Physiology of the endometrium and regulation of menstruation.

[1]Critchley, Hilary OD; [1]Maybin, Jacqueline A; [1]Armstrong, Gregory M.;
[1]Williams, Alistair R.W.

Affiliation:
[1]MRC Centre for Reproductive Health, The University of Edinburgh, The Queen's Medical Research Institute, Edinburgh, United Kingdom

Corresponding Author:
Hilary O D Critchley
MRC Centre for Reproductive Health
The University of Edinburgh
The Queen's Medical Research Institute
Edinburgh Bioquarter
47 Little France Crescent
Edinburgh EH16 4TJ
hilary.critchley@ed.ac.uk Tel: +44 131 242 6858, Fax: +44131 242 6441

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Abstract

The physiological functions of the uterine endometrium (uterine lining) are preparation for implantation, maintenance of pregnancy if implantation occurs, and menstruation in the absence of pregnancy. The endometrium thus plays a pivotal role in reproduction and continuation of our species. Menstruation is a steroid regulated event and there are alternatives for a progesterone-primed endometrium, i.e. pregnancy or menstruation. Progesterone withdrawal is the trigger for menstruation. The menstruating endometrium is a physiological example of an injured or “wounded” surface that is required to rapidly repair each month. The physiological events of menstruation and endometrial repair provide an accessible in vivo human model of inflammation and tissue repair. Progress in our understanding of endometrial patho-physiology has been facilitated by modern cellular and molecular discovery tools, along with animal models of simulated menses.

Abnormal uterine bleeding (AUB), including heavy menstrual bleeding (HMB) imposes a massive burden on society, affecting one in four women of reproductive age. Understanding structural and non-structural causes underpinning AUB is essential to optimise and provide precision in patient management. This is facilitated by careful classification of causes of bleeding.

We highlight the crucial need for understanding mechanisms underpinning menstruation and its aberrations. The endometrium is a prime target tissue for selective progesterone receptor modulators (SPRMs). This class of compounds has

78 therapeutic potential for the clinical unmet need of HMB. SPRMs reduce menstrual
79 bleeding by mechanisms still largely unknown.
80
81 Human menstruation remains a taboo topic and many questions concerning
82 endometrial physiology that pertain to menstrual bleeding are yet to be answered.
83
84

1. Introduction

The functions of the uterine endometrium are to prepare for implantation, maintain pregnancy if implantation occurs and to menstruate in the absence of pregnancy. The endometrium (uterine lining) thus plays a pivotal role in reproduction and maintenance of our species. These pivotal processes are steroid regulated, and the alternative pathways for a progesterone-primed endometrium are pregnancy or menstruation. The endometrium is a complex multicellular steroid-target tissue that in the absence of pregnancy sheds each month (menstruation), and is thereafter rapidly repaired without residual scarring or loss of function. Endometrial repair following endometrial breakdown and shedding involves resolution of inflammation, angiogenesis, tissue remodelling, and formation of new tissue. The constituent cell types within the endometrium include stromal, epithelial, vascular, and immune cells. There exists a dynamic cell-to-cell dialogue involving the endocrine and immune systems that is essential to ensure there is efficient endometrial shedding and subsequent re-epithelialization (repair of the injured mucosal surface) if pregnancy does not occur. The human endometrium and the physiological events of menstruation and endometrial repair provide an accessible *in vivo* human model of inflammation and tissue repair. Hence the menstruating endometrium may be considered a wonderful physiological example of an injured or “wounded” surface that is required to rapidly repair each month.

2. Menstrual cycle stage is governed by the prevailing endocrine environment with major impact on endometrial form and function.

During the human menstrual cycle the endometrium is exposed each month to sequential patterns of circulating ovarian sex steroids which are crucial to the

regulation of growth and differentiation of the endometrium. Chief players in this preparation for pregnancy are ovarian 17β-estradiol (E2) and pregn-4-ene-3,20-dione (progesterone; P4), the concentrations of which fluctuate in a well-characterized manner across the menstrual cycle **(Figure 1A).**

The endometrial endocrine environment is initially dominated by estradiol in the early, 'proliferative' phase of the menstrual cycle. At this stage of endometrial development vascular and endometrial tissues undergo extensive proliferation. The proliferative phase of the endometrial cycle has its counterpart in the ovarian 'follicular' phase, and following ovulation and formation of a corpus luteum, progesterone is secreted (49, 181).

During the progesterone dominant 'secretory' phase of the endometrial cycle (ovarian counterpart is the 'luteal' phase), progesterone production is required for the establishment and maintenance of pregnancy in the estradiol-primed endometrium (49, 62, 181).

The events that span the time from regression of the corpus luteum in the late secretory phase (the time of progesterone-withdrawal), through menstruation culminating in post-menstrual repair of the endometrium in the proliferative phase, may be termed the 'peri-menstrual' window and reflect the endocrine 'luteo-follicular' transition period **(Figure 1B)**.

Histologically the endometrium comprises a simple columnar epithelium overlying a multicellular stroma. The stroma hosts cellular components of connective tissue with

fibroblast-like stromal cells and contains a number of tubular glands contiguous with the luminal surface, spiral arteries and a fluctuating traffic of recruited innate immune cells (181). There is also substantial evidence for an endometrial adult stem cell population (89). These populations of endometrial progenitor stem cells may differentiate into stromal cells and epithelial cells and contribute to the efficient replacement and maintenance of the endometrium that is required to restore endometrial integrity with menstruation. In the human these progenitor stem cells are localized to the basal layer of the endometrium (205).

Endometrial stromal cells (ESCs) are a target for progesterone. The process in the endometrium termed decidualization indicates transformation of ESCs into specialized secretory “decidual cells” that provide both a nourishing and receptive cell microenvironment that is essential for embryo implantation and onward placental development (95). ‘Decidualization’ is a process involving transformation of ESCs from an elongated to a rounded morphology, with induction of expression of progesterone-dependent proteins, including prolactin, glycogen, tissue factor, insulin-like growth factor-binding protein 1 (IGFBP1); and the transcription factor Forkhead box O1 (FOXO1) in ESCs (31, 32, 72, 138, 139, 207). The endometrial decidualization response is initiated intracellularly by the production of cyclic adenosine monophosphate (cAMP; (32)), initially in perivascular endometrial stromal cells before decidualization spreads throughout the endometrial stroma. Decidualization also spreads under the luminal epithelium and is thus prominent in the region of the endometrium where an embryo may implant. In humans, decidualization is independent of embryo/endometrial contact (‘implantation’), and thus occurs ‘spontaneously’ in the presence of progesterone exposure. Recently

spontaneous decidualization has been described in a newly discovered menstruating rodent, the spiny mouse (16); (see later in description of mouse models of simulated menses, Section 5).

These functionally distinct phases of the menstrual cycle are critical for synchronization of the endometrium alongside oocyte maturation, fertilization and implantation of the embryo during the middle of the secretory phase when endometrial receptivity is likely to be optimal (229).

It is interesting that the human endometrium exhibits the most extensive decidualization response of any menstruating species studied to date (194). This feature may in turn play an important role in the extensive trophoblast invasion that takes place at the time of human implantation (82, 113). Crucially when trophoblast invasion is inadequate or defective in early pregnancy, miscarriage may be the consequence, and in later pregnancy problems such as fetal growth restriction and preeclampsia may manifest (40, 117, 140).

Increasingly, lines of evidence support defective decidualization responses as underpinning problems in early, mid and later pregnancy. Perturbed decidual cell-function in the early first trimester may be involved in the later development of preeclampsia. For example, aberrant expression of decidual-cell-derived chemokines/ -cytokines may result in altered migration, survival and adhesion of endometrial immune cells (uterine natural killer (uNK) cells, monocytes/ macrophages, T-cells). This may lead to abnormal interactions with endometrial epithelial and /or endothelial cells resulting in altered function (numbers, activation)

and impact upon trophoblast invasion and subsequent risk of preeclampsia development (113). It has also been proposed that “menstrual pre-conditioning” (33) protects against development of pre-eclampsia. Good lines of evidence provide support for the concept that both menstruation and pregnancy are inflammatory events associated with physiological ischemia-reperfusion tissue injury. The latter is particularly evident during pregnancy. Brosens thus proposed that cyclical menstruation protects uterine tissues from the excess inflammation and oxidative stress that is evident with deep placentation, and describes this as menstrual preconditioning (33).

Further development of this aspect of uterine / endometrial function lies beyond the scope of this review.

Circulating estradiol concentrations decrease during the late secretory phase, partly as a consequence of progesterone action. Progesterone promotes conversion of estradiol to a less biologically active form (estrone; E1) via induction of the steroid-metabolizing 17β-hydroxysteroid dehydrogenase enzymes (HSDs) (115, 133, 190, 237, 239), thereby regulating availability of estrogen ligands. Progesterone also reportedly reduces expression of the estrogen receptor (ER; (238)), and thereby further inhibits the actions of estradiol. The 17β-HSDs thus enable each cell to precisely control the intracellular concentration of each sex steroid according to local tissue needs (133). Decidualization appears to be a pre-requisite for menstruation and occurs spontaneously in women and old world primates. In contrast, other species only decidualize at implantation and do not menstruate.

The absence of pregnancy leads to regression of the corpus luteum (forming a scar-like structure in the ovary known as the corpus albicans) and a consequent sharp decline in circulating progesterone and estradiol concentrations (63). The withdrawal of estradiol and particularly progesterone initiates the onset of menstruation **(Figure 1 and 2)**, in which the upper, functional layer of the endometrium (functional zone; often also referred to as the functionalis) is broken down, shed and subsequently restored.

The withdrawal of progesterone effects a number of morphological changes in the endometrium, including tissue edema, increased endometrial blood flow, vessel permeability and fragility (81, 92, 181, 198) along with the trafficking of large numbers of leukocytes (55, 76, 81, 109, 116, 129, 165). Finn first hypothesized that menstruation was an “inflammatory event” over thirty years ago (81) and these phenomena in the menstrual endometrium are analogous to features of classical inflammation observed at other body sites.

Important molecular and cellular events accompany the morphological changes in the endometrium, and these include the focal activation of matrix metalloproteinases (MMPs) in regions of menstrual lysis (87, 144, 241) and the increased local endometrial expression of inflammatory mediators, for example, cyclooxygenase-2 (COX-2; (57, 119)), cytokines/chemokines (for example, IL-8, CCL-2 (57)) and an increase in local endometrial prostaglandin synthesis (222, 228). The nuclear factor (NF)κB pathway and E series of prostaglandin receptors and associated signalling pathways are modulated by progesterone withdrawal and implicated in the regulation of menstruation (see later in Section 7). There is evidence of perturbation of some of

these pathways in women who experience aberrant menstruation, most often experienced as heavy menstrual bleeding (HMB) (222).

3. Sex steroid regulation of the endometrium and a role for local glucocorticoids.

As well as ovarian-derived estradiol and progesterone regulating endometrial form and function, there is evidence that locally generated steroids, including other estrogens (96), androgens (97) and glucocorticoids, play important roles in endometrial function (159, 193).

The target cells for androgens are endometrial stromal cells. These cells are androgen receptor (AR)-positive in the functional layer during the proliferative phase and in the basal compartment throughout the menstrual cycle (97, 148). The AR is downregulated in endometrial stromal cells in the functional layer during the secretory phase, and upregulated in endometrial epithelial cells as circulating progesterone concentrations fall with the demise of the corpus luteum (pregnancy absence).

Locally generated glucocorticoids limit inflammation at other tissue sites. These effects are mediated by cortisol binding to the nuclear glucocorticoid receptor (GR) (42). In the endometrium GR is immuno-localized to stromal and endothelial cells, and uterine NK cells (107). The enzyme 11beta-hydroxysteroid dehydrogenase type 1 (11β-HSD1) produces cortisol by the enzymatic reduction of cortisone, and the reverse reaction is catalysed by 11β -hydroxysteroid dehydrogenase type 2 (11β-HSD2) (73).

Glucocorticoids are reported to inhibit angiogenesis (141, 221). Studies have described the endometrial expression of 11β-HSD1 which is upregulated at menstruation and coincides with maximal concentrations of GR mRNA in endometrial tissue homogenates (159). It is of interest that there is enhanced local inactivation of cortisol by 11β-HSD2 in the endometrium of women with HMB (193). In this study the level of *HSD11B2* mRNA was observed to be 2.5-fold higher in women with HMB when compared to women with objectively measured normal menstrual blood loss. This led to the hypothesis of lower local endometrial cortisol concentrations occurring in women with heavier menstrual bleeding experience. Inactivation of cortisol by 11βHSD2 may thus cause local endometrial glucocorticoid deficiency and hence increased angiogenesis and impaired vasoconstriction. Subsequently this observation has been translated in a double-blind response-adaptive parallel-group placebo-controlled trial (248). Therein, 'rescue' of luteal phase endometrial glucocorticoid deficiency in order to reduce menstrual bleeding has been explored with short-term administration of the oral synthetic glucocorticoid dexamethasone, which is relatively resistant to 11βHSD2 inactivation (ClinicalTrials.gov NCT01769820; EudractCT 2012-003405-98).

Estrogen receptor (ERα isoform) is located in nuclei of endometrial epithelial and stromal cells throughout the estrogen-dominant proliferative phase. Concentrations of ERα decline in epithelial and stromal cells of the endometrium through the progesterone-dominant secretory phase of the menstrual cycle, while progesterone receptor (PR) concentrations are maintained in the endometrial stroma, but decrease in the glandular epithelium (58, 60, 136, 224).

285

286 The progesterone receptor (PR) is located in nuclei of epithelial and stromal cells
287 during the proliferative phase. PR exists in at least two isoforms, PRA and PRB,
288 acting as transcriptional regulators of progesterone responsive genes (102, 103).
289 PR expression exhibits well described temporal and locational expression patterns
290 (136, 224). PR persists in the stromal compartment of the functional layer during the
291 secretory phase, particularly in the perivascular region.

292

293 Progesterone receptor expression is consistent with the functional roles for
294 progesterone at this time, *i.e.* differentiation and decidualization of the stromal cell
295 compartment in preparation for pregnancy. Immunohistochemical analysis of human
296 endometrium has revealed that PRA isoform is predominant during the secretory
297 phase, whereas the PRB isoform has been reported to decline in both stromal and
298 glandular cells during the latter half of the menstrual cycle (32, 167, 246). The
299 endometrial stromal cell thus remains responsive to progesterone throughout the
300 secretory phase. In contrast, the basal endometrial layer of the endometrium has
301 persistent PR expression across the menstrual cycle (136, 224).

302

303 Gene-microarray-based studies in both the human and the murine endometrium
304 have been reviewed in Dey et al. (2004) (67). Analyses of mid-secretory phase
305 endometrium, treated *in vitro* decidualized stromal cells, *ex vivo* progesterone and
306 PR antagonist-treated endometrium, and uterine tissues derived from PR-deficient
307 mice have all revealed a host of progesterone responsive genes with potential to
308 retain PR expression and exhibit maximal response to progesterone. In the context
309 of corpus luteum demise and preparation for menstruation, this primes the

endometrium to respond to progesterone withdrawal. Detailed reviews of ER and PR molecular biology and roles in female reproduction, particularly in the context of implantation, are available (20, 48, 188).

4. Endometrial cell heterogeneity.

There is an exquisitely coordinated interplay between circulating ovarian steroid hormones and their cognate receptors, with the multiple cell types (143, 152) that compose the endometrium. The consequence is function that is tightly regulated in both “time and place”, and when these endocrine-target cell communications are disturbed then common clinical consequences present, such as disorders of menstrual bleeding or problems associated with implantation.

Thus given the heterogeneous nature of the endometrium with its multiple cellular components, it is important to recognize that each endometrial cellular component, *i.e.* epithelial, stromal, vascular and immune, exhibits a unique gene expression profile. The cellular composition of endometrial samples therefore needs careful consideration when ”-omics” studies are undertaken involving whole-tissue samples. The data concerning, for example, endometrial receptivity associated gene expression profiles continues to be refined especially as technology and use of diagnostic platforms advances (68). The classic histological characteristics of the endometrium (181) at the specific time of endometrial sampling/ study have been demonstrated to impact upon tissue gene expression profiles (7, 36, 232). More recent data describe important alterations in the epithelial and stromal components between pre-receptive and receptive phases of the endometrium (229). Consequently, endometrial gene expression analysis is now a preferred gold

standard for prediction of the window of implantation (WOI) (7, 229). It should be noted however that the proportions of each cell type in an endometrial biopsy, which in turn will reflect the variation in proportions of cell components across the menstrual cycle, may influence data on gene expression profiles derived from whole tissue gene expression studies (229). The new era of single cell transcriptomics will herald exciting insights into endometrial function at the time of menstruation and implantation (242).

5. Modelling menstruation in the mouse.

Menstruation is a phenomenon that occurs naturally in only a few mammals. It is limited to women, old world primates, fruit bats, the spiny mouse and the elephant shrew. Study of human endometrial samples reveals important observational data regarding the menstrual process and its regulation. However, patient variability and disruption of the normal endometrial architecture during sampling may limit findings. To truly delineate causation, an animal model is required. Old world primate models have provided a significant contribution to our knowledge of endometrial function (see below) but are expensive and require large numbers to combat inter-animal variations (176). Current guidelines identify issues surrounding research and welfare of working with non-human primates. Mouse models are attractive due to genetic homogeneity, readily available reagents and relatively low cost. Three broad categories of mouse models of menstruation are currently available to researchers in this field.

