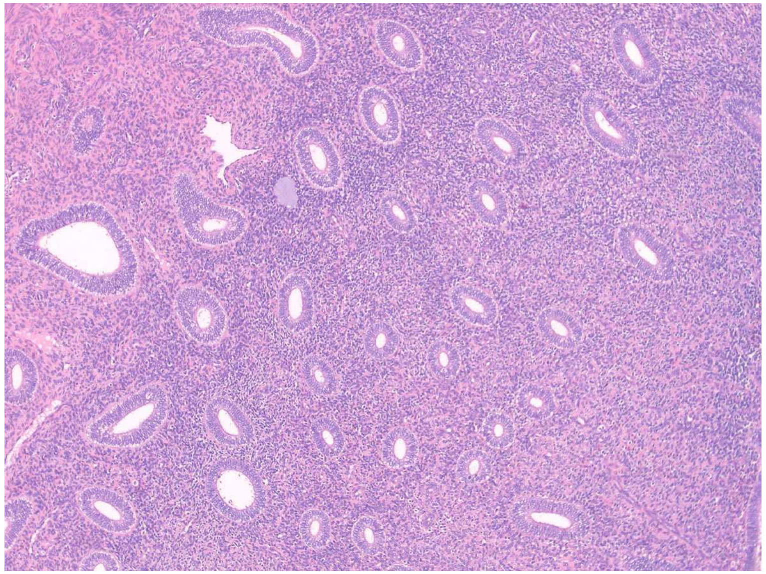
Endometrium – Cycle Morphology – Mid-Follicular

- Mid-follicular phase (estradiol levels increase) is characterized by:
- High endometrium with straight, occasionally tortuous tubular glands within edematous stroma
- Stroma in zona basalis is less compact
- Demarcation between functionalis and basalis less clear
- Increase in height of endo-metrium by stromal edema
- Basalis is similar to early follicular phase



Endometrium – Cycle Morphology - Late Follicular

- Late follicular phase is characterized by:
- Glands of functionalis with maximum of nuclear pseudo-stratification
- PAS-positive perinuclear cytoplasmic vacuoles indicated secretory activity
- Stromal edema diminishes but is persistent until ovulation

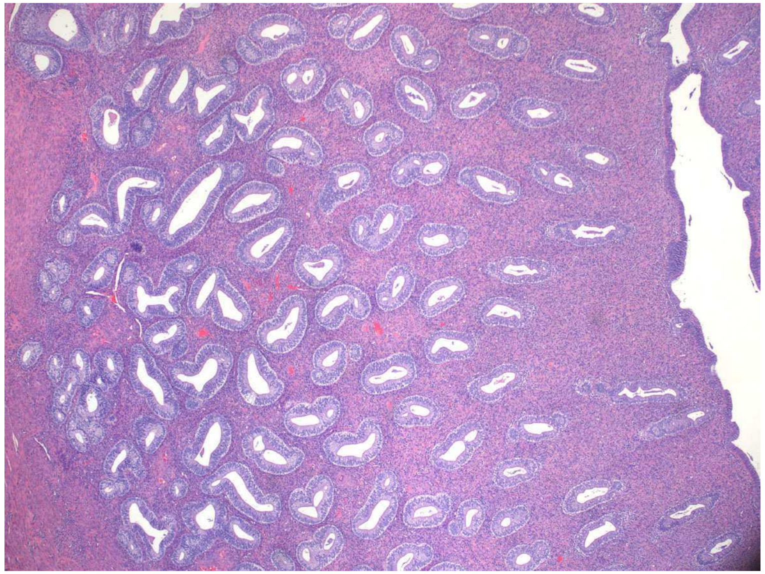


Endometrium – Cycle Morphology: Luteal Phase

- Morphological basis for implantation
- Estradiol level decline directly after ovulation
- Progesterone level gradually increases (dominance)
- Early luteal phase mean serum P:E2 ratio is 42:1

Endometrium – Cycle Morphology: Early Luteal Phase

- Portions of glands in functionalis are tortuous
- Glands lined by a columnar epithelium of medium height
- Characteristic presence of subnuclear vacuolation in glands of functionalis glycogen accumulation)
- Surface epithelial cells differ from epithelial cells lining the glands in that they are high columnar
- Loose stroma of functionalis
- Glands with increased sacculation
- Spiral arteries starts growing

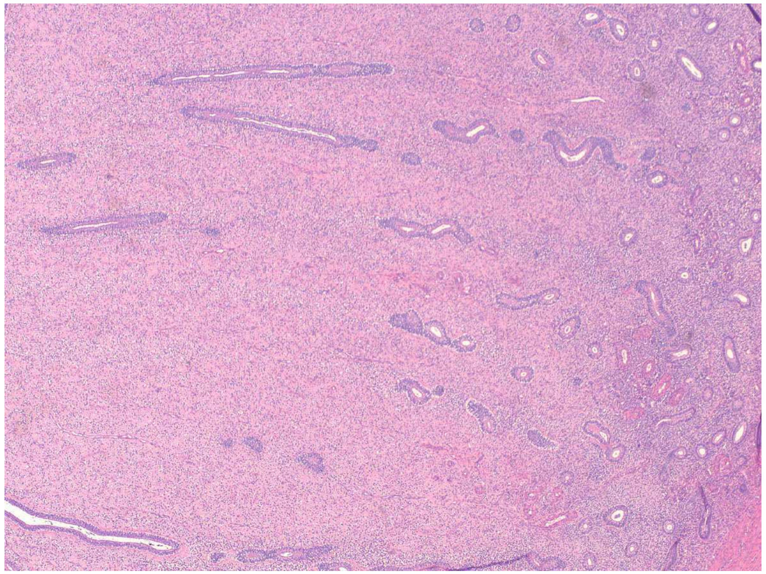


Endometrium – Cycle Morphology: Mid Luteal Phase

- Epithelium of tortuous glands is medium to high columnar, with PAS-positive cytoplasmic vacuoles
- PAS-positive, homogeneous to granular secretory material within the gland lumina
- Mitotic activity is completely absent in surface and glandular epithelium in functionalis.
- Stroma in functionalis is less loose compared to the early luteal phase
- Endometrial lymphocytes are abundant

Endometrium – Cycle Morphology: Mid Luteal Phase

- Hallmark is significant proliferative activity in deepest part of the glands in the zona basalis
- Epithelia in this zone are high columnar with nuclear pseudostratification and mitotic figures
- Significant amounts of glycogen may be in these cells
- Stroma of basalis is dense
- Spiral arteries are most prominent at the basalis–functionalis junction.



