"Menstruation: Science and Society"

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PII: S0002-9378(20)30619-0

DOI: https://doi.org/10.1016/j.ajog.2020.06.004

Reference: YMOB 13295

To appear in: *American Journal of Obstetrics and Gynecology*

Received Date: 20 March 2020

Revised Date: 13 May 2020

Accepted Date: 3 June 2020

Please cite this article as: Critchley HOD, Babayev E, Bulun SE, Clark S, Garcia-Grau I, Gregersen PK, Kilcoyne A, Kim JJ, Lavender M, Marsh EE, Matteson KA, Maybin JA, Metz C, Moreno I, Silk K, Sommer M, Simon C, Tariyal R, Taylor HS, Wagner GP, Griffith LG, "Menstruation: Science and Society", *American Journal of Obstetrics and Gynecology* (2020), doi: https://doi.org/10.1016/j.ajog.2020.06.004.

"Menstruation: Science and Society"

"Menstruation: Science and Society" Meeting, NIH: Eunice Kennedy Shriver National Institute of Child Health and Human Development, Bethesda, Maryland, USA 20-21 September 2018

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Disclosure: HODC has clinical research support for laboratory consumables and staff from Bayer AG and provides consultancy advice (but with no personal remuneration) for Bayer AG, PregLem SA, Gedeon Richter, Vifor Pharma UK Ltd, AbbVie Inc; Myovant Sciences GmbH. HODC receives royalties from UpToDate for article on abnormal uterine bleeding.

Financial Support: Some of the data herein were derived from research grants funded by the Medical Research Council (G0000066, G0500047, G0600048, MR/J003611/1); Wellcome Trust (083908/Z/07/Z); NIHR Efficacy and Mechanism Programme (12/206/52).

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Source of funding: This work has been supported by the NIH grants P01-HD57877 and R37-HD38691.

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Source of funding: Research Evaluation and Commercialization Hub (REACH); Center for Biotechnology's NIH award entitled "Establishing a Long Island Bioscience Hub"; National Heart, Lung, and Blood Institute of the National Institutes of Health-Award Number U01HL127522; The Endometriosis Foundation of America (EFA).

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Disclosure: KAM is co-investigator for Bayer Essure longitudinal research study and clinical trial (all funds for this research go to the site of research (WIH) – no personal compensation). KAM is scientific advisor for Myovant (advise on patient questionnaires related to AUB) – compensation goes to employer (CNEMG) – no personal compensation. KAM has received honoraria from ABOG, ACOG, and NIH for participating in working groups and meetings. KAM is HHS Office of Population Affairs Title X Grant Reviewer (received honorarium)

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Source of funding: Wellcome Trust grant (100646/Z/12/Z), Academy of Medical Sciences (SGCL13).

Disclosure: JAM declares no conflicts of interest.

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Disclosure: CM declares no conflicts of interest.

Source of funding: Research Evaluation and Commercialization Hub (REACH); Center for Biotechnology's NIH award entitled "Establishing a Long Island Bioscience Hub"; National Heart, Lung, and Blood Institute of the National Institutes of Health-Award Number U01HL127522; The Endometriosis Foundation of America (EFA).

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Disclosure: GPW declares no conflicts of interest.

Financial support: John Templeton Foundation grant 61329; NIH U54-CA209992; WSU19073

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Financial support: NIH U01EB029132-01; The John and Karine Begg Fund, and the Manton Foundation.

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Abstract

Women's health concerns are generally underrepresented in basic and translational research, but reproductive health in particular has been hampered by a lack of understanding of basic uterine and menstrual physiology. Menstrual health is an integral part of overall health as between menarche and menopause, most women menstruate. Yet for tens of millions of women around the world, menstruation regularly and often catastrophically disrupts their physical, mental, and social well-being. Enhancing our understanding of the underlying phenomena involved in menstruation, abnormal uterine bleeding (AUB), and other menstruation-related disorders will move us closer to the goal of personalised care. Further, a deeper mechanistic understanding of menstruation – a fast, scarless healing process in healthy individuals – will likely yield insights into myriad other diseases involving regulation of vascular function locally and systemically. We also recognize that many women now delay pregnancy and that there is an increasing desire for fertility and uterine preservation. In September 2018, the Gynecologic Health and Disease Branch of the Eunice Kennedy Shriver National Institute of Child Health and Human Development (GHDB NICHD) convened a two-day meeting, "Menstruation: Science and Society" with an aim to "identify gaps and opportunities in menstruation science and to raise awareness of the need for more research in this field". Experts in fields ranging from the evolutionary role of menstruation, to basic endometrial biology (including -omic analysis of the endometrium, stem cells and tissue engineering of the endometrium, endometrial microbiome, and AUB and fibroids), translational medicine (imaging and sampling modalities, patient-focused analysis of menstrual disorders including AUB, smart technologies/apps and mHealth platforms) to societal challenges in health literacy and dissemination frameworks across different economic and cultural landscapes shared current state-of-the art and future vision, incorporating the patient voice at the launch of the meeting. Here, we provide an enhanced meeting report with extensive up-to-date (as of submission) context, capturing the spectrum from how the basic processes of menstruation commence in response to progesterone withdrawal, through the role of tissue-resident and circulating stem and progenitor cells in monthly regeneration – and current gaps in knowledge in how dysregulation leads to AUB and other menstrual-related disorders such as

adenomysosis, endometriosis, and fibroids – to the clinical challenges in diagnostics, treatment, patient and societal education. We conclude with an overview of how the global agenda concerning menstruation, and specifically menstrual health and hygiene, is gaining momentum, ranging from increasing investment in addressing menstruation-related barriers facing girls in schools in low/middle-income countries, to the more recent "menstrual equity" and "period poverty" movements spreading across high-income countries.