The mouse model of simulated menstruation, already referred to herein, was originally developed by Finn and Pope in the 1980s (83). Refinement of the model's

reproducibility did not follow until some 20 years later. The prototype mouse model (83) utilized sequential administration of estradiol and progesterone injections in ovariectomized mice to prime the endometrium for artificial decidualization. This latter step involved an intra-uterine arachis oil injection to stimulate endometrial decidualization. The Finn and Pope (1984) model was highly successful, as usually only one mouse in six failed to exhibit a decidualization response at the end of the protocol. However due to the variability in response and the poor reproducibility concerning timing of decidualization responses it took two decades for this model to return to wider use and interest. In 2003, Brasted *et al*. (24) introduced the use of subcutaneous progesterone-releasing Silastic pumps (developed earlier by Cohen and Milligan; 1993 (46)), in place of progesterone injections. This use of Silastic implants to control the release of progesterone provided a method for the rapid withdrawal of progesterone from the uterus with surgical removal of the hormone pump. This step improved the reproducibility of the timings of the simulated menstrual events which follow progesterone withdrawal in this model. As a consequence of progesterone withdrawal, the endometrial environment is subject to a host of histological and molecular changes analogous to those observed morphologically in the human endometrium at the time of menses where there is shedding of the decidualized endometrium, recruitment of leukocytes (160) and visible menstrual-like bleeding (54, 201). Subsequently, all endometrial tissue is repaired, remodelled and regenerated. **(Figure 3).**

The critical requirement for a “decidualization step” in the simulated mouse model of menstruation was established during the development of this model of simulated menstruation, as progesterone withdrawal alone was shown to be insufficient for

induction of endometrial bleeding (24, 54, 83, 160, 201). The injection of arachis oil into the uterine lumen provided an induced injury during a prevailing endocrine environment of high systemic progesterone and resulted in pre-implantation decidualization of the mouse endometrium. This artificially created event was analogous to the naturally occurring mid-secretory phase phenomena in both the non-human primate (rhesus macaque) and women. This mouse model of simulated menses reinforced the importance of a decidualization step prior to progesterone withdrawal in the physiology of menstruation.

The further refinements of Brasted *et al.*'s (2003) mouse model of simulated menstruation has provided a model for the study of important features of human menstruation. These features include expression of inflammatory mediators, factors influencing endometrial/ menstrual bleeding and endometrial repair, (45, 54, 122, 123, 125, 160, 201, 254).

This model has been used to manipulate the inflammatory response (122) and hormonal control (123) at menstruation, and pharmacologically and genetically alter key factors involved at menses (157). These studies have confirmed and extended findings in human tissue, giving mechanistic insights into the regulation of menstruation and endometrial repair. The parallel observations reported in the 'peri-menstrual' human endometrium, and observations in the progesterone withdrawn mouse endometrium in the 'simulated menses model' **(Figure 3)**, provide support for the mouse model and ability to recapitulate events of human menstruation (11).

A xenograft model of menstruation also allows *in vivo* examination of the menstrual process (51, 105). In this model, fragments of human endometrium from the functional layer are xenografted to ovariectomized, immune-deficient mice. Treatment with estradiol and progesterone followed by removal of ovarian steroids resulted in menstrual breakdown of the xenografted human endometrium. The clear advantages of this model are the ability to standardize the hormonal variations to which human endometrium is exposed, and to treat and manipulate the endometrium in a way that would be unethical in human subjects. It also allows examination of the local versus systemic leukocyte response by identification of human and mouse cell contributions. However, transplantation of human endometrium will disturb the normal endometrial architecture and may alter vascular and immunological responses at menses. In addition, immune-deficient mice are required for transplantation, further modifying the local endometrial environment.

More recently the common or Egyptian spiny mouse has been identified as a novel model for menstruation (17). The spiny mouse was found to undergo spontaneous decidualization and menstruation with a cycle of an average of 3 days duration every 6-10 days. Prior to menses, endometrial transformation of the stroma corresponding to spontaneous decidualization was observed. As this rodent is susceptible to obesity and diabetes mellitus and has been previously studied as an animal model for these conditions, a limited selection of laboratory reagents is already available. The potential advantage of this mouse over the simulated mouse models is that it may permit study of multiple/ successive menstrual cycles and any pre-conditioning effects that menstrual cycles will have on endometrial physiology. In addition, like women, the spiny mouse produces cortisol as its circulating glucocorticoid, rather

than corticosterone in standard laboratory mice. The spiny mouse also appears to exhibit spiral arteriole remodelling in the decidualized endometrium (16), suggesting it may provide a model for vascular disorders of pregnancy, such as pre-eclampsia. Disadvantages include the variability in natural cycles that makes human and natural primate studies more difficult, and the current inability to genetically manipulate this mouse, limiting definitive mechanistic studies.

6. Modelling menstruation in the non-human primate.

Non-human primates are amongst the animal species that menstruate and undergo spontaneous endometrial decidualization. Several studies provide good evidence for the rhesus macaque as a model for human menstruation. The rhesus macaque and women share many molecular and histological similarities in the endometrium during the peri-menstrual window (luteo-follicular transition) and during menstruation. Both species display tightly coordinated spatially and temporally regulated increased levels of MMPs (27, 200) and VEGF expression during the menstrual phase (175), and these observations are preceded by progesterone withdrawal (28-30, 60, 175, 218, 220).

Although the similarities between humans and macaques support non-human primate studies for exploration of mechanisms underpinning menstruation, novel therapeutic approaches for management of menstrual complaints, and contraception strategies, there are ethical considerations concerning use of primates for research, and the monetary costs associated with their maintenance impose limits on the macaque's utility as a model for this aspect of women's health.

Alternative models have therefore been explored and the mouse models of human menstruation described above are attractive options for modelling and experimentally manipulating the events of menstruation.

7. Progesterone withdrawal: the trigger for menstruation.

The human endometrium is a physiological tissue site of repeated episodes of “injury and repair” (menstruation). The fall in circulating progesterone levels due to corpus luteum demise is the trigger for menstruation in women. Support for the crucial role of progesterone withdrawal in menstrual physiology is provided by studies in women where progesterone receptor (PR) antagonists administered during the secretory phase simulate the events of menstruation. The administration of the PR antagonist, RU486 (mifepristone) in the mid-secretory phase increased endometrial inflammatory mediators, including, cyclo-oxygenase (COX-2), nuclear factor (NF)κB and interleukin (IL)-8 (CXCL8; (56, 57).

Further support for the role of progesterone withdrawal and the induction of menstruation has been derived from contemporary studies in the non-human primate (rhesus macaque) (158, 175). In these non-human primate studies the menstrual cycle and menstrual bleeding were induced by the surgical removal of both ovaries followed by 14 days of estrogen priming prior to insertion of a progesterone implant in order to mimic the endometrial secretory phase. The maintenance of estradiol over the period of progesterone withdrawal (with progesterone implant removal) provided evidence for the dominant effect of progesterone withdrawal over that of estradiol withdrawal for the induction of menstruation.

484 The local endometrial events that follow progesterone withdrawal in women have
485 been considered to occur in two phases (128) **(Figure 4).** The first phase following
486 progesterone withdrawal is associated with increased local exposure of the
487 endometrium to cytokines and prostaglandins (PG) and is dependent upon an
488 efficient response of the decidualized perivascular stromal cells to declining levels of
489 progesterone, an anti-inflammatory hormone (39, 77). Chemokines, specific chemo-
490 attractant cytokines, are responsible for leukocyte traffic in the endometrium
491 (recruitment, migration and activation). The four sub classes of chemokines are
492 defined by the structure of their amino-terminal cysteine motif: C, CC, CXC and
493 CX_3C. Published data concerning *in vitro* studies of decidualized human endometrial
494 stromal cells describe the induction of inflammatory mediators following
495 progesterone withdrawal, including IL-6, the chemokines CCL11, CCL2, CXCL10,
496 CXCL8 and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF). This
497 cellular response to *in vitro* progesterone withdrawal and the induction of
498 inflammatory mediators is orchestrated by the actions of the NF-κB transcription
499 factor ((127); and reviewed in Evans and Salamonsen 2014 (77, 130).
500
501 NF-κB, is an inducible transcription factor that regulates expression of genes
502 involved in the inflammatory response. The NF-κB family comprises five members,
503 NF-κB1(p50), NF-κB2 (p52), RelA (p65), RelB and c-Rel which mediate target gene
504 transcription by binding to specific DNA elements as hetero- or homo-dimers (230).
505 NF-κB proteins are normally retained in the cytoplasm of cells in an inactive form by
506 inhibitory proteins, including IκB. Upon activation by extracellular stimuli, such as, IL-
507 1 and TNF (184), ubiquitin-dependent degradation of IκB by the proteasome occurs
508 and this leads to translocation of NF-κB to the nucleus. Activation of NF-κB involves

several protein kinases and subsequent degradation of an endogenous inhibitor, IκBα.

In the context of menstruation and endometrial function, progesterone has an inhibitory effect upon the NF-κB pathway (128, 130) by increasing IκB or by competing for NF-κB gene recognition sites. Withdrawal of progesterone following corpus luteal regression removes the inhibition and results in increased NF-κB activity and consequent endometrial cytokine and chemokine production. The NF-κB transcription factor is also reported to be important for control of matrix metalloproteinases (MMP)-1, MMP-3 and MMP-9 production (43), thereby providing a pathway by which progesterone-withdrawal promotes endometrial MMP activity.

The second phase of local endometrial events following progesterone withdrawal **(Figure 4)** is a consequence of increased cytokine production and is followed by an influx of innate immune cells into the local endometrial environment and accompanied by activation and release of MMPs with destruction of the extracellular matrix (ECM). This second phase is considered to be independent of progesterone receptor actions (**Figure 4**): a hypothesis which has been subsequently supported by studies in the ovariectomized non-human primate (rhesus macaque) and in a mouse model. In the non-human primate, progesterone implants were removed at the end of the simulated menstrual cycle and thereafter replaced at staggered time-points from 12 to 72 hours after the initial P-implant withdrawal (218). The study in the macaque demonstrated that replacement of progesterone up to 24 hours after withdrawal prevented menstruation and also prevented the expected increases in endometrial MMP1,-2 and -3. In contrast, progesterone replacement 36 hours after

earlier removal had no effect on menstrual bleeding and only partially blocked the production of MMPs with notably less endometrial MMP2. Replication of the ability to block local endometrial progesterone withdrawal events in a mouse model of simulated menstruation (247) has also been reported. These more recent mouse studies have also demonstrated a “temporal progesterone deprived threshold” (247), during which time period the event of menstrual bleeding is inevitable.

The decidualized stromal cells characteristic of the pre-menstrual endometrium are a key component of endometrial sensitivity to circulating hormones. This may offer a mechanism whereby progesterone dependent decidualized endometrial stromal cells (which express the PR) influence the recruitment of innate immune cells prior to menses. In support, a study by Evans and Salamonsen (77) mimicked the luteal phase by decidualizing cells over a 12 day period of exposure to estradiol and progestin (medroxyprogesterone acetate; MPA). Subsequent hormone withdrawal demonstrated an anticipated increase in NF-κB, along with an expected production of inflammatory cytokines and chemokines. The increase in inflammatory mediators was abrogated with administration of a NF-κB inhibitor.

8. Menstruation as inflammation.

Inflammation refers to a generic response mounted by the immune system to noxious external stimuli in order to eliminate infection, regenerate injured tissue and restore physiological function. Inflammation is classified into ‘acute’ and ‘chronic’ responses. The former is a short-lived, initial response to injurious stimuli and the latter is a prolonged response that involves the persistence of certain immune cells at the site of inflammation alongside host-mediated tissue destruction and/or fibrosis,

559 ultimately risking a loss of function (34). Throughout the body it is critical that
560 inflammation is efficiently resolved upon successful removal of noxious stimuli, in
561 order to limit tissue damage and to restore tissue integrity and function. At a cellular
562 level, acute inflammation involves rapid recruitment of neutrophil granulocytes,
563 followed shortly thereafter by inflammatory monocytes which differentiate into
564 macrophages, proliferate *in situ* and orchestrate the continuing inflammatory
565 response and its resolution (98). Molecular mediators of acute inflammation
566 produced locally and by recruited leukocytes include prostanoids (prostaglandins
567 and prostacyclins), lipid mediators, complement, cytokines and chemokines. The
568 inflammatory mediators, which are produced locally in the endometrium during the
569 luteo-follicular transition, *i.e.,* during the time of progesterone-withdrawal-induced
570 endometrial shedding (menstruation), may act individually or in concert to determine
571 the inflammatory response by inducing vasodilation, modifying cell adhesion
572 molecule expression and ultimately recruiting leukocytes (50, 57, 88, 120, 162, 212).
573
574 The resolution of inflammation in the endometrium is no less tightly coordinated than
575 the inflammatory response elsewhere. As at other tissue sites in the body the
576 synthesis of pro-inflammatory mediators is curtailed and existing inflammatory
577 mediators are degraded in order to limit leukocyte recruitment and restore vascular
578 integrity. Endometrial prostanoids are catabolized via a series of oxidation and
579 reduction reactions (8, 75), and the clearance of chemokines is orchestrated through
580 chemokine receptor dependent scavenging by apoptotic leukocytes (10). The local
581 resolution of inflammation also involves the phagocytosis of apoptotic neutrophils.
582 The recognition of apoptotic cells by macrophages during phagocytosis modulates
583 macrophage phenotype and promotes the release of anti-inflammatory and pro-

584 resolution cytokines, such as IL-10 and TGF-β (114), along with lipid mediators, for
585 example, lipoxins and resolvins (34).
586
587 The utility and validity of the mouse model of menstruation to human menstruation
588 has been described in a recent study where the events of apoptosis and
589 inflammation in the human endometrium across the peri-menstrual window, have
590 been characterized in endometrium from women for menstrual phase and bleeding
591 status, alongside study of the mouse endometrium following progesterone-
592 withdrawal in a mouse model of induced menstruation (11). The spatial and
593 temporal regulation of apoptosis in the normal human endometrium were
594 demonstrated to be recapitulated in the endometrium of the mouse model of induced
595 menses, as endometrial apoptosis (as assessed by cleaved caspase-3 expression)
596 was extensive prior to the onset of breakdown, shedding and bleeding in both
597 women and mice.
598
599 Furthermore increases in menstrual-phase inflammatory chemokine transcription
600 (CXCL8, CCL2, Cxcl1) and in inflammatory cytokine transcription (TNF, IL6, Tnf)
601 were observed in both the well-characterised normal human endometrium, and
602 recapitulated in the endometrium of the mouse menstruation model (11). These
603 increases in chemo-/cytokine(s) expression in response to progesterone-withdrawal
604 in human and mouse were accompanied by substantial increases in neutrophil
605 numbers upon the onset of menstruation/ simulated mouse menses. A similar
606 leukocyte response with mononuclear phagocytes localising to areas undergoing
607 active repair has also been described in the mouse model of menses (53).
608

609 Menstruation is a physiological model of self-limiting inflammation. The menstrual
610 endometrium displays many classic hallmarks of inflammation, including tissue
611 oedema and influx of immune cells. The inflammatory process that occurs in the
612 endometrium at menstruation is tightly regulated to prevent loss of function (55, 116).
613
614 The proposal that menstruation is an inflammatory event (81) has been further
615 supported over the past three decades by many important discoveries pertaining to
616 endometrial molecular and cellular changes that accompany inflammation and
617 include: focal activation of MMPs; (87, 144, 241)), increased COX-2 expression (57,
618 119), increased prostaglandin concentrations (228) and involvement of local tissue
619 hypoxia (52, 59, 93, 154, 157); see later.
620
621 An upregulation of local endometrial inflammatory chemokines and cytokines
622 coincides with the withdrawal of progesterone. There are well documented
623 increases in CCL2 (MCP-1), CXCL8 (IL-8), IL-6, TNF and cyclooxygenase-2 (COX-
624 2) expression in the late secretory and menstrual phases of the menstrual cycle (11,
625 57, 119, 162).
626
627 In the human endometrium, CXCL8 mRNA concentration and protein (9, 154) are
628 present across the menstrual cycle in stromal cells (9, 154), perivascular cells and
629 expression is maximal following progesterone withdrawal (162). Maximal
630 endometrial transcription and expression of the cytokine IL-6 across the menstrual
631 cycle has been reported in the late secretory phase (243) with the protein localized
632 to endometrial glands and secretions (244). TNF also likely plays a role in
633 menstruation and has been described in human endometrial stromal and epithelial

cells (231). Levels of endometrial *TNF* mRNA are described as increased during the late secretory phase (243). In none of these earlier studies however were menstrual-phase samples studied. More recently Armstrong et al reported increased *CCL2*, *CXCL8*, *IL6* and *TNF* transcription in menstrual phase human endometrium (11) from women with objectively measured normal menstrual blood loss. These findings were recapitulated in a simulated model of mouse menses, *i.e.,* increased transcriptional changes in *Tnf* (TNF) and in the neutrophil chemokine gene *Cxcl1* (KC; GRO-α). CXCL1 is the mouse homologue of the human CXCL8 (183, 225).

The chemokines maximally produced in association with menstruation perform a range of functions, not only recruiting leukocytes but also contributing to local cell proliferation and angiogenesis. For example, CXCL8 (IL-8), alongside its well-documented leukocyte chemotactic properties, is active in local angiogenesis (131) and the induction of chemotaxis and proliferation of vascular smooth muscle cells (255).