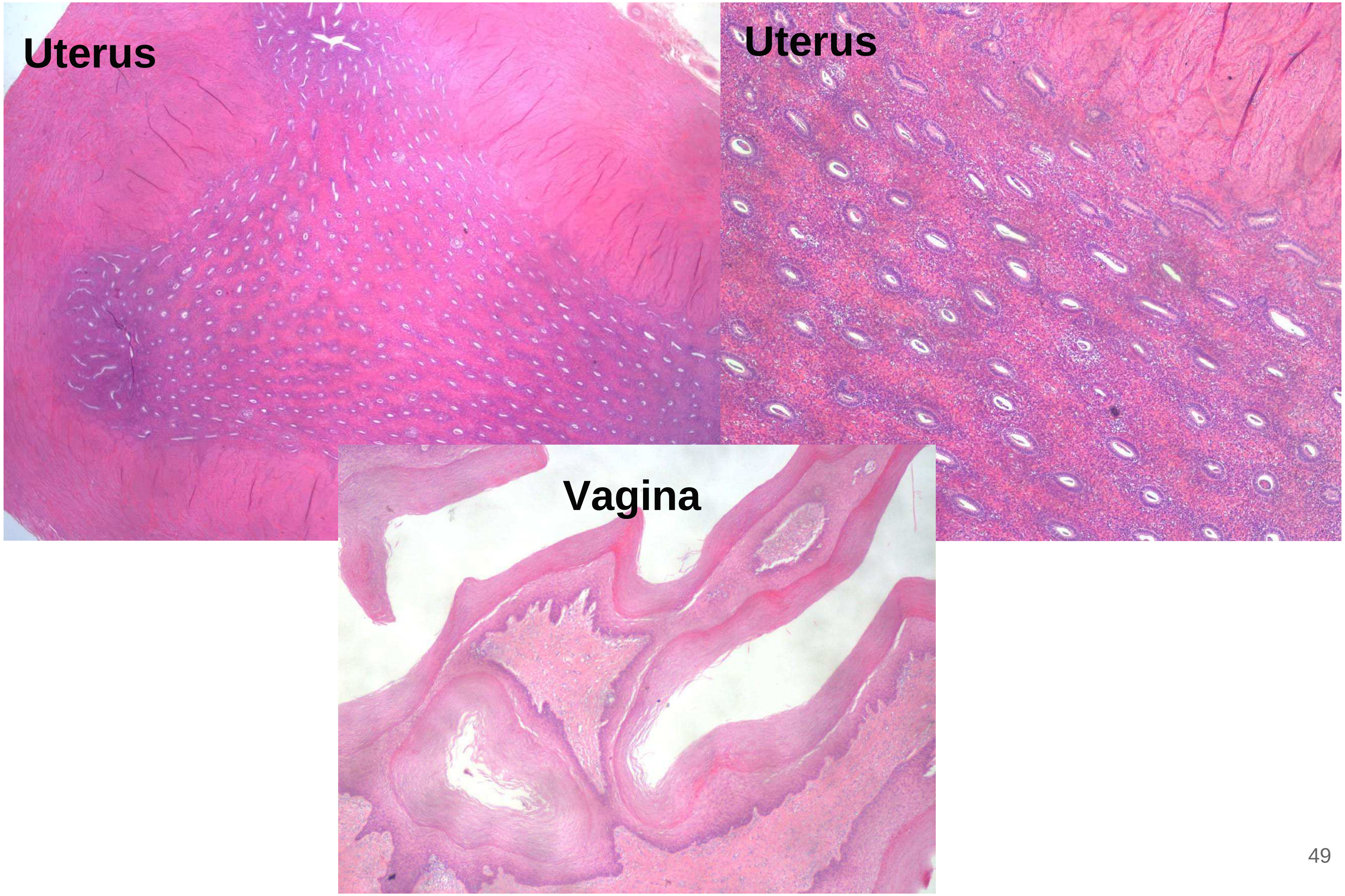
Endometrium – Cycle Morphology: Late Luteal Phase

- Spiral arteries fully developed
- Stromal cells adjacent to spirals pseudodecidualized
- Glands in zona functionalis less tortuous with large amounts of homogeneous, PAS-positive material
- Epithelial cell lining glands are columnar to cuboidal
- Surface epithelium is low columnar with small cyto-plasmic protrusions on luminal surface
- In area underneath surface epithelium, the stroma can be edematous
- Hemorrhages and fibrin leakage
- Numerous endometrial lymphocytes
- Mitotic activity absent in glands and stroma
- Spiral arteries prominent
-

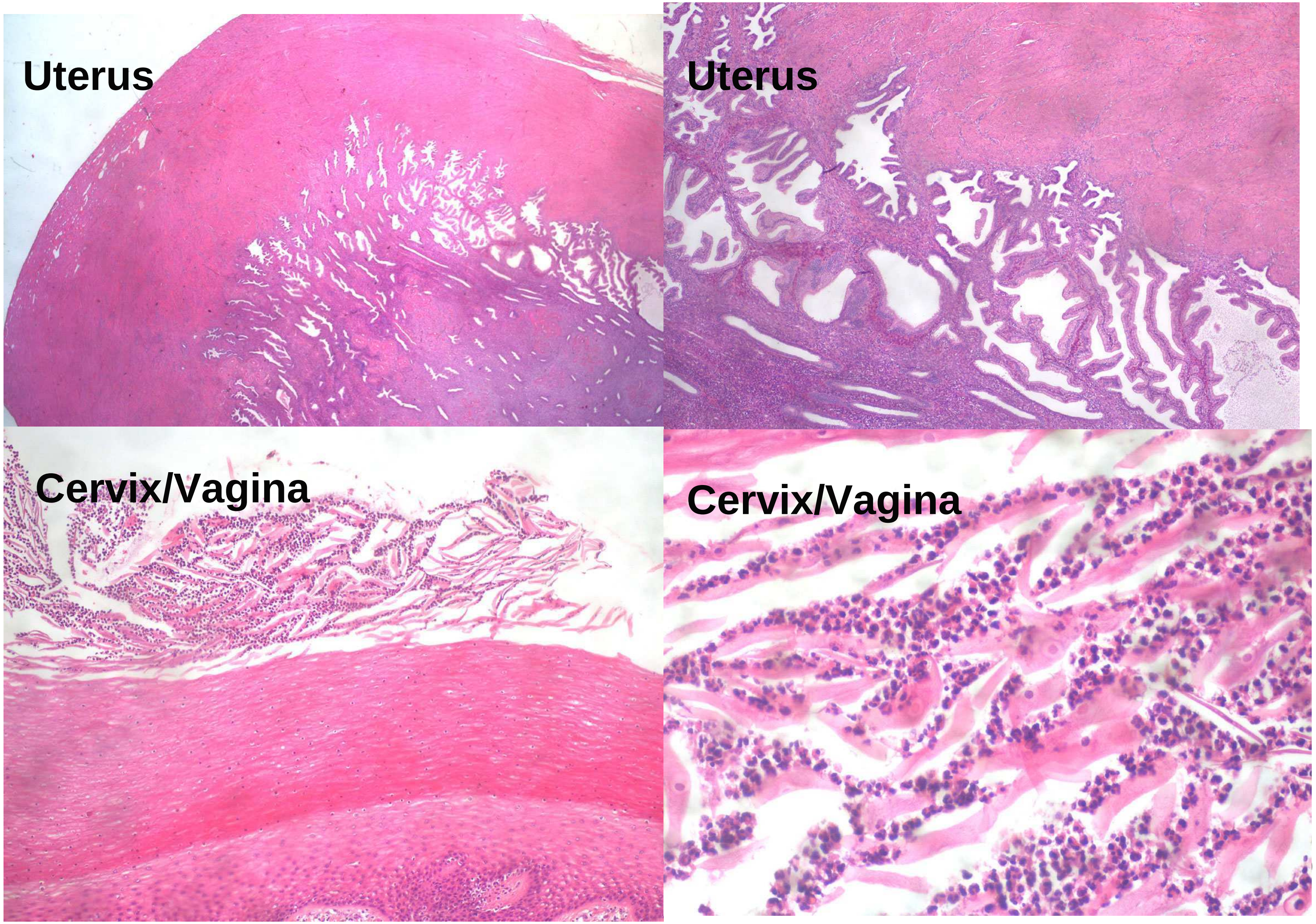
Endometrium – Cycle Morphology: Late Luteal Phase

- In zona basalis, blunt ends of glands are distinct
- Some glands filled with eosinophilic material.
- Spiral arteries still prominent.

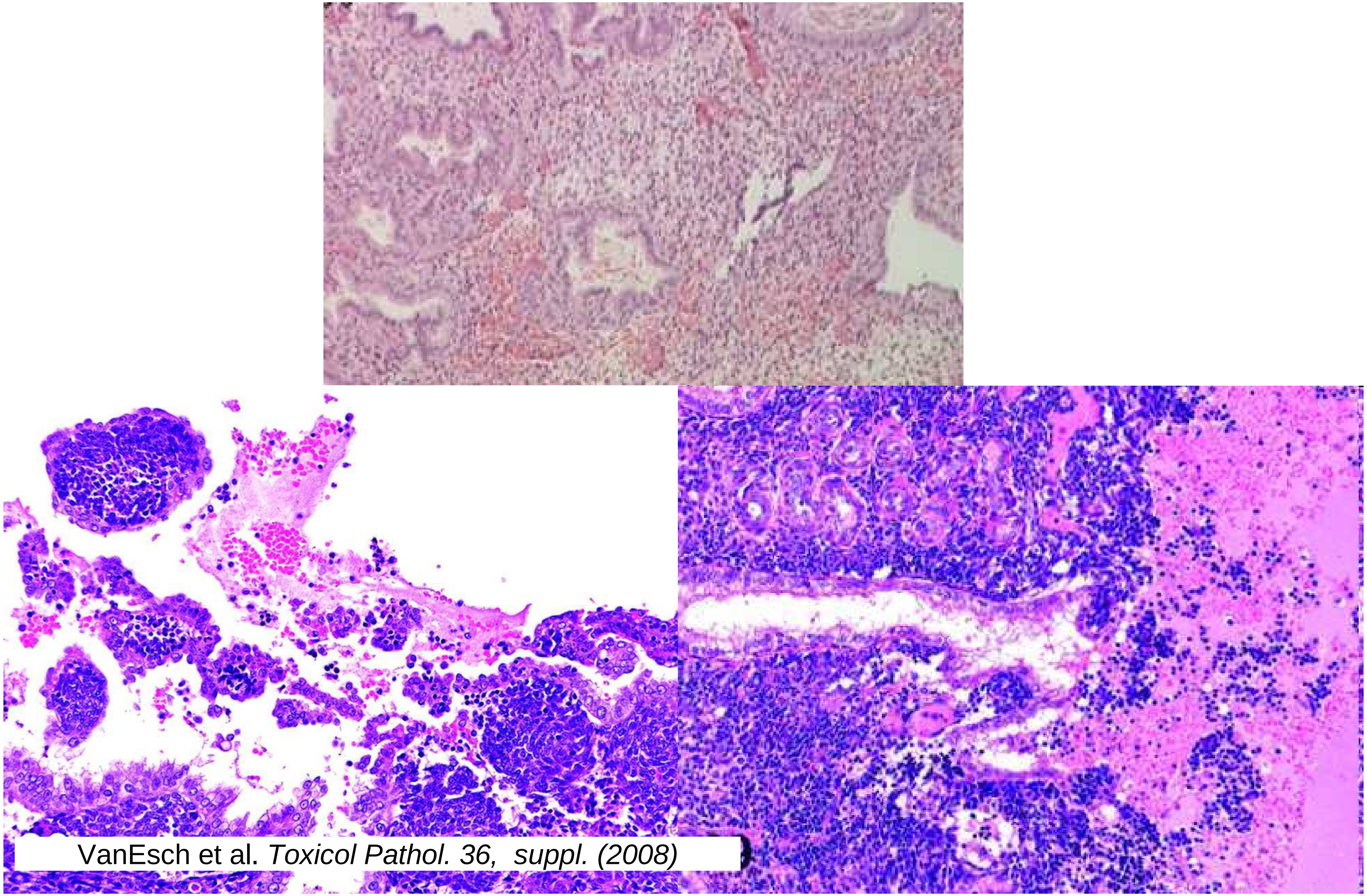
Classical Cycle Morphology: Proliferation