9. Endometrial Leukocyte Traffic.

Endometrial leukocytes play crucial roles in endometrial function including not only the breakdown of endometrial tissue, but also endometrial repair (204) and embryo invasion (214). Leukocytes recruited during the peri-menstrual window are integral components of the endometrial repair process. The pivotal role for local endometrial immune cell population(s) has been demonstrated by the finding that the depletion of neutrophil granulocytes retards endometrial repair in a mouse model of simulated menstruation (122). It is of interest too that in a mouse model of myocardial injury,

wound healing was disturbed by the depletion of macrophages: a similar situation might reasonably be inferred if macrophages are depleted or deficient at the time of human menstruation, albeit this phenomenon is yet to be demonstrated (240).

It is possible that local availability of bioactive glucocorticoids also plays a role in immune cell vascular cell interactions in the endometrium during tissue repair at menstruation, and such action may be either direct or indirect via the functions of tissue resident macrophages (235). Endometrial macrophages express the glucocorticoid receptor but not the progesterone receptor (235). There are data that draw attention to the importance of local cortisol in regulating paracrine actions of macrophages in the human endometrium. *In vitro* studies have demonstrated that culture with supernatants from cortisol-treated peripheral blood monocyte-derived macrophages alters endometrial endothelial cell transcription of angiogenic genes, e.g. *CXCL2*, *CXCL8*, *CTGF*, and *VEGFC*. CXCL2 and CXCL8 protein has also been detected in endometrial macrophages *in situ* (235). The endometrial expression of these factors was observed to be maximal during the menstrual phase and this is consistent with these factors playing a role in endometrial repair at this time.

Endometrial leukocyte populations vary in number across the menstrual cycle (152). Original reports described the endometrial leukocyte population as varying between 8.2% (35) and 10 – 15% (126) of the endometrial stromal cell compartment in the proliferative phase when leukocyte numbers are lowest. In contrast immune cell numbers vary from 20 – 25% (126) to 40 – 45% of the endometrial stromal cell population immediately before onset of menstruation (23, 203) when leukocyte numbers are most abundant. There are however very significant challenges in

684 quantification of endometrial immune cell populations owing to the major impact of
685 endocrine ovarian cycle stage along with vast heterogeneity in cell distribution
686 patterns in the multicellular endometrium. Attempts to address challenges of
687 quantification of specific immune cell types in the endometrium, for example, uterine
688 NK cell measurements are reported and have relevance for clinical care (135). Such
689 measurements are undertaken in women in the context of infertility care but do not
690 have known relevance for menstruation/ menstrual bleeding complaints. Any
691 meaningful clinical test for uNK cell measurements, whatever the clinical context
692 would require a standardised method in order establish normal ranges for uNK cells.
693 This will be a challenge due to the heterogeneous localisation of uNK cells
694 throughout the endometrium and, as noted above, menstrual cycle related increases
695 in cell numbers.
696
697 Neutrophil granulocytes are recruited into the endometrium in substantial numbers
698 immediately prior to menstruation (191) and this leukocyte recruitment is coincident
699 with progesterone withdrawal (11). Neutrophils contain high levels of MMPs and
700 endometrial neutrophils may activate MMPs *in situ* (88), thereby contributing to
701 endometrial breakdown at menstruation.
702
703 Macrophages are present in the endometrium throughout the menstrual cycle and
704 increase modestly in number with onset of menstruation (11, 152, 203, 234).
705 Phagocytic clearance of apoptotic cells by macrophages is necessary for the
706 resolution of inflammation at other tissue sites (206), and hence is likely to be
707 important for resolution of menstrual-related inflammation in the endometrium. Post-

menstruation, macrophages clear cellular debris (92) and contribute to the remodelling and repair of the functional layer of the endometrium (156).

Macrophage populations in the endometrium are considered to represent contributions from two distinct sources, these being, *in situ* proliferation of resident macrophages (64, 118, 161) and macrophages derived from monocytes recruited into the tissue (70, 209). Macrophages are known to proliferate *in situ* in other inflammatory contexts, such as in the presence of IL-4 in Th2 inflammatory responses (118) . In the context of menstrual endometrium, evidence for *in situ* proliferation comes from experiments in which human endometrium was xenografted into immunocompromised mice (105). The mice were subjected to a hormone environment simulating the human menstrual cycle, and human macrophage numbers were highest on day 28 of this simulated cycle. Tissue-resident human endometrial macrophages express the β-isoform of the oestrogen receptor (104) and GR immunoreactivity is present in macrophages in the human endometrium (235).

Among the chemotactic stimuli by which monocytes are brought into the endometrium is CCL2 (MCP-1), which is highly expressed in late-secretory-phase endometrium (119). Endometrial macrophage populations are reported to be relatively stable across the menstrual cycle, with numbers increasing only in the late secretory/menstrual phase and early proliferative phase of the menstrual cycle (25). It is of note that the authors of this study combined tissue samples of the late secretory and menstrual phases into a single group. In a more recent study (47) macrophage abundance was described as significantly increased only during the menstrual phase.

Armstrong and colleagues characterized patterns of macrophage localization across the peri-menstrual window (luteo-follicular transition), and described innate immune cell trafficking towards apoptotic glands *(***Figure 5**) and subsequent movement outward into the stroma (11). (**Figure 6**).

Resident and recruited leukocyte populations are therefore critical to endometrial breakdown via expression and activation of MMPs, and to its repair, modulating local expression of angiogenic factors and clearing cellular debris. Numbers of these leukocytes, among which neutrophils and macrophages are the most abundant, are highest before and during menstruation.

10. Mesenchymal-epithelial transition as a critical factor in determining endometrial function.

The repeated (physiological) repair and remodelling characteristic of the human endometrium has been the subject of recent attention in the context of mesenchymal-epithelial transition (MET) and epithelial-mesenchymal transition (EMT). The pivotal physiological event of menstruation is an excellent example where transition between a mesenchymal and epithelial cell phenotype is required for successful endometrial function. A recent review of the topic details the historic perspective of studies pertaining to transition of cell phenotypes in the context of reproduction (185) and background studies in the field of wound healing. The menstruating endometrium is after all a physiological example of an injured "wounded" surface that is required to rapidly repair each month.

Both mesenchymal and epithelial cell markers have been characterized. The phenotype of a mesenchymal cell is spindle shaped, multipolar, with invasive and migratory properties. In contrast the epithelial phenotype is that of a polygon, with apico-basolateral polarization, and cell-to-cell adhesion properties. Mesenchymal markers include, vimentin, fibronectin, Snail 1 and 2, smooth muscle actin (137, 185) and N-cadherin, epithelial markers include, cytokeratins (8,9,18) and E-cadherin (137, 185). In the context of endometrial regeneration, N-cadherin, has been reported as a specific surface marker for endometrial epithelial progenitor cells studied in vitro. Furthermore in the same study, examination of full-thickness (lumen to endometrial-myometrial junction) hysterectomy sections revealed that N-cadherin was a marker for such progenitor cells in the glands within the basal layer of the endometrium (178).

At menstruation the upper functional layer of endometrium is shed. As described earlier this event is triggered by progesterone withdrawal and a coordinated sequence of pro-inflammatory events (55, 152, 202, 204). Endometrial regeneration follows and evidence is increasing for the existence of stem/ progenitor cell populations located in both the upper functional layer as well as the lower basal layer close to the endometrial/ myometrial junction (41, 90, 149, 186), and in perivascular locations in both the basal and functional layers. Regeneration of the endometrium thus requires several types of stem/ progenitor cells including circulating cells derived from the bone marrow (5, 71, 233). A stem cell population derived from menstrual effluent has also been characterized and reported to exhibit angiogenic and inflammatory properties (6, 187). Cells from the endometrial functional layer are also present in menstrual effluent. The transcriptome from laser captured cells

783 derived from the endometrial functional and basal layer has been described (87).
784 This transcriptome study identified gene products associated with tissue degradation
785 (for example, matrix metalloproteinase and plasmin systems) along with gene
786 products of apoptosis. Cells from the functional layer were enriched in gene
787 products associated with extracellular matrix biosynthesis (collagens and their
788 processing enzymes). These data are consistent with the hypothesis that cell
789 fragments of the functional layer contribute to endometrial regeneration during late
790 menstruation. Further discussion on endometrial stem/ progenitor cells lies beyond
791 the scope of this review (65, 90).
792
793 The role for MET during endometrial regeneration has been studied in a mouse
794 model of simulated menstruation (54). In this model decidualization is artificially
795 induced and progesterone withdrawn by removal of an implant. The mouse
796 endometrial stromal cells located close to areas of luminal epithelial repair were
797 identified as expressing both epithelial and stromal cell markers. Furthermore, and
798 in support of an underlying MET process involved in endometrial regeneration,
799 mesenchymal markers were noted to decrease and markers of epithelial cells to
800 increase (54).
801
802 **11. Local mechanisms that limit normal menstrual bleeding: vasoconstriction;**
803 **role for hypoxia; vascular and epithelial repair and hemostasis.**
804 As already highlighted, progesterone withdrawal induces many classic hallmarks of
805 inflammation in the endometrium. The influx of innate immune cells and release of
806 matrix metalloproteinases results in shedding of the functional layer at menstruation.
807 This endometrial injury must be tightly regulated and rapidly repaired to limit

menstrual blood loss. Curtailment of endometrial bleeding (menstruation) thus requires timely resolution of inflammation, vasoconstriction of specialised endometrial spiral arterioles, an efficient hemostatic response including repair of damaged vasculature, luminal re-epithelialization of the remnants of the denuded basal endometrium (an injured mucosal surface) and stromal expansion (151, 152, 202). The unique, scarless repair process associated with post-menstrual repair is essential to ensure fertility potential is maintained and menstrual bleeding is limited. However, the mechanisms and regulation of endometrial repair remain poorly understood (157).

Close to 80 years ago classic experiments in a non-human primate model explored the physiological roles of hypoxia and sex steroid withdrawal (145). Autologous transplants of rhesus macaque endometrium into anterior eye chamber permitted visualisation of the events of menstruation directly through a slit lamp ophthalmoscope. Transient and intense vasoconstriction of the spiral arterioles and focal bleeding was observed 4 to 24 hours prior to menstruation, following steroid (progesterone) withdrawal. These observations were later revisited over 30 years ago by Baird and colleagues (4), where endometrium was transplanted into the hamster cheek pouch, an immune-privileged site, enabling direct observation of the impact of addition and withdrawal of steroids on blood vessel vasoconstriction and vasodilatation.

Since these publications, the crucial role of hypoxia in the endometrium has continued to be a subject of intense debate. The literature in this field has been inconsistent and utilized a variety of *in vitro*, *ex vivo* and *in vivo* models. As the

radius of a blood vessel is the major determinant of resistance to flow, even a small increase in vessel radius will have a significant impact on flow, *e.g*. a two-fold increase in vessel radius leads to a sixteen-fold decrease in resistance to flow (153). **(Figure 7A)**. Efficient vasoconstriction of endometrial arterioles is necessary to limit menstrual blood loss.

Endometrial mediators of vasoconstriction

Decreased vasoconstriction at menses may be due to defective production of vasoactive factors following progesterone withdrawal. Prostaglandin (PG) F2α and endothelin-1 (ET-1) are two endometrial factors with known vasoconstrictive properties (15, 147). Women with heavy menstrual blood loss have been shown to have a significantly decreased PGF2α/PGE2 ratio (223) and decreased prostaglandin F (FP) receptor expression (222). Excessive PGE2 production at the expense of PGF2α may result in less constriction of the spiral arterioles prior to menstruation. Women with HMB have also been shown to have decreased endometrial expression of the potent vasoconstrictor ET-1 and increased expression of its metabolizing enzyme, neural endopeptidase (147). **(Figure 7B)**.

Alternatively, decreased vasoconstriction at menstruation may be due to aberrant spiral arteriole maturation throughout the preceding menstrual cycle. Vessel wall circumference and focal discontinuities have been noted to be larger in the endometrium of women with HMB than normal controls (163). Women with heavy bleeding had significantly reduced vascular smooth muscle cell proliferation in spiral arterioles during the mid-late secretory phase when compared to normal controls (2). Furthermore, smooth muscle myosin heavy chain, a contractile protein used as a

858 marker of vascular smooth muscle cell maturation, has also been reported to be
859 significantly decreased in spiral arterioles of women with HMB (1). Additional data in
860 this context comes from study of smooth muscle content and differentiation stage of
861 vascular smooth muscle cells (VSMCs) in endometrial blood vessels in women with
862 normal and reported heavy menstrual bleeding (21). Therein, expression of the
863 VSMC differentiation markers, smoothelin and calponin, was observed to be
864 dysregulated in endometrial blood vessels in women with heavy bleeding.
865 Smoothelin and calponin are regulators of vascular tone and vascular contractility
866 (21). Endometrial arteriogenesis requires coordinated maturation of VSMCs,
867 underlying endothelial cells (ECs) and surrounding extracellular matrix (ECM).
868 Spatial and temporal differences in protein levels of EC markers and components of
869 the ECM in endometrial vessels and superficial myometrium have also been
870 reported in women with HMB (22). Such vascular abnormalities may result in
871 suboptimal vasoconstriction at menses and significantly increase menstrual blood
872 loss **(see Figure 7B)**.
873
874 Decreased vasoconstriction of specialized endometrial spiral arterioles will thus not
875 only impact on menstrual blood flow but may also increase duration of bleeding due
876 to delayed endometrial repair. Indeed, women with HMB have been reported to
877 bleed for 2 days longer (157). The processes involved in vascular and epithelial
878 repair are discussed below.
879
880 *Endometrial hypoxia*
881 In addition to its impact on blood flow, vasoconstriction at menstruation may
882 contribute to induction of hypoxia in the menstrual endometrium. Markee's studies of

endometrial explants in the anterior chamber of the rhesus monkey suggested that the vasoconstriction observed was consistent with the presence of hypoxia in the menstrual endometrium (145). This led to the hypothesis that hypoxia was required for the initiation of menstruation. However, subsequent *in vitro* and *ex vivo* studies demonstrated menstrual breakdown occurred despite the absence of hypoxia (86, 256).

Hypoxia Inducible Factor (HIF) is the master regulator of the cellular response to hypoxia, with well-described roles in mitogenesis, angiogenesis, apoptosis, inflammation and metabolism at other tissue sites in the body (210, 211). It is composed of two subunits: the alpha subunit, rapidly degraded when oxygen is abundant, and the beta subunit, constitutively expressed (210). In hypoxia, αβ dimerization occurs and HIF translocates to the nucleus to induce transcription of genes with hypoxic response elements, including those involved in angiogenesis, energy metabolism and tissue remodelling. All the latter processes underpin menstruation.

In the presence of abundant oxygen, HIF-1α, the alpha subunit is hydroxylated by prolyl hydroxylase (PHD) enzymes. These enzymes initiate rapid degradation of HIF-1α by the proteasome. In hypoxic conditions, oxygen-dependent PHD enzymes are inactive and HIF-1α protein remains stable. HIF-1α is consequently able to bind to HIF-1β, the beta subunit. The transcription of downstream targets of HIF takes place and enables adaptation to an hypoxic environment. The alternative binding partner for HIF-1β is HIF-2α and this subunit has overlapping as well as distinct target genes. (195).

HIF-1α has been detected in the human endometrium but it is limited to the peri-menstrual phase (59, 217). Women with objectively measured HMB had significantly decreased endometrial HIF-1α protein and its downstream targets at menstruation versus women with normal loss, consistent with a defective hypoxic response. This was modelled in the mouse (157) by pharmacologically and genetically decreasing HIF-1 at menstruation and resulted in significantly delayed endometrial repair (a surrogate marker for menstruation in this mouse model).

Much uncertainty has existed regarding the presence and role of hypoxia at menstruation. The mouse model of menstruation (described in detail above; Section 5) has shed new light on this issue. The mouse model of simulated menses has enabled the use of pimonidazole, a marker of oxygen partial pressures of <10mmHg. Intense pimonidazole staining was detected in the uppermost endometrium following progesterone withdrawal (52, 79, 157). Transient hypoxia occurred during tissue breakdown and was not detected following repair of the denuded endometrial surface. This provided definitive evidence that physiological hypoxia occurs in the menstrual endometrium of this mouse model of simulated menses. To determine its role, menstrual hypoxia was prevented by incubation of mice in a hyperoxic chamber at the time of progesterone withdrawal. This delayed endometrial repair following shedding, indicating that hypoxia is required for normal repair at menstruation (157).

This study lends important further support for a pivotal role for hypoxia/ hypoxia inducible factor (HIF) in the menstruation process. There was demonstration using pharmacological stabilisation of HIF-1α with DMOG administration of the rescue of

delayed endometrial repair in the hypoxia-deficient mice. DMOG is a prolyl-hydroxylase (PHD) inhibitor that stabilises HIF-1α even in normoxia; (110). This study provides strong support for a role for HIF-1 in the endometrium (157). Furthermore the potential utility of PHD inhibitors (HIF-1α stabilisers) as a briefly administered non-hormonal treatment for women with heavy menstrual bleeding (HMB) has been proposed.