Classical Cycle Morphology: Secretion



Classical Cycle Morphology: Menstruation



VanEsch et al. *Toxicol Pathol. 36, suppl. (2008)*

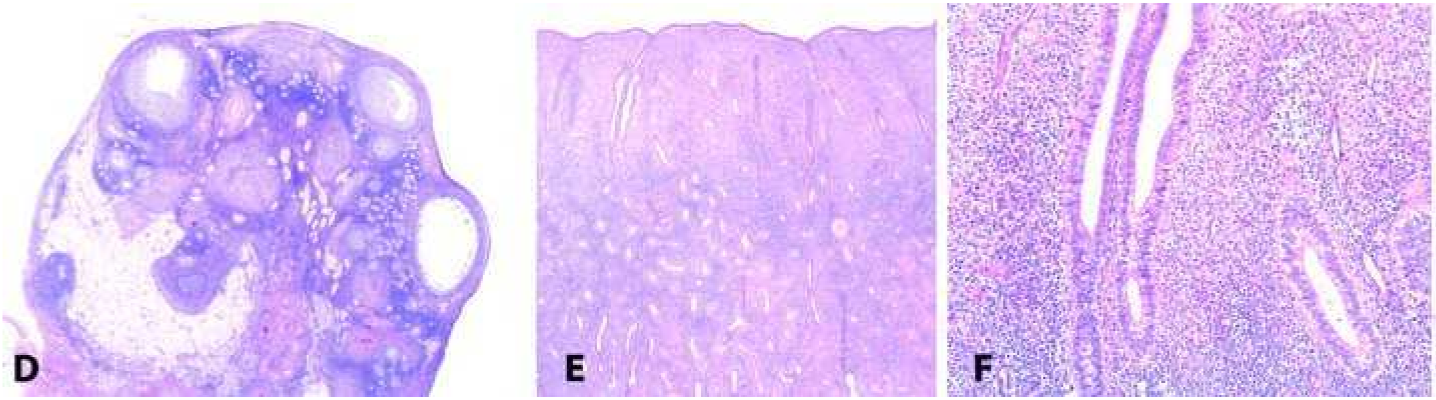
Endometrium – Cycle Morphology: Regeneration

- Estradiol levels low, hence regeneration mediated by other factors
- First signs in remaining upper parts of glands of the functionalis (starts already during late menstruation) phase.
- Proliferation and migration from remaining portion of glands in zona functionalis and upper basalis
- Epithelium starts to proliferate from stumps of remai-ning glands
- Newly formed surface epithelium is flat

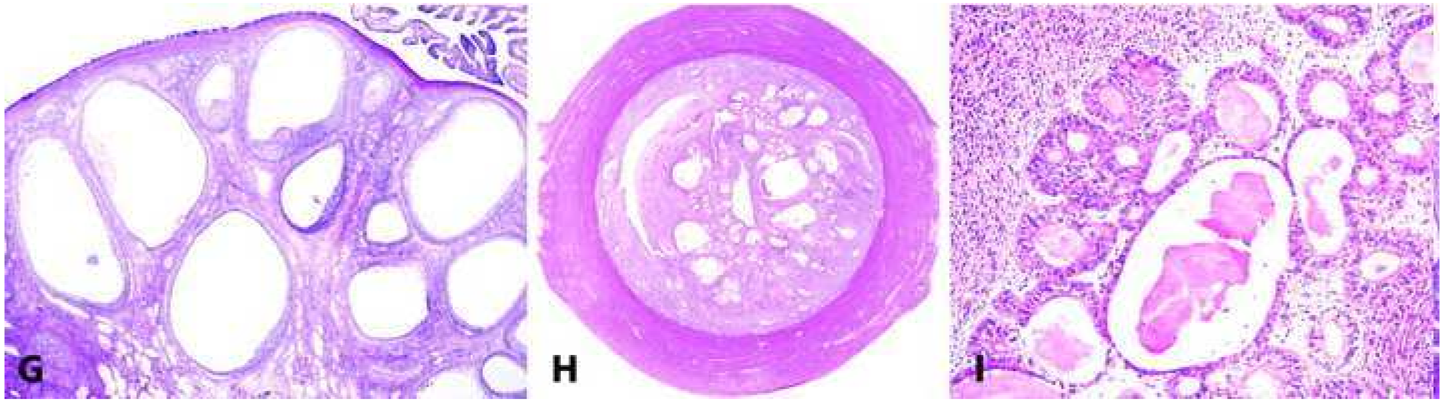
Uterus – Bacground Lesions

Clin et al, Toxicol Pathol. 36, suppl. (2008)

- **Irregular uterine bleeding (common)**
- **Anovulatory Cycles**

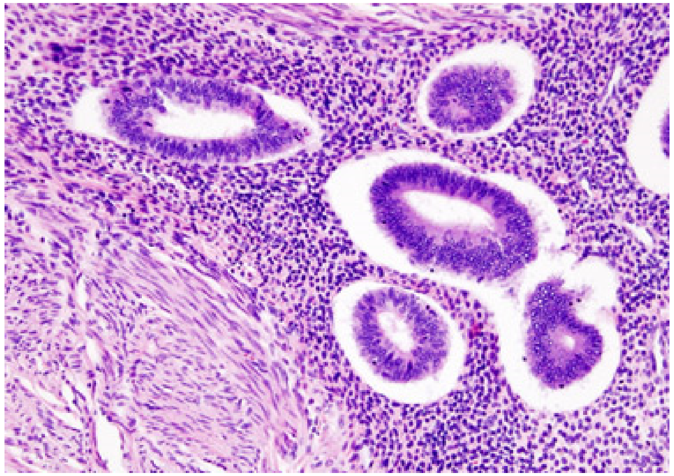


- **Polycystic Ovary Syndrome**

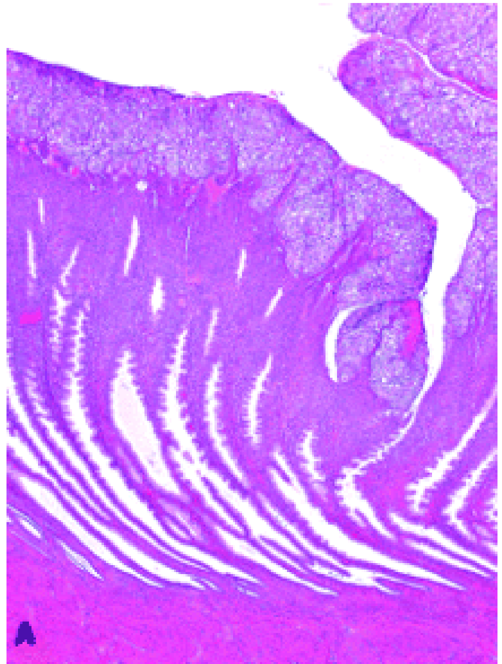


Uterus - Lesions

- **Endometriosis**
- **Adenomyosis**



- **Epithelial Plaque (epithelial proliferative response of the endo-metrial surface in ear-ly implantation)**



- **Endometrial Polyps,**
- **Endometrial Hyperplasia**
- **Neoplasia**

Clin et al, Toxicol Pathol. 36, suppl. (2008)

Cervix - Histology

- Stratified squamous epithelium overlying a stroma composed of dense connective tissue and smooth muscle.
- In contrast to vaginal mucosa, the cervical squamous epithelium lacks prominent rete and often has less superficial keratin
- Squamous mucosa is divided into three layers:
  - germinal basal/parabasal zone
  - stratum spinosum of intermediate cells
  - superficial zone of mature keratinocytes.