Endometrial vascular and epithelial repair

Messenger RNA and protein of the angiogenic factor VEGF are increased in human endometrial tissue during menstruation. In the present context it is notable that HIF-1α is necessary for hypoxia-induced increases in VEGF in human endometrial epithelial cells (155). A complementary study where the angiogenic factor VEGF was blocked with VEGF Trap in the non-human primate (rhesus macaque) and an alternative mouse model of simulated menstruation (pseudo-pregnancy followed by decidualization induction and ovariectomy to trigger menses) revealed inhibition of new blood vessel development and delayed repair (re-epithelialization) of the denuded endometrial surface during menstruation (79). Further supporting evidence for a role for HIF in the physiological event of endometrial repair at menstruation comes from the demonstration that HIF-1α directly binds to the VEGF promoter during menstruation in a mouse model (44). These experimental observations provide good evidence that hypoxia regulates HIF-1α *in vivo* to coordinate timely repair of the injured endometrial mucosal surface at menstruation.

Endometrial hemostasis

At other tissue sites, disruption of blood vessels after injury results in adherence of platelets to collagen on the injured basement membrane. Platelet aggregation stimulates the coagulation cascade and formation of a fibrin clot. In contrast, platelet involvement in endometrial vascular repair is relatively low. Vasoconstriction and activation of the clotting cascade are more important in achieving hemostasis post-menstruation (94). The coagulation cascade is activated by two pathways: extrinsic and intrinsic. Each culminates in the conversion of factor X to Xa, which catalyzes the conversion of pro-thrombin to thrombin, ultimately leading to the formation of a fibrin clot. Disorders that interfere with systemic haemostasis significantly impact on menstrual blood loss, reviewed in (66). Von Willebrand disease is the most common of these disorders, with a prevalence of 13% in women with a complaint of HMB (213).

Degradation of the fibrin clot is mediated by the fibrinolytic system. Fibrinolysis involves conversion of plasminogen to active plasmin, promoting the degradation of fibrin deposits. Tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA) drive the production of plasmin. In contrast, plasminogen activator inhibitor (PAI) inhibits fibrinolytic activity. The human endometrium contains t-PA and u-PA, as well as PAI and the uPA receptor (100, 180). Women suffering from HMB have raised levels of t-PA activity on the second day of bleeding when compared to those with normal menstrual blood loss (100), consistent with an overactive fibrinolytic system. Further evidence for this over activation of the fibrinolytic system comes from the efficacy of tranexamic acid as a non-hormonal treatment for HMB administered during the first few days of heavy menses. This

antifibrinolytic reduces t-PA and PAI levels in women with HMB and results in a 58% reduction in blood loss (101).

Tissue formation and remodelling are key processes in wound repair. Endometrial repair post-menses is usually scar-free and rapidly occurring over three to five days (78, 157). An interesting study of menstrual fluid (MF) was performed in which menstrual effluent was collected on cycle day 2 in a menstrual cup from women with a normal bleeding duration of 3-5 days. It was demonstrated that MF enhanced wound healing in an *in vivo* porcine wound model as well as *in vitro,* and identified MF influence on cell migration as an important process in the authors' models of wound repair (78).

Study of menstrual endometrium with scanning electron microscopy has revealed that regrowth of the epithelium occurs first, prior to stromal expansion (142). This study suggested epithelial cells grow from the necks of the endometrial glands and spread to meet migrating cells from other glands, forming a new luminal surface. Examination of BrdU and PCNA staining in the mouse model of simulated menstruation suggested that re-epithelialization of the uterine surface arises from progenitor cells residing in the glandular epithelial cells (124). A study of human endometrium using dynamic hysteroscopic and microscopy techniques revealed that endometrial shedding and regrowth are piecemeal and occur simultaneously in different areas of the uterus (91). This study indicated that re-epithelialization arose from the denuded stromal cells rather than from residual glands. These studies highlight that our understanding of the endometrial repair process still remains in its infancy. Considering the ability of the endometrium to repeatedly repair without

scarring or loss of function, it is of strategic importance that these processes and their regulation are delineated.

12. The impact and classification of abnormal uterine bleeding (AUB).

Abnormal uterine bleeding (AUB) (169, 170), including heavy menstrual bleeding (HMB) is associated with debilitating symptoms, iron deficiency anemia(IDA). AUB imposes a significant clinical burden. 20% of the 1.2 million referrals to specialist gynaecologist services in the UK concern women with HMB (179, 197). A US study has reported financial losses of >$2000/patient/year due to work absence and home management costs (85). As a consequence of advances in access to contraception, women in high income countries may now anticipate at least 400 menstruation events in their lifetime. Previously women experienced ~40 menstrual bleeds in a lifetime due to repeated pregnancy and prolonged lactational amenorrhea (216). Thus as women menstruate more often the opportunity for development of menstrual abnormalities has increased as has the burden of menstrual-cycle related complaints, including those associated with menstrual bleeding experience.

Medical treatments for AUB are available but are often discontinued due to lack of efficacy or side effects, signifying a need for personalised, non-hormonal treatments for HMB. All progestin-only methods are associated with irregular and often unpredictable endometrial spotting or bleeding (3). Unscheduled uterine bleeding is one of the commonest reasons for discontinuation of use of progestin-only methods. This includes the levonorgestrel-releasing intrauterine system (LNG-IUS; (199)). A national 4-year audit of HMB reported that 43% of women received surgery in the year following first attendance at hospital (196, 197). In England and Wales the

largest percentage increase in fertility rates recently reported was for women aged 40 and over and this rate has more than tripled since 1981. In 2015, the fertility rate for women aged 40 and over rose above the rate for women aged under 20 (182, 196, 197) meaning fertility-ending surgery is not always an acceptable option for women with problems of menstruation. In addition, surgery introduces risk of bowel/bladder/ureteric damage, haemorrhage and infection. Problems with menstruation represent a clinical area of major unmet need and personalized, efficient medical treatments that permit women to retain their fertility/uterus are wanted. Delineation of the physiology of menstrual initiation and cessation and the endometrial aberrations present in women with AUB will aid the identification of novel diagnostic and therapeutic strategies for such disorders.

The management of this common, life-limiting complaint will not improve without appropriate diagnosis of the underlying disorder. The plethora of potential causes and lack of universally accepted nomenclature surrounding AUB has previously hindered progress in this clinically important field. However, in 2011 a FIGO (The International Federation of Gynecology and Obstetrics) classification system for AUB was published after a multistage development process involving an international group of clinician-investigators from 6 continents and over 17 countries (168). This group advised elimination of inconsistent, confusing terms such as dysfunctional uterine bleeding and menorrhagia with replacement by more simplified terms, such as heavy menstrual bleeding or irregular heavy menstrual bleeding. In addition, the PALM-COEIN classification system was introduced (169, 170). PALM represents the structural disorders of polyps (AUB-P), adenomyosis (AUB-A), leiomyoma (AUB-L) and malignant/pre-malignant conditions (AUB-M) and COEIN the non-structural

causes such as coagulopathy (AUB-C), ovulatory dysfunction (AUB-O), endometrial dysfunction (AUB-E), iatrogenic causes, for example associated with progestin-only and other hormonal treatment approaches (AUB-I), and not otherwise classified (AUB-N). Endometriosis is included in the AUB-N category as it may sometimes cause AUB. The endometrial phenotype of endometriosis is an area of active research and the subject of recent reviews (89, 146), hence will not be discussed further.

Structural causes of AUB ("PALM"); Figure 8;

Endometrial or cervical polyps are usually benign proliferations of epithelium and stroma with variable vascular, glandular, fibromuscular and connective tissue components. They commonly present with irregular uterine bleeding but why they occur and how they cause AUB is poorly understood. They may represent localised foci of non-shedding endometrium that enlarge through multiple cycles. They are amenable to surgical removal and the introduction of tissue retrieval devices has enabled efficient hysteroscopic removal in the outpatient setting.

Adenomyosis (FIGO classification, AUB-A) (170) is defined by the presence of ectopic endometrial glands and stroma within the myometrium. Its prevalence ranges from 7 – 27%, with a significant variation in reporting due to the challenges of diagnosis (150, 174). Like the eutopic endometrium, the ectopic endometrial deposits undergo cyclical bleeding in women with adenomyosis, leading to significant pain. The effect of adenomyosis on the endometrium and the mechanisms causing AUB are not well understood. AUB-A is particularly challenging as it is often resistant to medical treatment, and surgical options

(ablation or hysterectomy) are unacceptable to those wishing to preserve their fertility. Delineating the endometrial mechanisms causing AUB-A may reveal novel therapeutic strategies for these women. There is some evidence that progesterone receptor levels are reduced in endometrium of women with adenomyosis (18, 19) and could lead to decreased responsiveness of the endometrium to progesterone. An increased expression of the oestrogen receptor (ER) subtype, ER-B, with reduced expression of PR has also been identified in endometrium from women with adenomyosis (108). Progesterone is anti-proliferative and counteracts estrogenic impact during the proliferative phase of the menstrual cycle, therefore these defects could partially contribute to the HMB experienced by those with adenomyosis.

Leiomyomas (uterine fibroids; AUB-L) are the most common benign tumours in women of reproductive age and have a cumulative lifetime risk of up to 80% (12). They are symptomatic in approximately 50% of women (12) and may cause bleeding disturbances (HMB or irregular bleeding), pressure symptoms (on bladder and bowel), pelvic pain and subfertility. Leiomyomas form as a consequence of the proliferation of uterine smooth muscle cells and associated collagen matrix. They are extremely heterogeneous in size and location and new evidence from genome wide association studies has identified genetic subgroups leading to fibroid formation (reviewed in (226)). Their impact on endometrial function has received attention due to their association with subfertility and AUB. Submucosal fibroids are associated with a blunted decidualization response and aberrations in progesterone-regulated genes such as HOXA10 (69, 192). In addition to a negative impact on implantation, defective preconditioning in the secretory endometrium may significantly impact on endometrial function during menstruation and increase blood loss. Vasoconstriction

may also be impaired at the time of menstruation in women with fibroids, with fibroid tissue expressing altered levels of endothelin receptors and prostaglandin F2α when compared to normal myometrium (164, 189). As discussed above, even a small decrease in vasoconstriction will significantly increase menstrual blood flow (153). Further examination of the endometrial impact of fibroids will inform future strategies for fertility-preserving management of AUB-L.

Non-structural causes of AUB ("COEIN"); See Figure 8

These disorders are not readily identified by imaging of the pelvis and require skilled history-taking and examination of the patient to enable appropriate investigation and diagnosis. The classification category, AUB-C includes those women with an inherited coagulopathy and disorders of hemostasis. Local regulation of endometrial hemostasis has been discussed above and requires the input of a haematologist for appropriate management (100, 121, 132). Bleeding due to "AUB-O" (ovulatory dysfunction) may be a consequence of extremes of reproductive age or body mass index, or due to underlying conditions such as polycystic ovarian syndrome. As the defect is at the level of the ovary, hormonal therapies are often effective. "AUB-E" includes disorders of local endometrial coagulation, vascular function and/or inflammation and have been discussed above (Sections 8, 11). . A detailed understanding of the regulatory factors involved in the pivotal reproductive event of menstruation and the aberrations present in those with AUB will facilitate specific correction and more precise, personalised treatments. Only by understanding the physiology and pathology of menstruation can medical treatment strategies for AUB become more effective and acceptable, decreasing the reliance on non-specific surgical interventions that remove fertility.

13. Targeting the progesterone receptor (PR) for therapy: Class effects; clinical applications; mechanisms of action of PR ligands (SPRMs).

There is currently a great deal of clinical interest in selective progesterone receptor modulators (SPRMs), a family of compounds with actions at the PR. These compounds have therapeutic potential in a range of unsolved problems of benign gynecology (245). One of the most significant effects of SPRMs clinically is that they rapidly induce amenorrhea (absence of bleeding), by mechanisms that remain largely unknown. **(Figure 9A).** The endometrium is a prime target tissue for SPRMs. There is a range of potential actions, from the pure agonist effect of progesterone and synthetic progestins to the pure antagonist effect of synthetic pharmaceutical agents such as onapristone, ZK137695. Several compounds including asoprisnil and mifepristone (RU486) show a mixed partial agonist and antagonist effect **(Figure 9B)**.

The first SPRM to undergo clinical development was mifepristone (RU486), which in addition to its use in medical termination of pregnancy (236) was recognised to have a range of potential applications: in emergency contraception (99); as a non-estrogen containing daily oral contraceptive pill (134); in prolonged release contraceptive devices (106); and in treatment of uterine fibroids (215), endometriosis and adenomyosis. Clinical development depended not only on demonstration of efficacy, but also on the safety profile, and there was concern about the long-term effects of mifepristone on endometrium, which in fact still applies to all SPRMs at the present time.

The effects of mifepristone on endometrium are dependent on dose and duration of administration. At doses of 10 mg/day or over, follicular development is inhibited by suppressing gonadotrophins, and the ovarian secretion of estradiol is minimal. At low dose (2 mg/day or less) mifepristone inhibits or delays ovulation without inhibiting follicular development, and circulating levels of estrogens tend to be in the mid-follicular range (14). Endometrium is thereby exposed to unopposed estrogen, and in early studies it was hypothesized that this could lead to endometrial hyperplasia or carcinoma. Indeed a number of reports in women described effects consistent with endometrial hyperplasia, with widespread cystic gland dilatation and overall thickening (74, 171). At very high dose (400 mg daily for 6 months) massive simple hyperplasia of endometrium was reported which reversed at cessation of treatment (177).

However it became evident that the situation in reality was more complex, and that mifepristone and other SPRMs exert a paradoxical anti-proliferative effect specifically on endometrium. This was originally studied in primate experiments, and the anti-proliferative effect shown to be specific to endometrium (111, 253). Studies in women demonstrated that SPRM-treated endometrium showed glandular epithelium with quiescent or inactive appearances and reduced mitotic activity, the cystic glands more resembling postmenopausal cystic atrophy than hyperplasia (38, 61). The antiproliferative effect was also described in endometrial cell lines (172, 208).

Mitotic activity in the endometrium is driven by estrogen, with progesterone exerting inhibitory effects, so it was somewhat paradoxical to find that anti-progestins have an anti-proliferative effect in the endometrium. Even at high estrogen concentrations,

SPRMs are able to suppress endometrial proliferation in primates (219, 253) and in women (38). The mechanism is unknown, and postulated explanations have included downregulation or ER, or inhibition of estrogen-induced proto-oncogenes. Evidence from primate experiments suggests that the endometrial androgen receptor AR may play an important role in these effects. Endometrium responds to SPRM treatment in primates and women by upregulation of AR, and it is significant that in the primate, the endometrial anti-proliferative effect can be prevented by administration of the antiandrogen flutamide (26).

It has become clear that the endometrium responds to treatment with different SPRMs in a similar way, with slight differences between agents that are believed to depend on the degree of agonist/antagonist effect at the PR **(Figure 9B)**. Morphological changes vary with duration of treatment, but are remarkably similar between different agents given for durations up to 3 months. The principal effects on endometrial glands are the development of cystic glandular dilatation, associated with disordered architecture of non-dilated glands. Glands show a non-physiological secretory effect, which includes the development of apocrine-type luminal protrusions and abortive cytoplasmic vacuolation. It is characteristic that glandular epithelium appears quiescent, with reduced nuclear stratification and few mitotic cells, reflecting the anti-proliferative effect. The endometrial stroma is compact and shows no evidence of pre-decidual change. **(Figure 10 A-C)**. These changes are now recognised to be a “class effect” of SPRMs, a spectrum of morphological changes none of which is specific, but which in combination had not previously been seen in any other situation or with any other agent. The changes have been termed “progesterone receptor modulator associated endometrial changes” or PAEC (112,

173). Characteristic histological changes have been described with mifepristone (84), asoprisnil (252), onapristone (37), ZK230211 (lonaprisal) (106), telapristone acetate (173), and ulipristal acetate (251).

The natural history of development, and the consequences of PAEC in endometrium are of considerable clinical significance, as many of the potential clinical indications ideally require continuous long-term administration. Use of SPRMs continuously for 6 months or more leads to endometrial thickening, the extent of which correlates with the degree of cystic dilatation of endometrial glands (13, 227). Although morphologically the glandular epithelium adopts an increasingly quiescent or atrophic appearance with increasing duration of continuous treatment, the increased endometrial thickness often leads to clinical concern, resulting in invasive investigations and treatment. Consequently, current models of SPRM treatment adopt an interrupted schedule, in which the agent is administered for 12 weeks and then withdrawn, after which the endometrium is allowed to undergo menstrual shedding. Evidence from clinical trials of UPA indicates that in the great majority of women, the endometrium undergoes reversal to a physiological pattern, even after up to 8 interrupted courses (80). Some safety concerns remain around the long-term endometrial effects of SPRMs, but the focus of current safety issues centres more around potential hepatotoxicity, as several agents including most recently ulipristal acetate have been associated with severe liver injury in a very small proportion of women (166).

Much remains to be learned about the mechanisms of action of SPRMs on the endometrium, in particular the mechanisms of the antiproliferative effect and of

amenorrhoea. It is likely that altered expression of sex-steroid receptors **(Figure 10 D-F)** and progesterone-regulated genes are important in mediating these effects. Not only is there evidence for altered overall levels of expression of sex steroid receptors in the endometrium, there are also significant alterations in the pattern of expression between endometrial glands and stroma. For example, there have been shown to be increased overall mRNA concentrations of AR, PR, PRB and ESRI (ERα) in ulipristal acetate (UPA)-treated endometrium compared to pre-treatment secretory phase (249). In this study, immunohistochemical localization of PR and PRB showed intense nuclear expression in glands, with minimal expression in stromal cells, contrasting with pre-treatment proliferative phase endometrium, which showed intense nuclear staining in both glands and stroma. With AR, pre-treatment secretory phase endometrium showed intense nuclear staining in glands but not stroma; ERα immunoexpression showed less dramatic alterations, although changes in intensity were described.