Cervix

(A) prepubertal

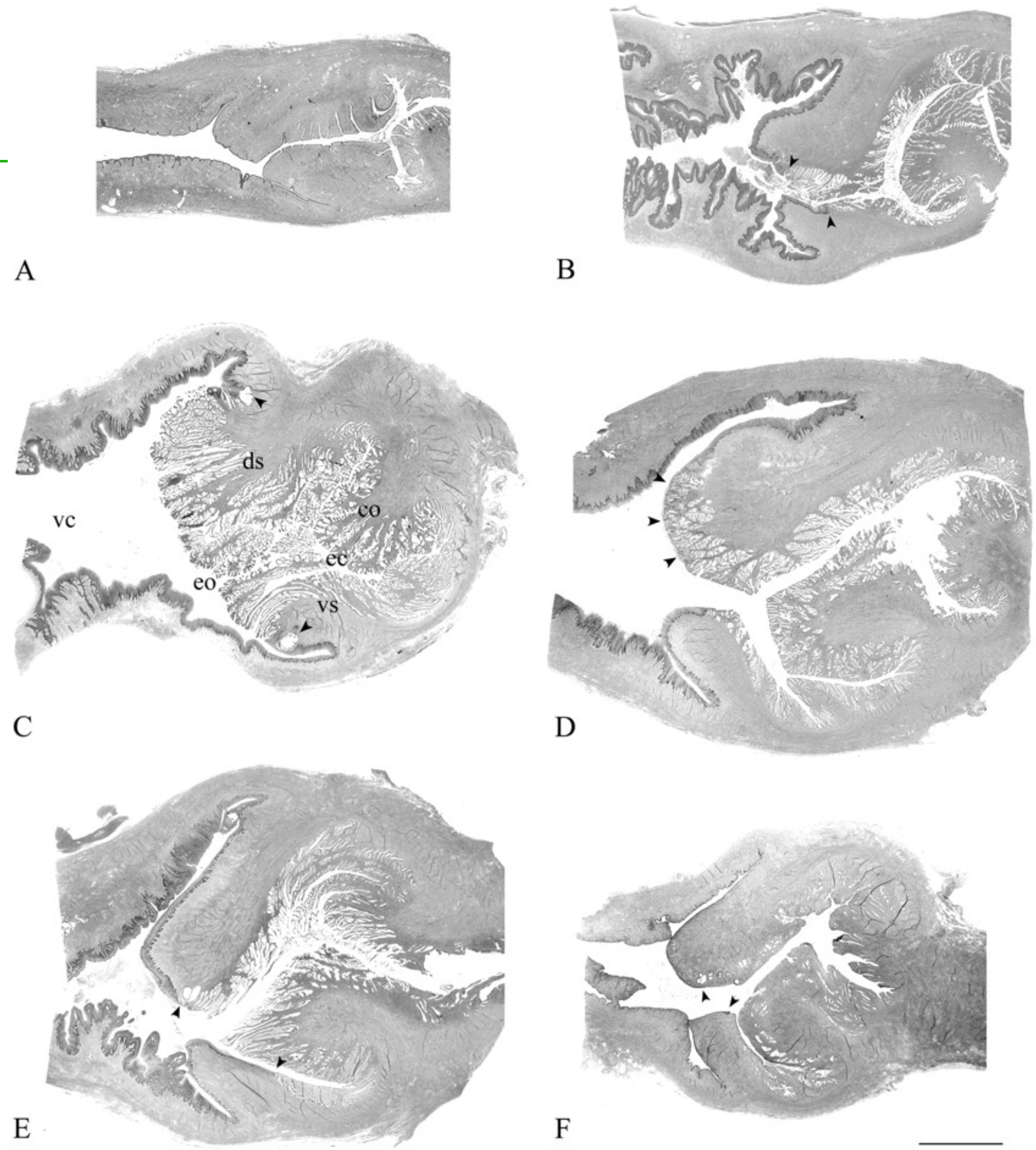
(B) early pubertal

(C) premenopausal

(D) premenopausal

(E) premenopausal

(F) ovariectomized

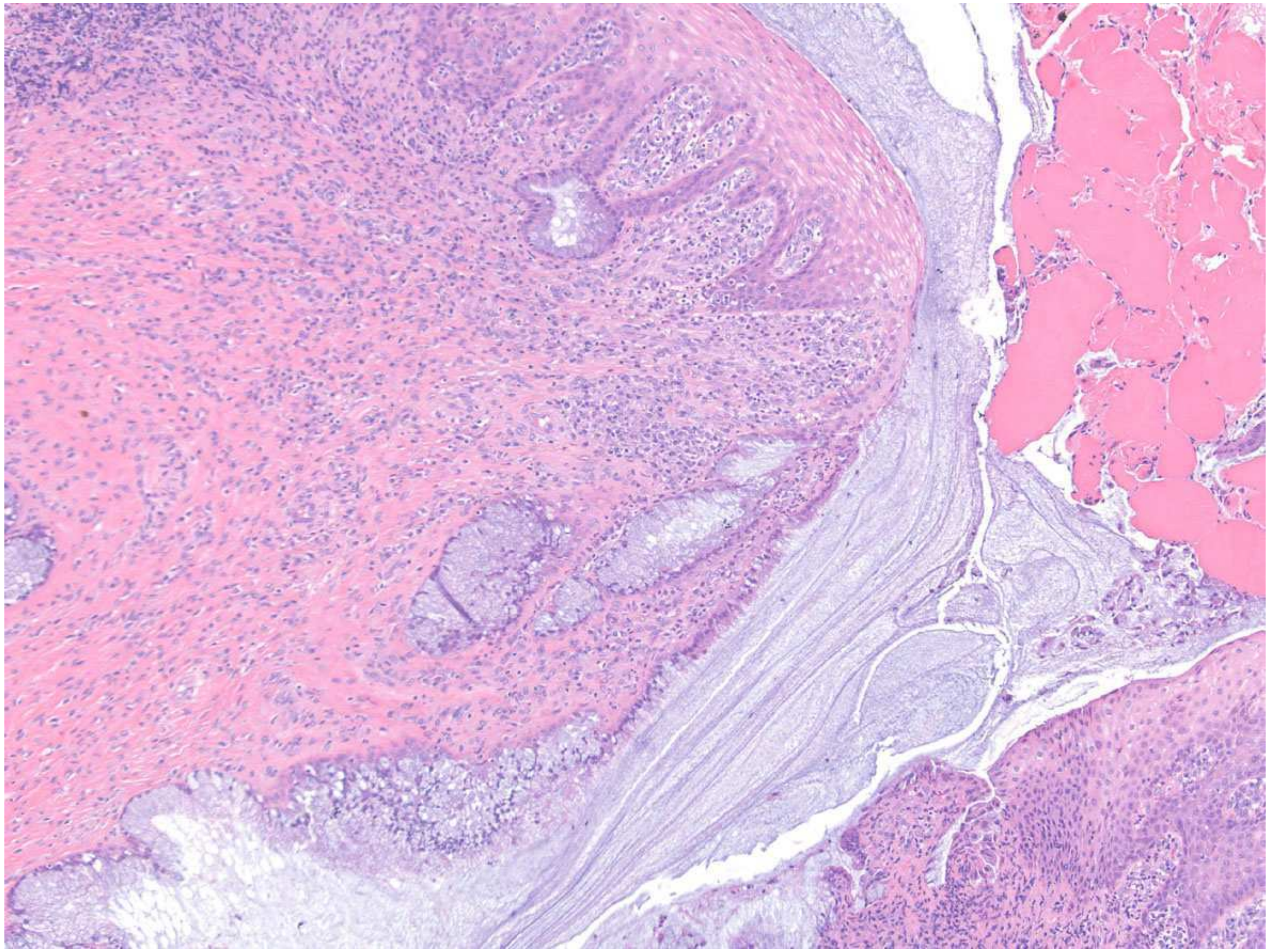


VanEsch et al. *Toxicol Pathol. 36, suppl. (2008)*

Cervix - Development

- Prior to puberty, squamous and glandular epithelia are atrophic, cervical shelves and colliculi are rudimentary, and squamocolumnar junction (SCJ) is indistinct (where squamous epithelium abruptly shifts to tall columnar glandular epithelium)
- Estrogen exposure (about 2-3 y of age) induces marked increase in vaginal and cervical squamous maturation and keratinization and moderate increase in endocervical gland hypertrophy
- At this early pubertal state (i.e., prior to ovulation), SCJ remains within the endocervical canal
- With complete menstrual cycle activity, the glandular cervix becomes enlarged shifting the original SCJ to the exocervix (called ectopy)

Cervix - Squamocolumnar junction (SCJ)

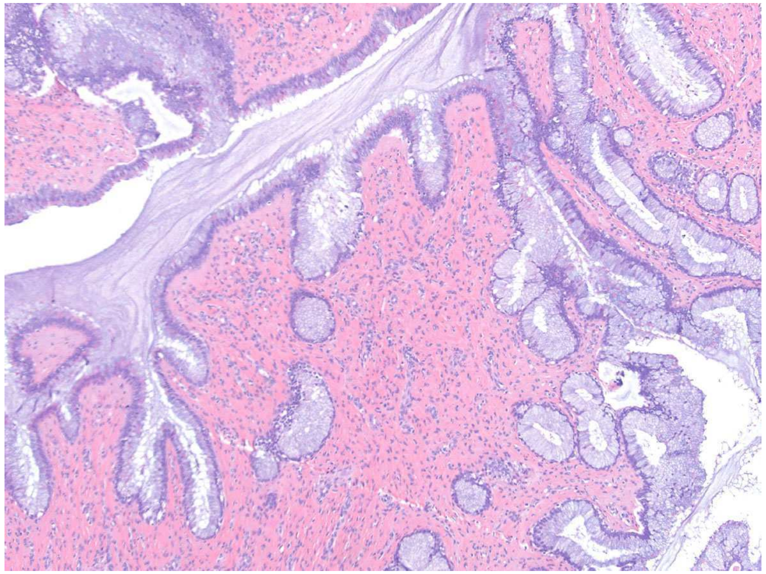


Cervix – T-Zone

- T-zone forms a second “functional” SCJ
- expands with increasing age
- composed of squamous epithelium lacking superficial maturation and the abundant intracytoplasmic glycogen seen in normal maturing keratinocytes of the spinosum
- Squamous metaplasia occur in response to factors, including the lower pH of the vagina, hormones, local infection, inflammation, and microtrauma
- Squamous metaplasia also common in basal portion of endocervical glands cycling macaques (estrogen is a key factor)

Cervix - Endocervix

- Mucosa composed of simple columnar epithelial cells lining main canal and colliculi and form infolded glandular structures
- In cycling animals, cells have basal nuclei and clear to pale eosinophilic vacuolated cytoplasm
- Primary roles of secretory and ciliated columnar cells is to secrete and distribute mucus
- Secretory activity peaks in luteal phase (cells may reach about 50 µm in height