Interesting observations in human endometrium exposed to SPRM administration (asoprisnil) have provided support for a role for the IL-15 pathway in the complex interplay between endometrial stromal cells, innate immune cells (uterine NK cells), and endometrial spiral arteries with effects on both arteriole morphology and menstrual bleeding (250). Whether such disturbances of normal endometrial function as just described are evident with all SPRM class members is yet to be determined. SPRMs do however provide an invaluable tool to modulate progesterone- progesterone receptor interaction and thereby enhance our understanding of endometrial physiology and in particular in the enigmatic area of regulation of menstrual bleeding.

14. Concluding comment and future directions.

The pioneering studies on endometrial physiology and its regulation by steroids that still underpin much of our knowledge of the menstruation process were undertaken nearly eighty years ago. Much progress in our understanding of endometrial pathophysiology has been made, facilitated by modern cellular and molecular discovery tools, along with animal models of simulated menses. However, four decades later this quote from 1982 (over 35 years ago) "despite renewed interest over the last few years, the mechanism of menstrual bleeding still remains something of an enigma" (4) remains valid today. Our understanding of endometrial biology has progressed but many questions pertaining to this remarkable physiological model of repeated injury and repair so fundamental to the continuation of our species, remain.

Human menstruation persists to this day as a taboo topic and many questions concerning menstrual physiology are still unanswered. Unfortunately, negative attitudes around menstruation continue, especially where women have a lower social status and where reproductive health education is lacking. This, alongside the previously confusing classification and terminology surrounding menstruation, has hindered progress in this field. As the FIGO classification system for AUB gains traction, inconsistencies will decrease and the utility of studies in this area will increase. This will have an inevitable positive impact on women suffering from this debilitating condition.

The endometrium is a physiological tissue site of repeated episodes of “injury and repair” (menstruation) governed by the prevailing endocrine environment and sequential steroid exposure. Hence, it may also serve as a model for injury/ repair of an injured mucosal surface without scarring that may inform biology of other tissue sites in the body (152). Following menstruation, the “injured” endometrium undergoes rapid repair and tissue restoration in preparation for the next menstrual cycle. Endometrial inflammation needs to be resolved alongside cellular proliferation and angiogenesis in order to regenerate a functional endometrium ready for implantation. Therefore addressing the unanswered questions in this field may not only improve the lives of many women, but may also have considerable translational impact in other tissue sites.

It remains of longstanding interest, and is still unexplained, why menstruation only occurs in species whose endometrium spontaneously decidualizes prior to implantation. In contrast, in those species where the endometrium does not decidualize until embryo/endometrial contact is established, the withdrawal of progesterone does not affect endometrial breakdown or bleeding. The impact of endometrial preconditioning in the preceding cycle on menstruation is also unknown. Do aberrations in decidualization, vascular remodelling and/or the immune response significantly impact bleeding during menstruation? The mechanisms of menstrual cessation remain undefined. Are haemostatic factors or vasoconstrictive properties more influential? Or is it the reforming of the luminal epithelium that is required for cessation of bleeding? Does the migratory capacity of cells in the menstrual fluid play a role in luminal re-epithelialization? What regulates these local mechanisms? Why does the endometrium not scar under physiological conditions and why does it

scar in conditions like Asherman's Syndrome (acquired uterine condition when scar tissue (adhesions) form within the uterine cavity)?

In addition to the many questions surrounding endometrial physiology, there are numerous unknowns regarding endometrial pathology. We do not yet know the endometrial impact of leiomyomas (fibroids) or adenomyosis or the mechanisms whereby they lead to AUB. Furthermore, we do not yet understand fully the mechanisms of action of many of our current medical treatments, for example, SPRMs.

Without this knowledge, we cannot deliver effective treatment to the many women currently suffering from AUB. Unravelling the physiology and pathology of endometrial disorders will generate personalised therapeutic strategies that preserve fertility and limit side effects, improving the lives of millions of women worldwide.

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Figure legends

Figure 1. The human menstrual cycle. (A) Estradiol is the dominant hormone acting on the endometrium during the proliferative phase (ovarian equivalent = follicular phase). The secretory phase occurs subsequent to ovulation, when the corpus luteum secretes progesterone (ovarian equivalent = luteal phase). (**B)** Perimenstrual window (luteo-follicular transition): rearrangement of the traditional menstrual cycle to focus on the significant endocrine and endometrial changes that occur during menstrual breakdown and repair.

Figure 2. **Progesterone withdrawal: the trigger for menstruation.** Menstruation is a steroid regulated event. There are alternatives for a progesterone-primed endometrium, i.e. pregnancy or menstruation. Withdrawal of progesterone as a consequence of corpus luteum demise, in the absence of pregnancy, is the trigger for menstruation. Progesterone withdrawal is associated with an influx of innate immune cells, increased expression of inflammatory mediators, including prostaglandins, increased vessel permeability and tissue breakdown. This inflammatory event manifests as menstrual bleeding. (*Adapted from Jabbour et al 2006; (116)*)

Figure 3. Comparison of the human menstrual cycle and the simulated mouse model of endometrial breakdown and repair. The mouse uterus is bicornuate, in comparison to the human “pear-shaped” uterus. Human endometrium undergoes

2201 spontaneous decidualization, whereas a transcervical oil injection is required to
2202 induce decidualization in a primed mouse uterus. The intense vascular remodelling
2203 that occurs during the late secretory phase is not observed in the mouse model.
2204 However, the menstrual processes of bleeding, shedding and repair are comparable
2205 and an influx of neutrophils and macrophages has been detected in both human and
2206 mouse endometrium. Also, markers of hypoxia have been detected in human and
2207 mouse menstrual endometrium. Furthermore, the critical period of P4 withdrawal is
2208 present during menses in humans and this mouse model. E_2: estradiol, P4:
2209 progesterone, equiv: equivalent.
2210
2211
2212 **Figure 4**. **Critical period of progesterone (P) withdrawal**. (*Adapted from Kelly,*
2213 *King and Critchley 2001; (128)).*
2214 The *reversible first phase* is associated with increased local exposure of the
2215 endometrium to cytokines and prostaglandins (PG) and is dependent upon an
2216 efficient response of the decidualized perivascular stromal cells to declining levels of
2217 progesterone, an anti-inflammatory hormone.
2218 The *irreversible second phase* of local endometrial events following progesterone
2219 withdrawal is a consequence of increased cytokine production and influx of innate
2220 immune cells into the local endometrial environment, accompanied by activation and
2221 release of MMPs with destruction of the extracellular matrix (ECM). This phase is
2222 considered to be independent of progesterone receptor actions.
2223
2224

Figure 5 Apoptosis precedes menstrual shedding in the glands and stroma of the late-secretory-phase human endometrium. *Upper panel:* Blue box in diagram depicts late secretory phase.
Lower panel: Representative immunofluorescent photomicrograph of cleaved caspase-3 (red; apoptosis marker, cleaved caspase-3 positive immunoreactivity) staining in late secretory phase human endometrium (menstrual cycle day 29). Nuclear counterstain: Sytox Green (green; Molecular Probes Inc.).

Figure 6 Neutrophils and macrophages are abundant in the shedding menstrual-phase human endometrium. *Upper panel:* Blue box in diagram depicts menstrual phase.
Lower panel: Representative immunofluorescent photomicrograph of CD68 (blue; macrophage marker, CD68 positive immunoreactivity) and elastase (red; neutrophil marker, elastase positive immunoreactivity) staining in menstrual phase human endometrium (menstrual cycle day 1). Endometrial glands are indicated by **G**, macrophages by **M** and neutrophils by **N**. Nuclear counterstain: Sytox Green (green; Molecular Probes Inc.).

Figure 7. Endometrial processes during menstrual breakdown and repair.
(A) Physiological processes. Following progesterone withdrawal, the mature, specialised endometrial spiral arterioles contract due to increased vasoactive factors (e.g. endothelin and prostaglandin (PG)F2α). This vasoconstriction is imperative for limitation of menstrual blood loss, as demonstrated by Poiseuille's equation where Vessel resistance (R) is directly proportional to the length (L) of the vessel and the viscosity (η) of the blood, and inversely proportional to the radius to the fourth power (r4). The local endometrial environment is exposed to inflammation and

hypoxia and repairs without loss of function. **(B)** Aberrant processes occur to cause abnormal blood loss. Immature endometrial vessels and a reduction in vasoconstrictive factors result in a larger vessel radius during menses, significantly increasing menstrual blood loss. In addition, this may prevent the physiological hypoxic response required for normal endometrial repair post menses.

Figure 8. The PALM COEIN classification system for abnormal uterine bleeding (AUB). P = polyps; A = adenomyosis; L = leiomyoma (Fibroid); M = malignancy; C = coagulopathy; O = ovulatory dysfunction; E = endometrial; I = iotrogenic; N = not otherwise classified. See Refs. (169, 170)

Figure 9. Mechanisms of action of progesterone receptor ligands (selective progesterone receptor modulators; SPRMs) that impact upon endometrial bleeding.

A. Control of endometrial bleeding with SPRMs may be effected by: (i) an endometrial anti-proliferative effect of SPRMs; (ii) decrease in uterine/ endometrial blood flow; (iii) disturbance of the complex interplay between endometrial stromal cells, innate immune cells, and endometrial spiral arterioles (250).

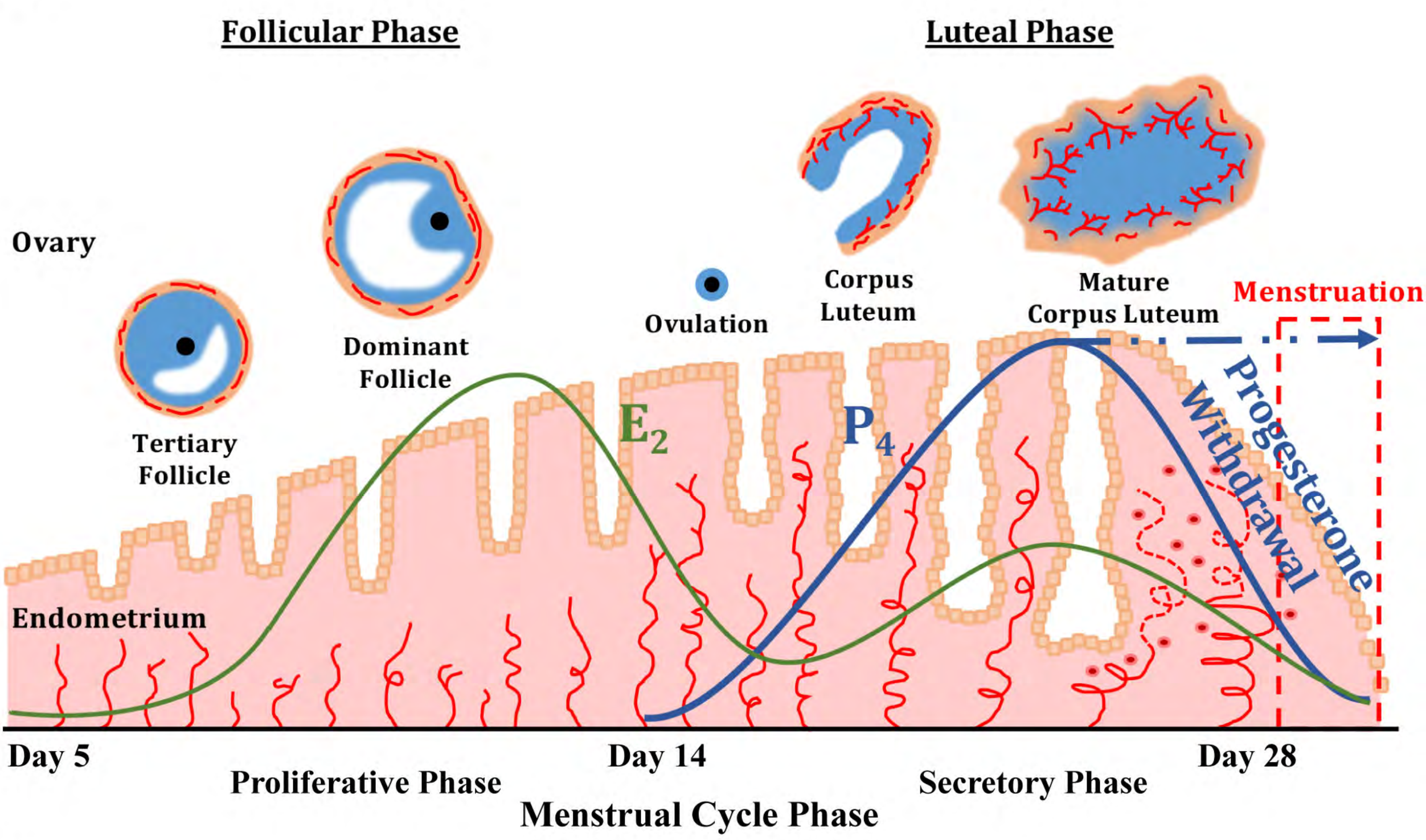
B. Spectrum of progesterone receptor modulators from agonist to antagonist. Selective progesterone receptor modulators (SPRMs) are a class of synthetic ligands for the progesterone receptor, with agonist, antagonist or mixed effects on progesterone-target tissues, for example the endometrium.

Figure 10. Selective progesterone receptor modulators (SPRMs) induce progesterone receptor associated endometrial changes (PAEC; A-C) and modulate steroid receptor expression in human endometrium (D-F).

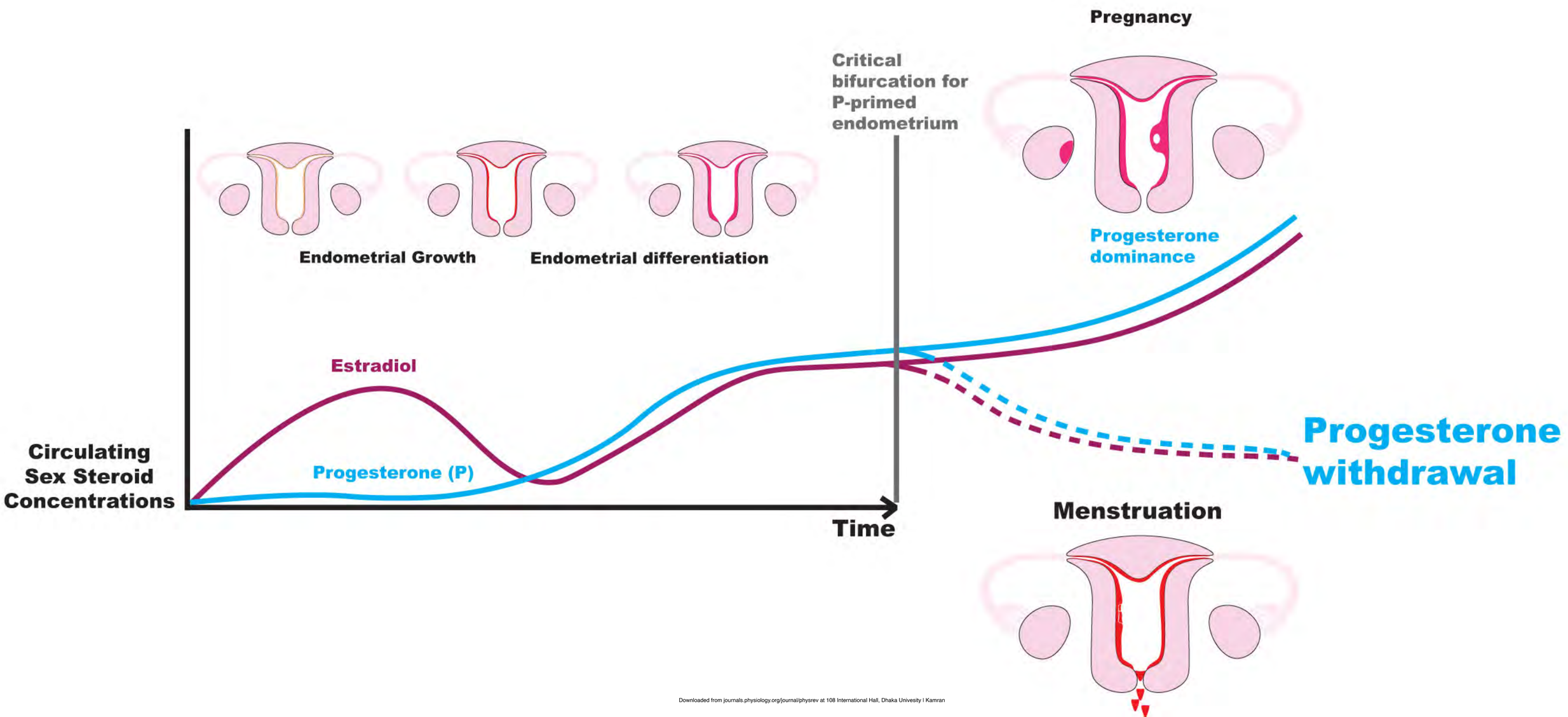
Proliferative phase endometrium (**A and D**); secretory endometrium (**B and E**); following treatment with SPRM (**C and F**). SPRMs induce PAEC. Endometrial morphology is characterised by non-physiological secretory appearance or inactive stroma with dilated cysts (**C**).

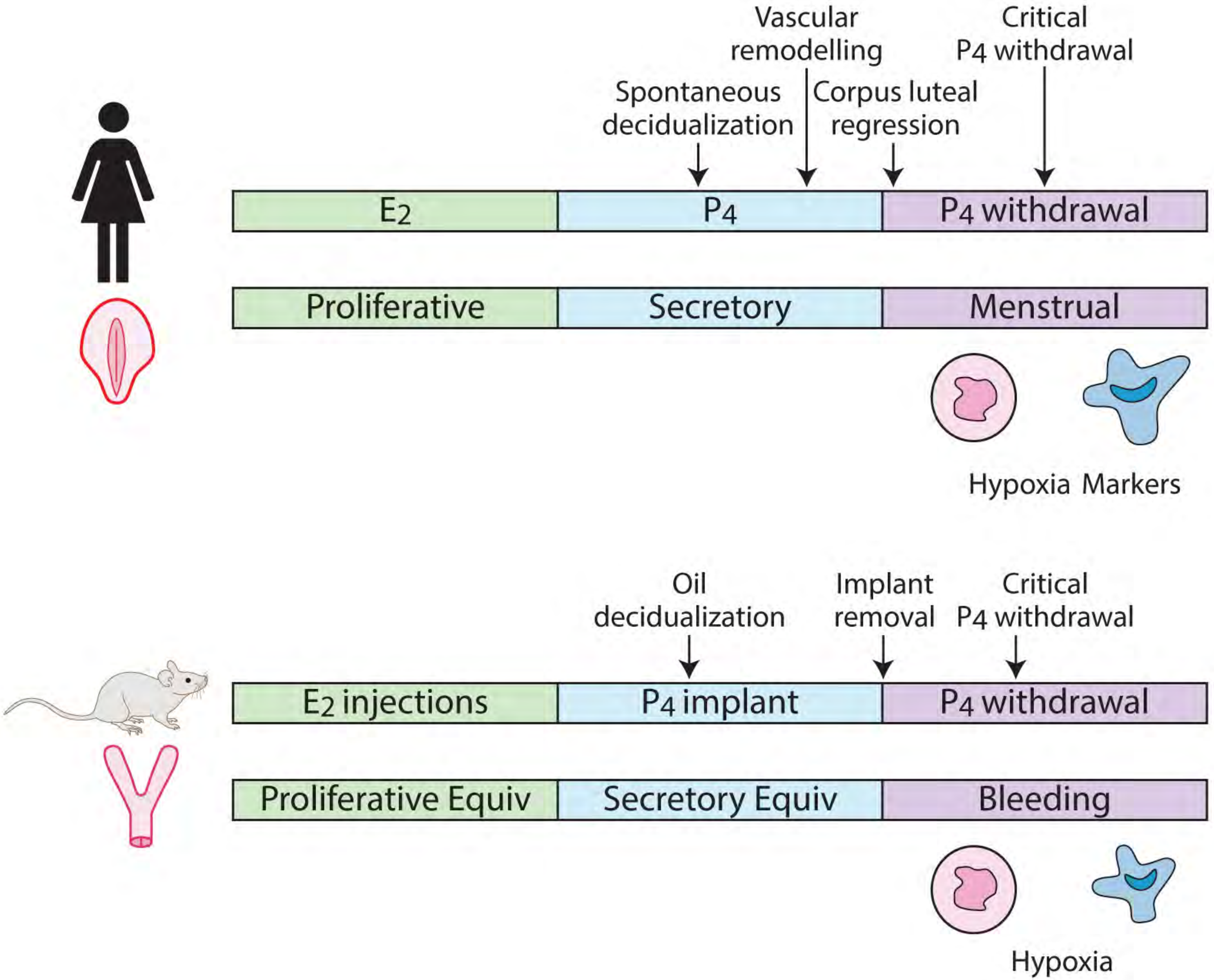
Images of progesterone receptor (PR) immuno-reactivity in human endometrium in proliferative (**D;** positive (brown) immunostaining in the glandular epithelium and stromal cells) and secretory (**E;** positive immunostaining only in stromal cells) endometrium and, after administration of a SPRM (**F**). Note intense positive (brown) immunostaining in the glandular epithelium and reduced immuno-reactivity in stromal cells (**F**).

A



B





Decidualization and pregnancy

Progesterone increases threshold for inflammatory response.

Progesterone with cAMP induces decidualization.

Progesterone concentration

Declining progesterone allows increasing NFkB expression.

Prostaglandins rise in perivascular cells. Removal of repression of NFkB events.

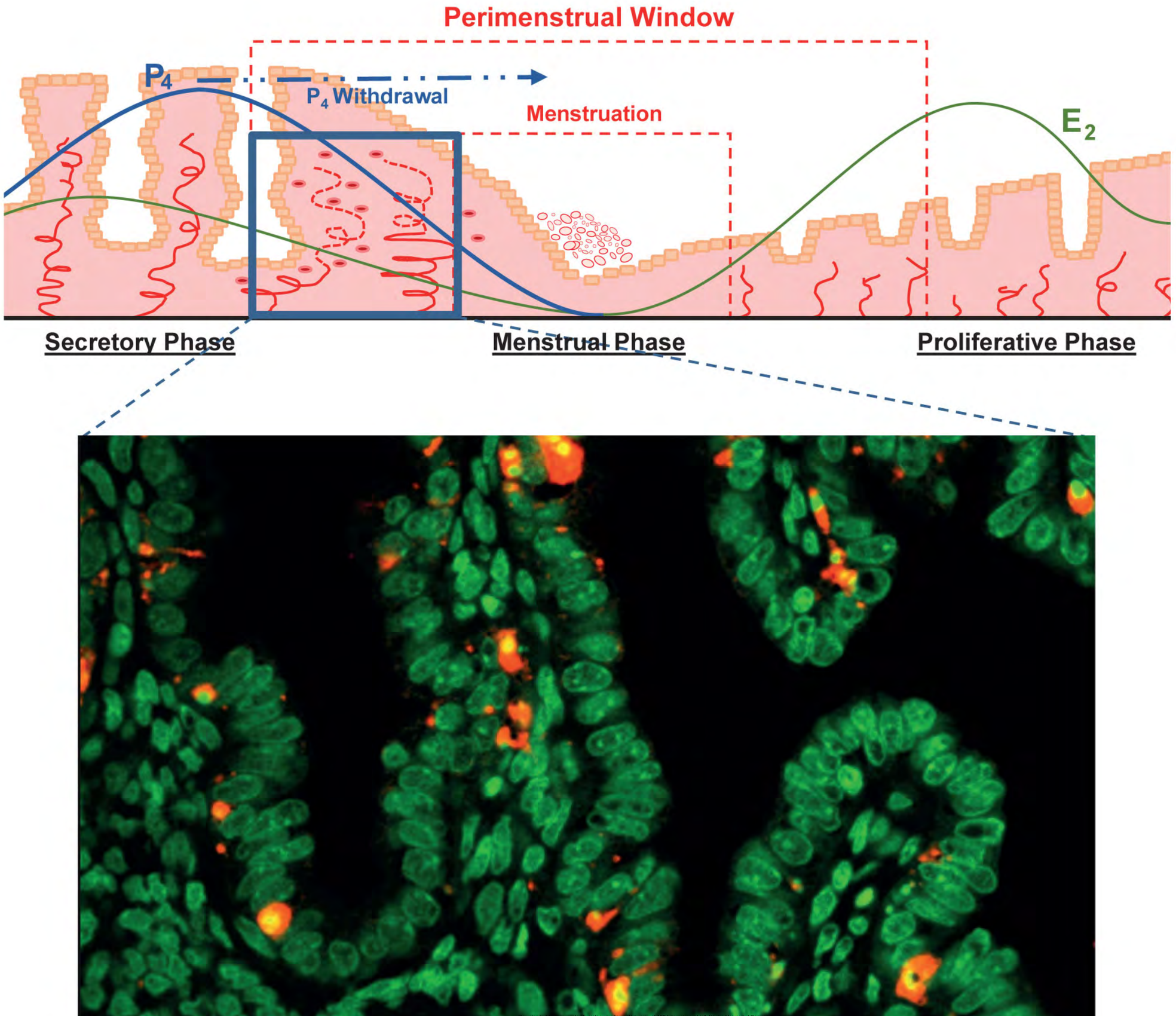
Reversible (First phase)

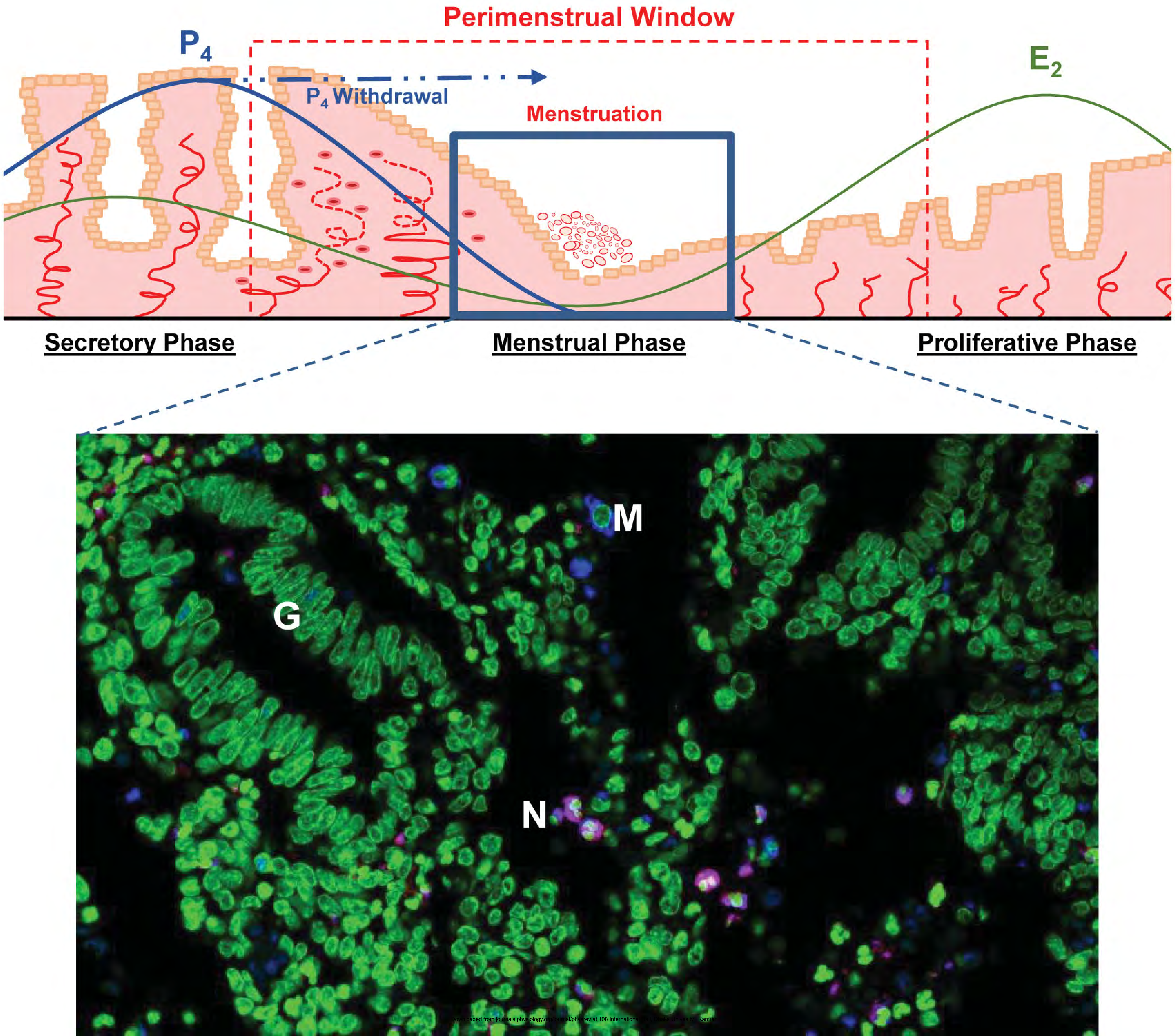
Edema and cellular influx.

MMP activation Tissue sloughing

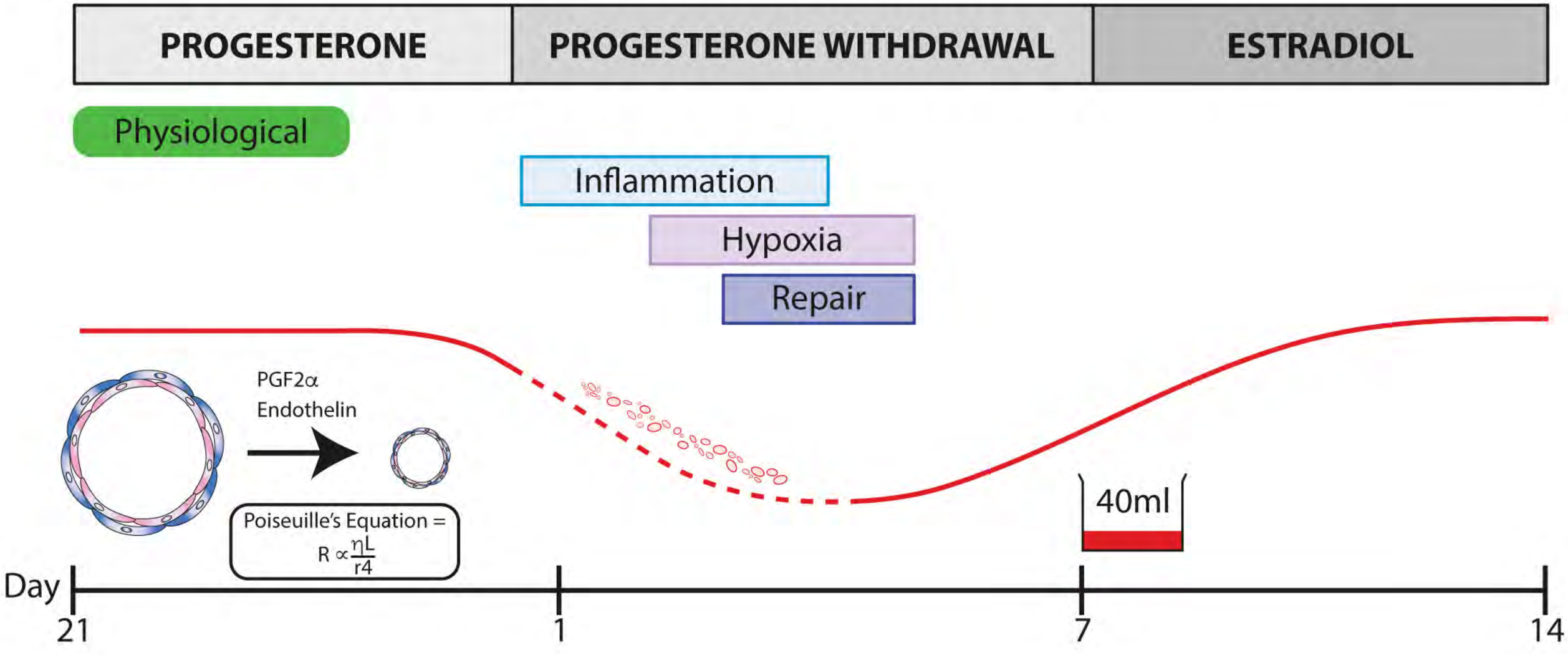
Irreversible (Second phase)

Menstruation

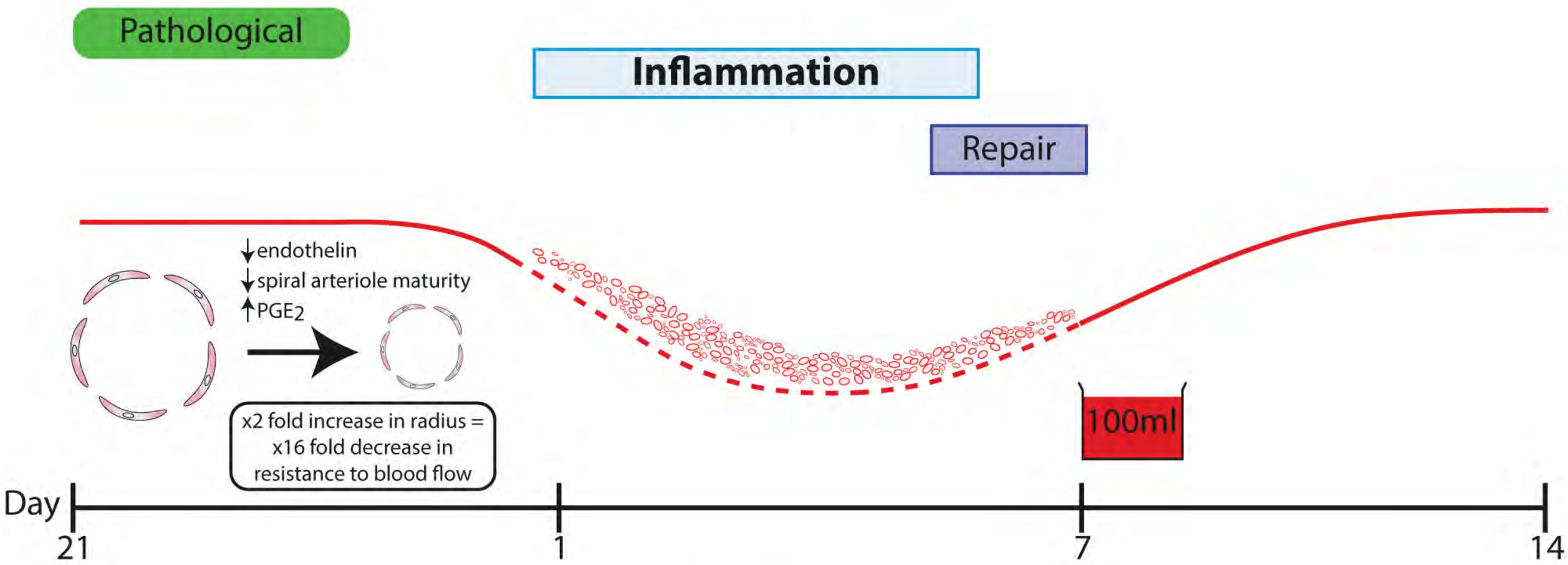




A



B



Key: R = resistance to flow; η = viscosity; r = radius of vessel; L = length of vessel.