Cervix – Endocrine Regulation: Estrogen and Progesteron

- Ovarian hormones induce keratinization in vagina and adjacent exocervix
- Cells are particularly sensitive to estrogen (basal cell proliferation, maturation, desquamation)
- SCJ increases in thickness under estrogen (but not keratinized, characteristic: accumulation of intracyto-plasmic glycogen in cells of spinosum)
- Loss of estrogen causes diffuse atrophy of squamous epithelium
- Estrogens and progestogens induce marked hypertro-phy and mucus secretion of endocervical glands
- Estradiol induces a more profuse, watery, alkaline mucus
- Progesterone induces more viscous, acidic mucus

Cervix-Lesions

Spontan:

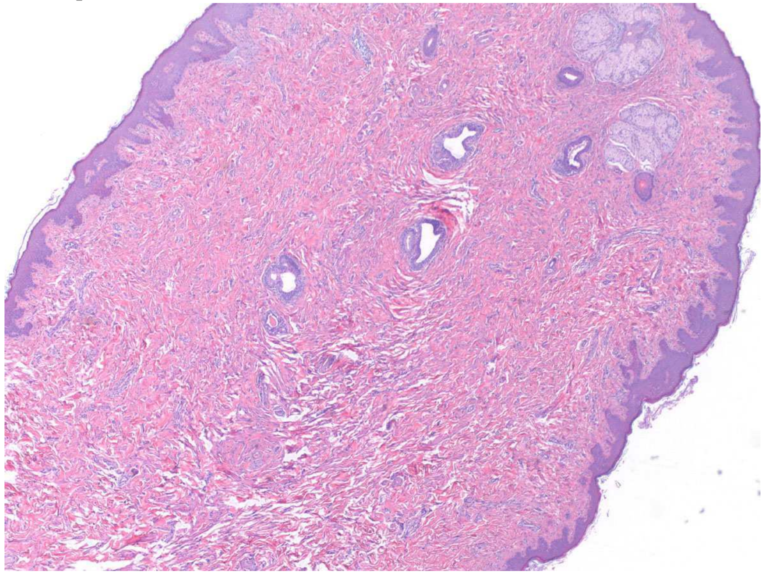
- Lymphoid follicular hyperplasia
- Mucus-filled nabothian cysts
- Subacute to chronic vaginitis cervicitis,
- Endocervical polyps
- Vaginal adenosis
- Less common endometriosis

Induced:

- Methylcholanthrene: cervical dysplasia in rhesus
- Diethylstilbestrol: increased incidence of vaginal adenosis, and squamous metaplasia, but not adeno-carcinoma).

Mammary Gland

- Macaques have 2 pectoral mammary glands
- 5-7 lactiferous ducts exiting each nipple with varying degrees of communication between corresponding ductal and lobular units
- Occasional small clusters of glandular tissue in nipple
- Growth/differentiation depend on ovarian and local production of steroid hormones, GH or IGF
- Secretory stimuli include prolactin and placental lactogen



Mammary Gland - Endocrine Regulation: Estrogen, Progesteron, Androgens

- Development depend on ovarian steroid production
  GH/IGF Axis
- Growth hormone (systemically and locally)
- GH and IGF are critical in proliferation and differenti-
  ation

Mammary Gland - Endocrine Regulation: Prolactin, Placental Lactogen, Tissue Hormones

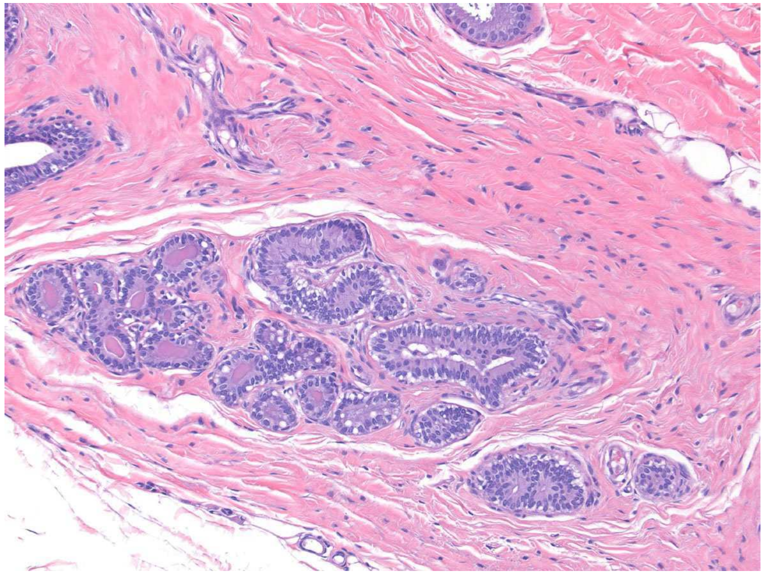
- **Prolactin not obligate for mammary growth and development but required for lactation**
- **Exogenous administration causes insignificant increase**
- **Galactorrhea reported in conjunction with prolactin-producing neoplasms of the pituitary gland in macaques**
- **Placenta Lactogen (Somatomammotropin)**
- **GH-related placental lactogen derived (*placental lactogen* does not impair lactation)**
- **Intratissue production of sex steroids and growth factors is important (enzymatic systems for conversion of precursors to more bioactive estradiol (aromatase and steroid sulfatases)**

Mammary Gland – Puberty

- Nipple development in macaques is distinctive and precedes regular menstruation by several months
- Pubertal development of the breast starts with rudimentary ductal tree early in life, followed by elongation branching of major ducts
- Lobular development during puberty
- Pubertal development of mammary tissues in male macaques is not well described; but transient glandular development (gynecomastia) in more than 50% of normal adolescent males

Mammary Gland – Adult-Non Lactating

- Homogeneous pattern of mature type 2 lobules
- Estrogen receptors α and β with the latter being more abundant
- Effect of menstrual cycle on proliferation in breast is controversial: cycle-related changes are small
- Ductal tissues proliferate more during luteal phase
- Lobuloalveolar tissues epithelium with higher proliferation during late follicular phase



Mammary Gland - Lactation

- Gestation in macaques approx. 150 days
- Extensive growth and differentiation under estrogens, progestogens, chorionic gonadotropin, placental lacto-gen, prolactin
- Change in volume of glandular tissue is 10-20x, as a re-sult of both epithelial proliferation and secretory distent-ion of ductal and alveolar system
- Lobuloalveolar units markedly increase in number/size
- Macaques lactate for approx. 12 months and during this time, ovulation is suppressed
- Single offspring (neonate typically with strong prefer-ence for one nipple or the other

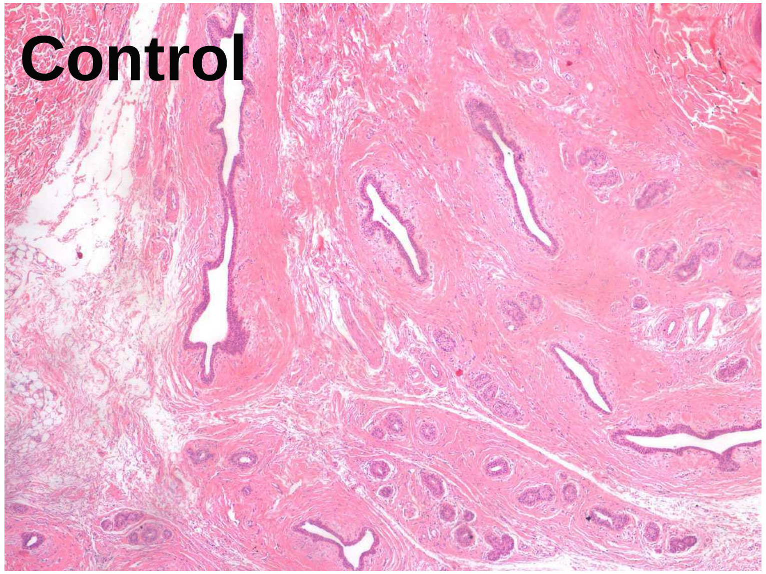
Mammary Gland - Senescence

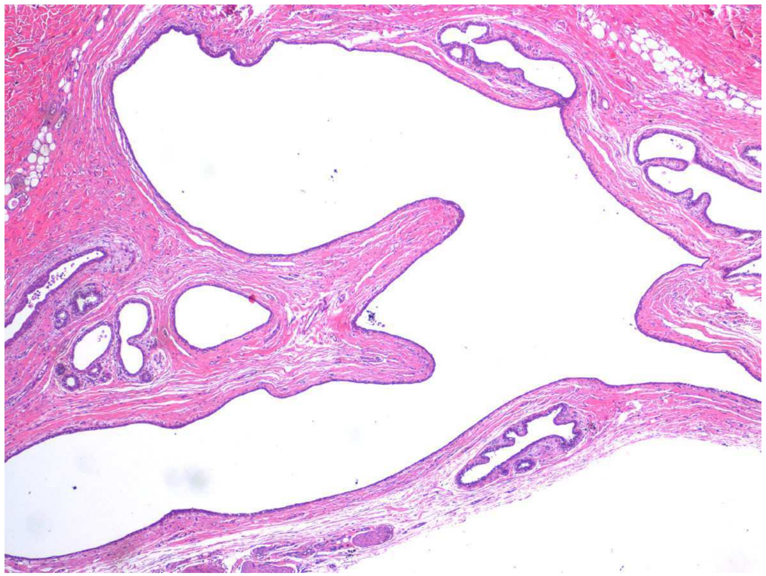
- Regression into ductal network with marked lobular atrophy and little proliferative activity
- Substantial variation in the amount of tissue remaining
- Estrogen and progesterone receptors expression per-sists (surgically postmenopause: at least 6-7 y)
- Breast is responsive to exogenous hormonal stimulat-ion by estrogens and progestogens beyond 25 y

Mammary Gland – Spontaneous Findings

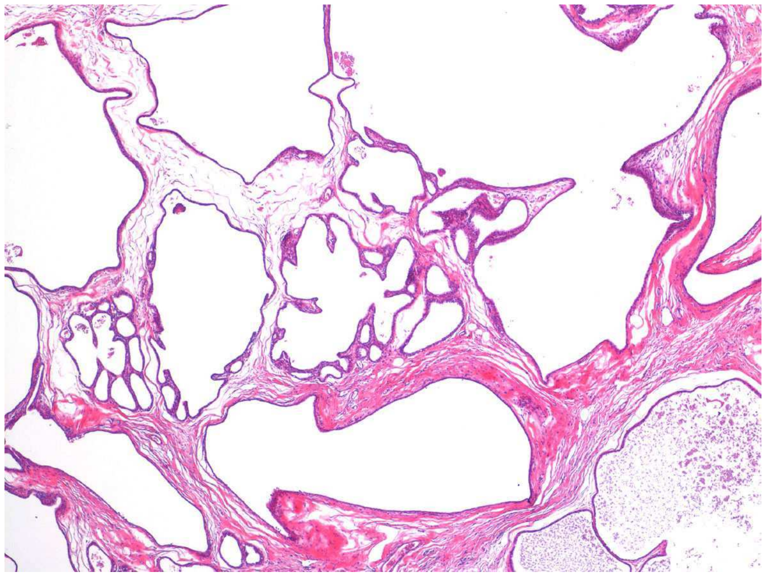
- Cystic change
- Columnar cell change
- Apocrine metaplasia
- Ductal hyperplasia,
- Lobular hyperplasia
- Neoplasms (carcinoma)

Mammary Gland – Induced Findings: GH





Induced cystic change



Summary

- Macaques excellent models for preclinical testing of female reproductive toxicants
- Often species of choice (e.g. of biologicals)
- Cynomolgus is the current predominant species
- By functional and morphological aspects, the ovary, uterus, cervix and vagina of cynomolgus is representative for conditions in human.
- Cycle monitoring easy (may be complicated by hormone measure)
- In contrast to rodents, primates have long life span of corpus luteum (>2 weeks)
- Unlike in rodents, prolactin does not play important role during luteal phase
- Luteolysis does not involve uterine signal

Summary

- Similarities of mammary gland physiology in monkeys to human,
- Evaluation of mammary glands is very important due to test items possibly mimicking sex steroids.
- Relevant effects known from estrogenic, progestogenic, androgenic, GH and other receptor interaction or indirect mechanisms