Structural Causes 'PALM'

Polyp

Adenomyosis

Leiomyoma

Malignancy and Hyperplasia

Non-Structural Causes 'COEIN'

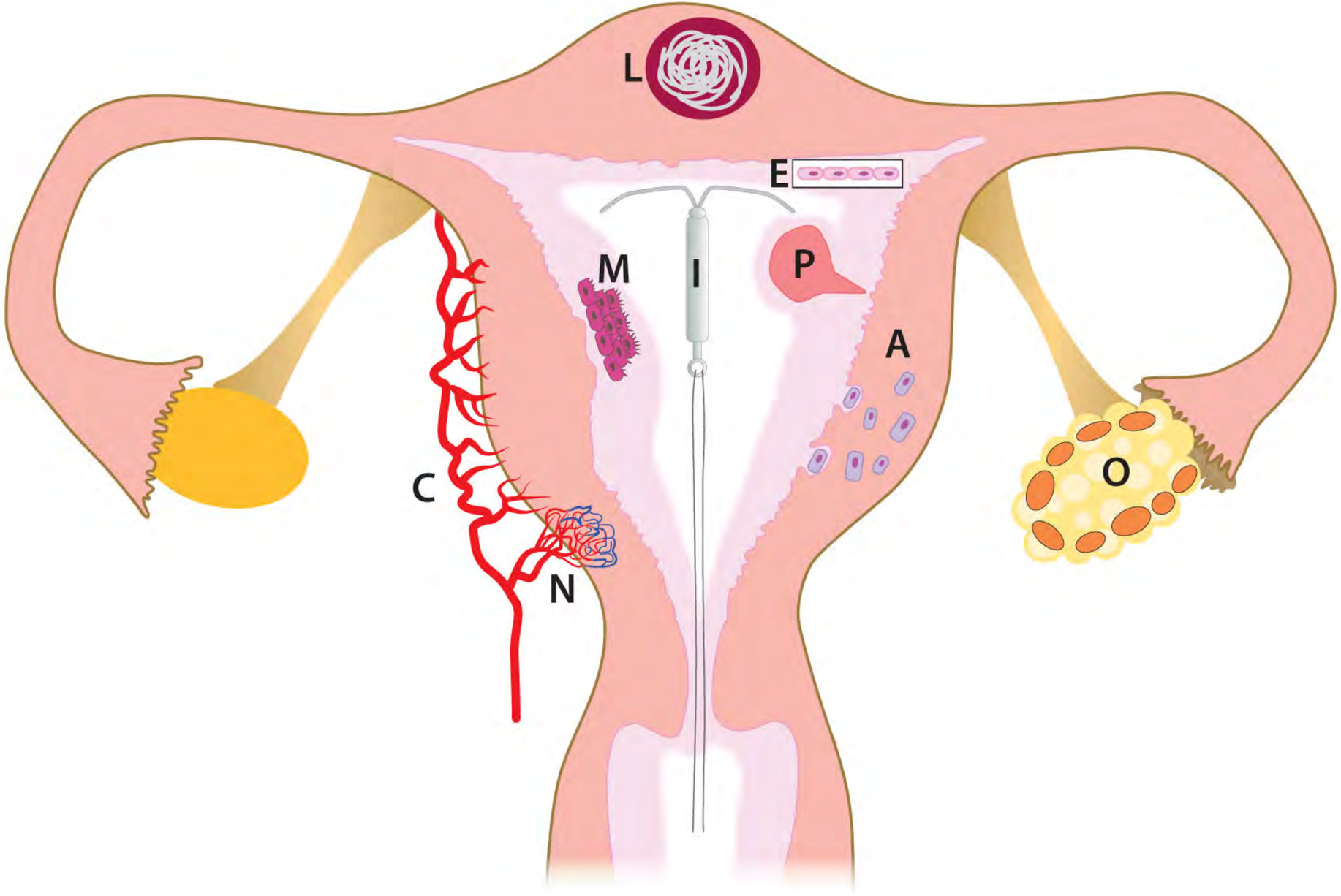
Coagulopathy

Ovulatory dysfunction

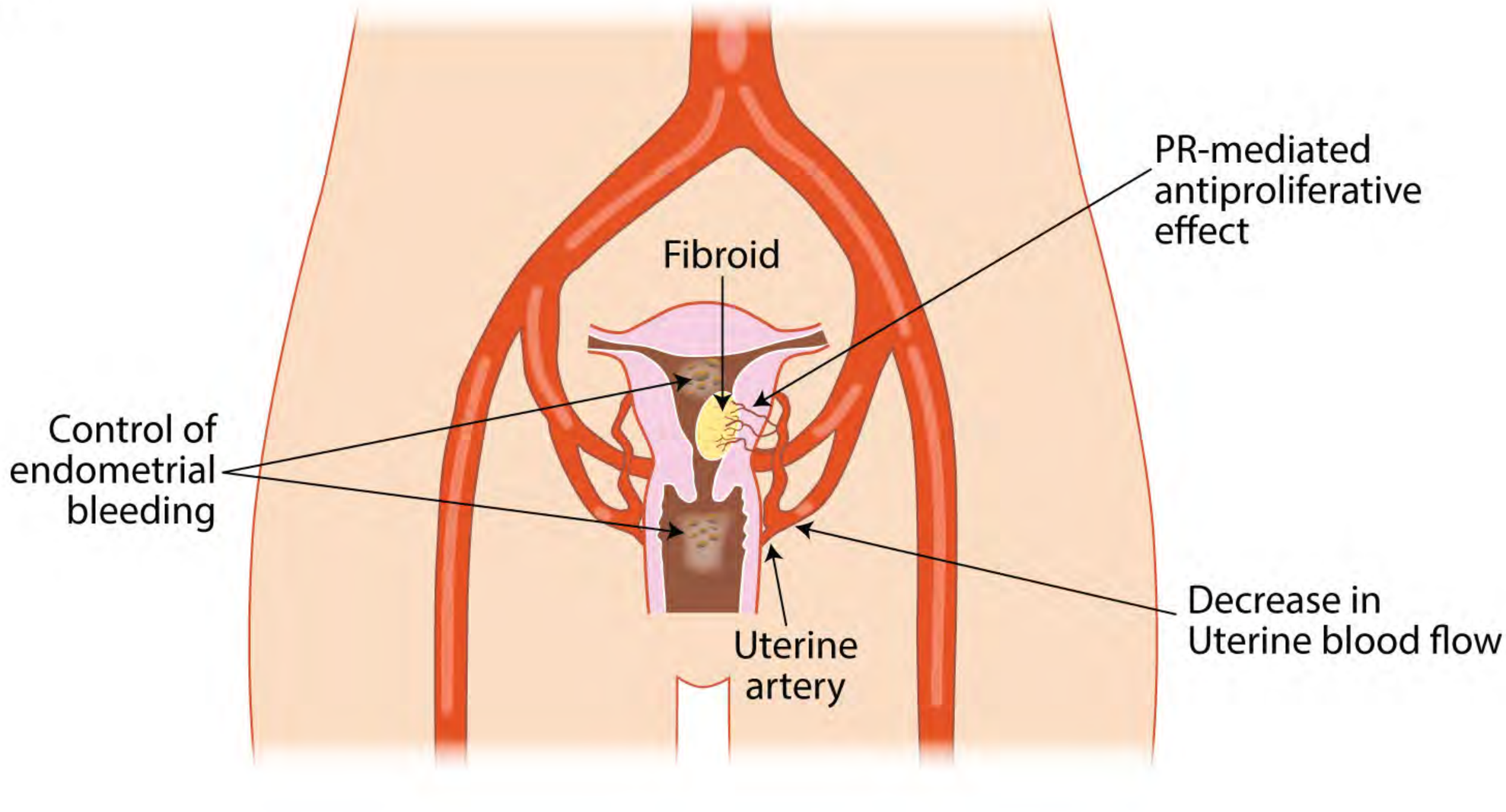
Endometrial

Iatrogenic

Not otherwise classified (eg AVM)

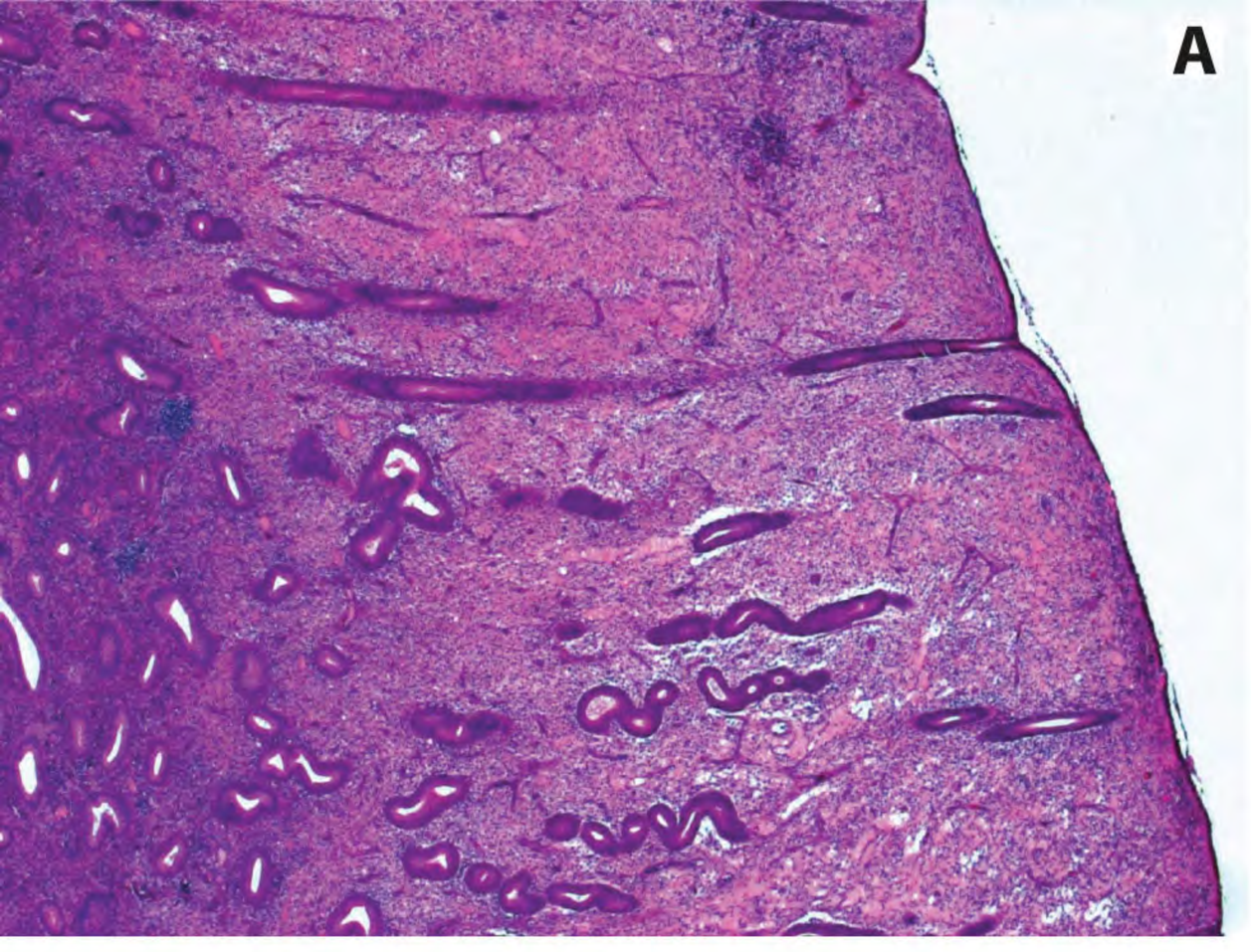


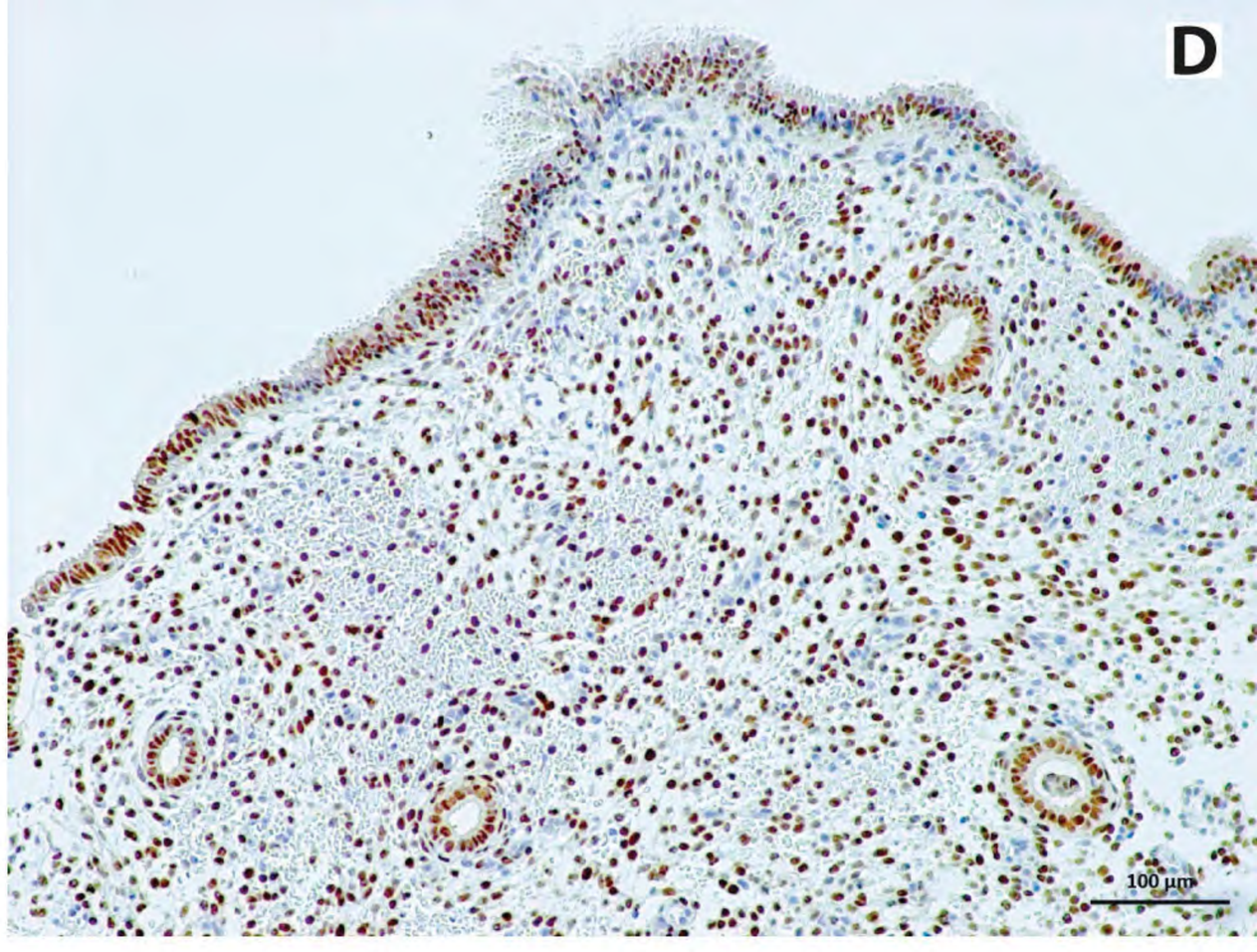
A

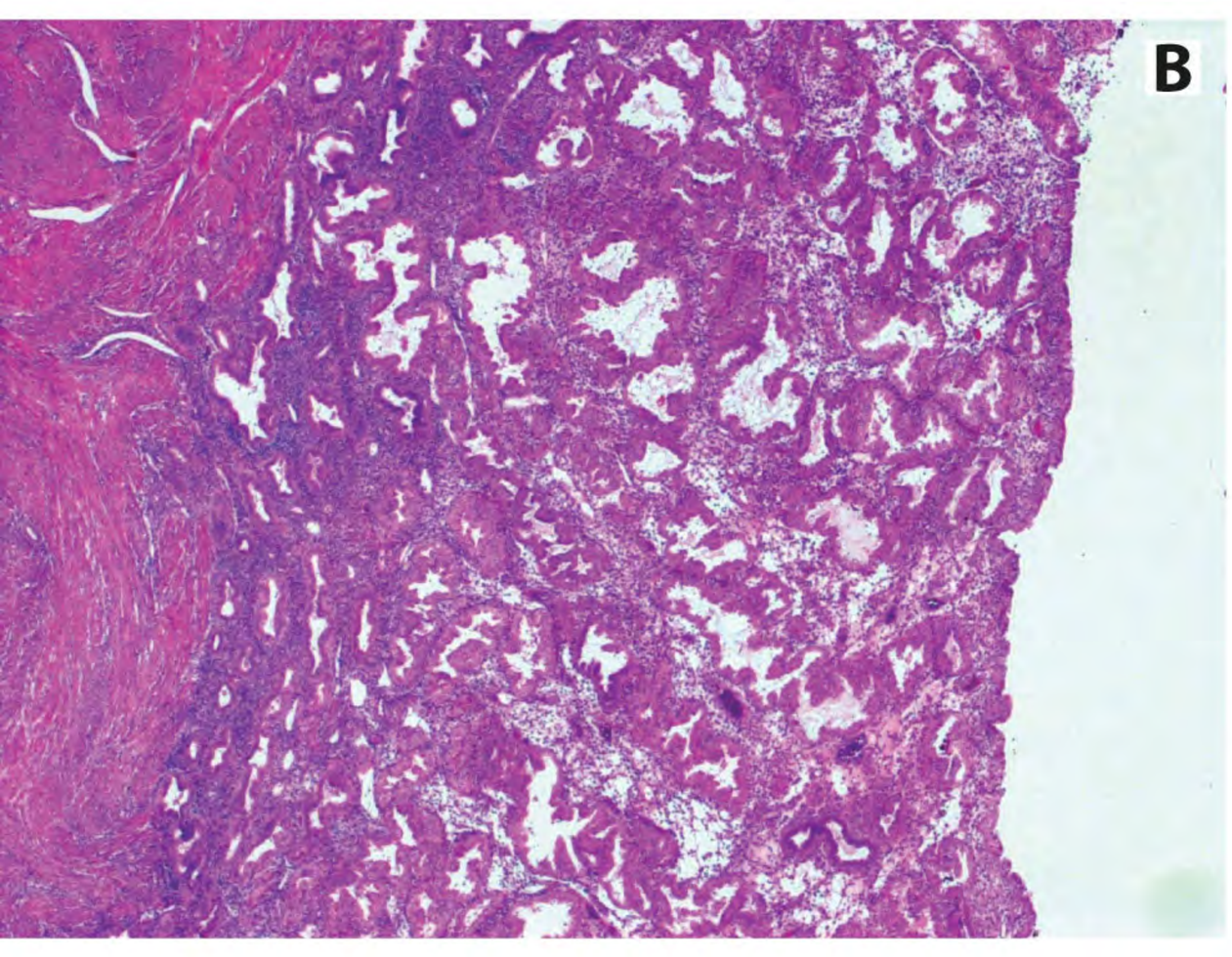


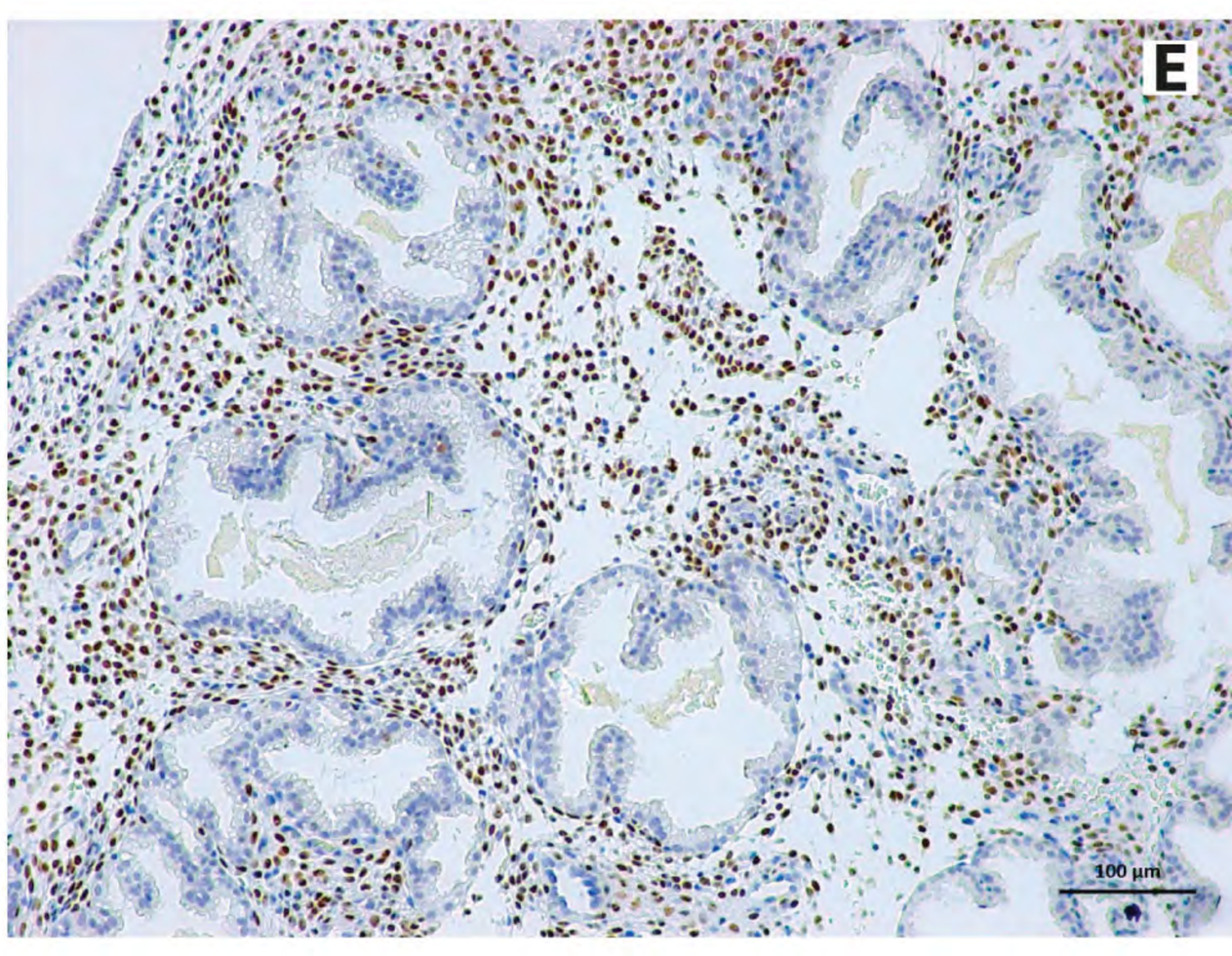
B

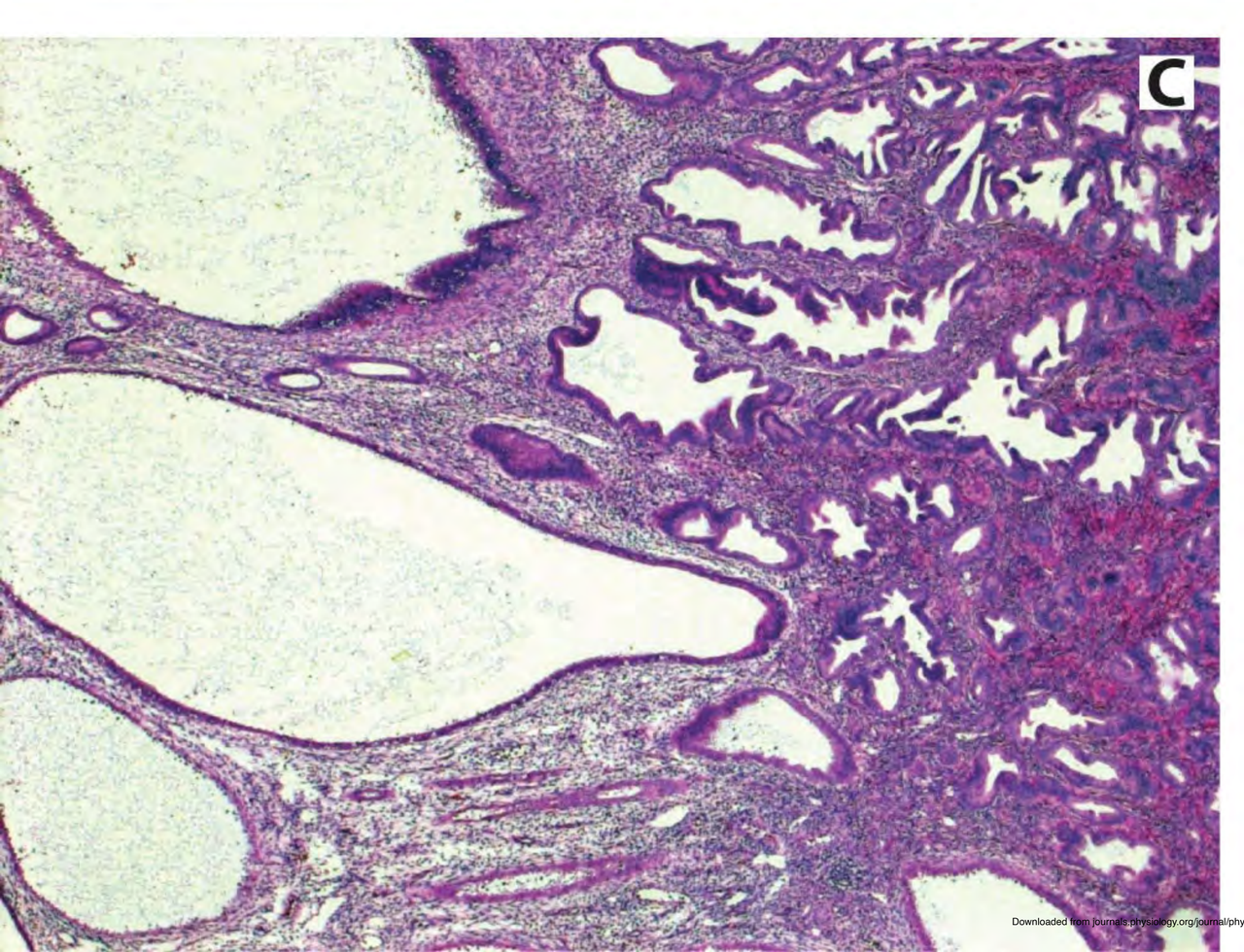
Key: PR = progesterone receptor
LNG = levonorgestrel
UPA = ulipristal acetate

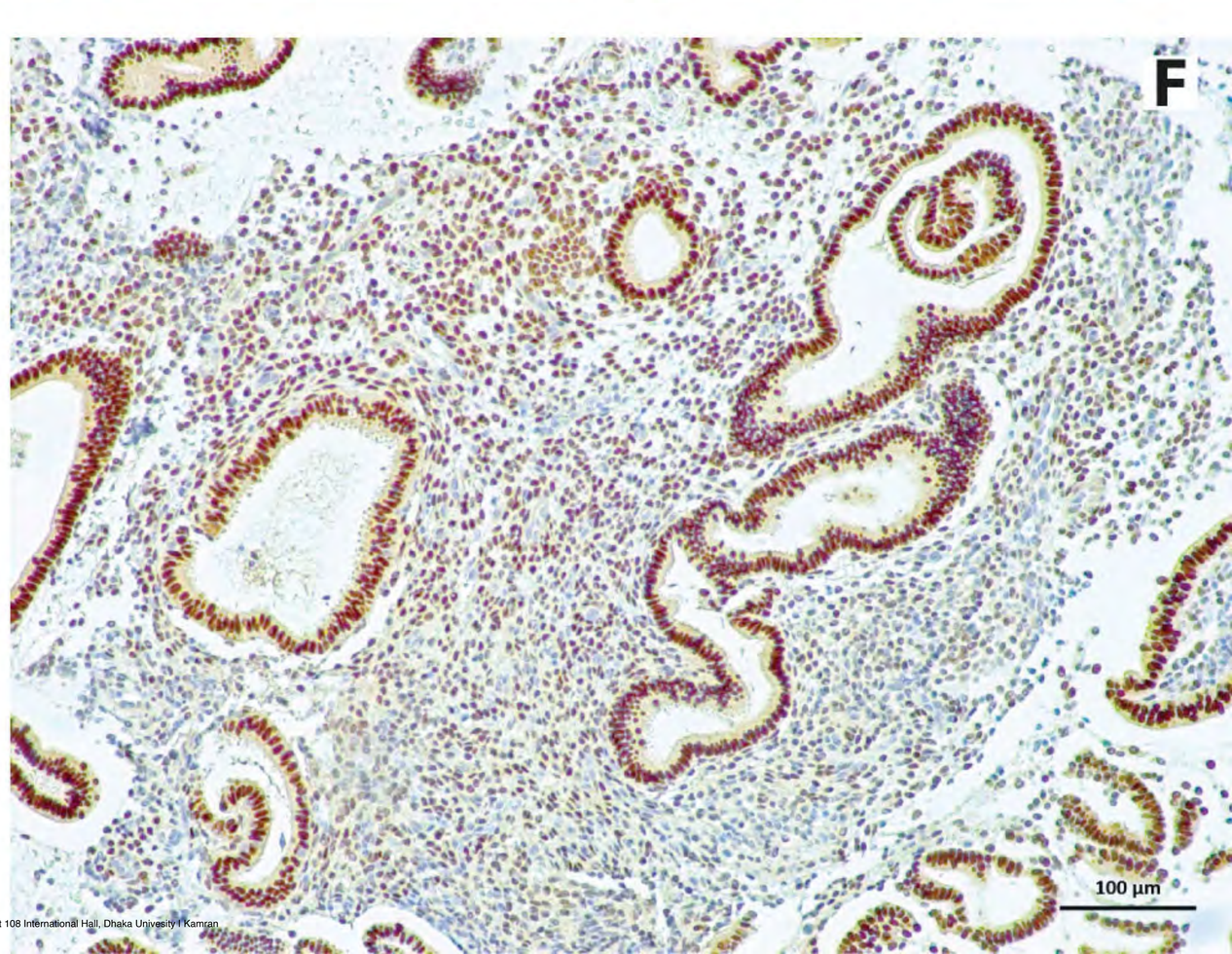


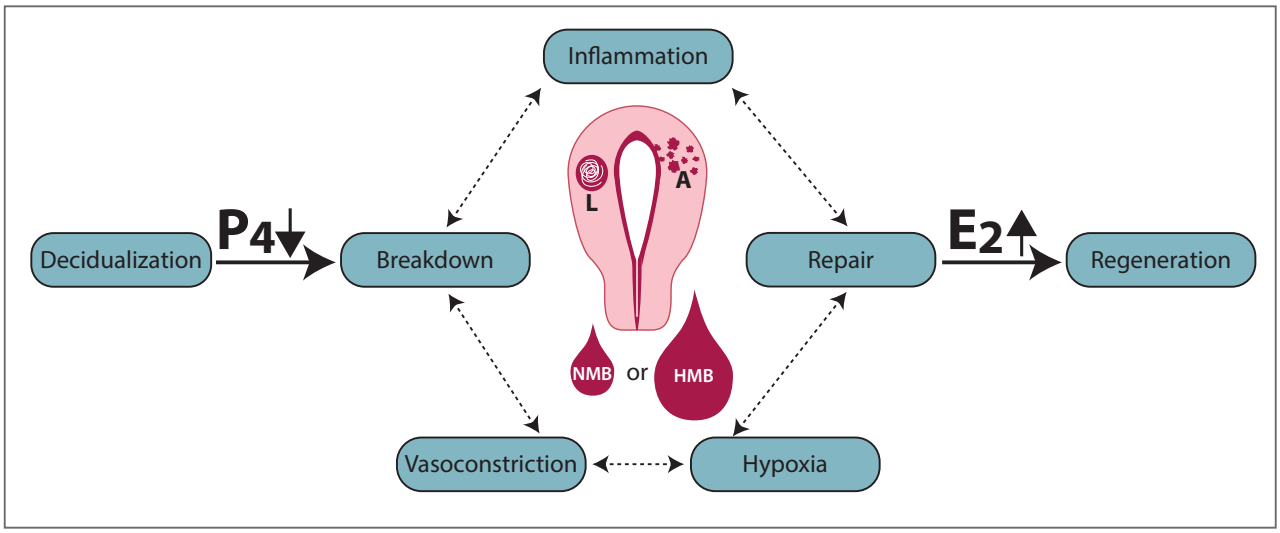












Legend for graphical abstract.

The Phoenix-like Endometrium: the regulation of menstruation.

Each month the human endometrium is exposed to sequential patterns of circulating ovarian sex steroids (estradiol [E2]; progesterone [P4]) which are crucial to the regulation of growth and differentiation of the endometrium (decidualization of an E2-primed endometrium). In the absence of pregnancy, regression of the corpus luteum results in progesterone-withdrawal which is the trigger for menstruation. The menstruating endometrium is the visible consequence of coordinated events of vasoconstriction of the spiral arteries; local inflammation and tissue breakdown (upper layer of endometrium is shed). Much remains to be understood about the tightly regulated mechanisms that underpin menstruation and subsequent endometrial repair and regeneration. Hypoxia likely plays a pivotal role in endometrial repair processes. The menstruating endometrium is thus a physiological example of an injured or “wounded” surface that is required to rapidly repair each month. Blood loss from the uterus may be of normal quantity (NMB), however a quarter of women of reproductive age experience heavy menstrual bleeding (HMB). Structural features may be present within the uterus, for example, leiomyoma/L; fibroids) and/or adenomyosis (A) and are often associated with the symptom of HMB.