Introduction

Twenty-five years have passed since the National Institutes of Health (NIH) mandated that women and minorities be included in all government-funded clinical studies unless their exclusion could be justified. Clearly, this policy has led to numerous women's health research programs. However, women and women's health concerns continue to be underrepresented in research. Most recently, the 2019-2023 *Trans-NIH Strategic Plan for Women's Health Research was initiated to improve the health of women by advancing rigorous research relevant to advancing women's health, including sexual and reproductive health.* Despite focused initiatives like these, d*i*agnostic development for improving women's reproductive health has been hampered by a lack of understanding of basic uterine and menstrual physiology. A PubMed search of the term "menstruation" yielded less the 1,000 publications between 1941 and 1950, followed by a peak of more than 6,000 publications between 1971 and 1980 (note: *Our Bodies, Ourselves*, a book addressing women's health topics, including menstruation and birth control, was published in 1973), and a then a stable trough with less the 4,000 publications per decade over the past three decades spanning 1991 through 2019 (Figure 1A). By contrast, a PubMed search of the term "menstrual blood" yielded 1 publication during 1941-1950, followed by a steady increase over time to just over 400 publications in the last decade (Figure 1B). For reference PubMed searches of "peripheral blood" and "semen" yielded almost 100,000 and 15,000 publications, respectively, over the past decade.

In September 2018, the Gynecologic Health and Disease Branch of the Eunice Kennedy Shriver National Institute of Child Health and Human Development (GHDB NICHD) convened a two-day meeting to "identify gaps and opportunities in menstruation science and to raise awareness of the need for more research in this field". Leaders in the field with expertise in endometrial biology, omic analysis of the endometrium and menstrual effluent, new imaging or sampling modalities, smart technologies/apps and mHealth platforms, menstrual health, and health literacy and dissemination frameworks were invited to participate as speakers and discussants to critique and summarize new discoveries and avenues of future research surrounding menstruation. This meeting encompassed normal menstrual health and endometrial

function and the potential of diagnostics for abnormal functioning and disease. To provide a broad perspective on menstruation science, this meeting included investigators and stakeholders across multiple disciplines, including population health and public health sectors, and considered carefully the broader societal implications of menstrual health. This manuscript summarizes the presentations and discussions that took place at the 2018 Menstruation Science and Society Meeting hosted by the Gynecologic Health and Disease Branch (GHDB) of the NICHD.

1. Toward a better understanding of Menstrual Health: Menstrual Health Literacy and Communication

Kristen A. MATTESON, MD, MPH, Missy LAVENDER, MBA, Erica E. MARSH MD, MSCI, Kami SILK, PhD

I Introduction

According to the World Health Organization, "health" is "a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity".[1] For women, menstrual health is an integral part of overall health because, between menarche and menopause, most women menstruate and menstruation can have significant impact on physical, mental, and social well-being.[2] Normal menstruation is currently defined as as cyclic bleeding that occurs from the uterine corpus between menarche and menopause. It can be described in terms of 4 simple domains: how frequently the woman has episodes of bleeding; the regularity or predictability of these episodes; the duration of bleeding episodes; and the volume or heaviness of bleeding.[3-5] Not all women experience "normal" menstrual bleeding; up to 30% of women will experience alterations in the volume or pattern of menstrual blood flow, which is defined as the symptom of abnormal uterine bleeding (AUB) which can be caused by multiple etiologies, and sometimes more than one etiology at the same time.[3, 6] In addition many women will have other symptoms such as pain, dysmenorrhea, anxiety, depression, and fatigue associated with their menstrual cycle that require attention in order for them to achieve early diagnosis of reproductive health issues such as endometriosis, premenstrual syndrome, and premenstrual dysphoric disorder and attain optimal health. In research and in clinical care, a better understanding of what the norms of menstrual health are and how a "lack" of menstrual health impact women's quality of life is needed. Furthermore, for positive health and well-being outcomes, everyone – men and women as well as clinicians- need to understand menstrual cycles and menstrual health, which can be achieved through menstrual health literacy initiatives and improved health communication.

Menstrual health and menstrual health literacy are extremely broad topics with multiple stakeholders and diverse areas of active investigation and contributors. Adapted from the broader health literacy definition, menstrual health literacy refers to the level of capacity a person has to obtain, process, and understand basic information about menstruation so they can make appropriate health decisions.[7] This section of the manuscript summarizes the presentations and discussion that took place related to menstrual health and menstrual health literacy at the 2018 Menstruation Science and Society Meeting hosted by the Gynecologic Health and Disease Branch (GHDB) of the NICHD. We summarize only areas of menstrual health and literacy that were part of the presentations and active discussions at the NICHD GHDB meeting, which were largely focused on the bleeding aspect of menstrual health.

II Progress in menstrual health terminology and menstrual health literacy and communication

Progress in menstrual health terminology.

Standard terminologies related to menstrual bleeding, and specifically AUB, represent real progress for clinical care and research. Ill-defined terminologies to describe symptoms, signs, and diagnoses associated with abnormal uterine bleeding led to communication challenges in clinical care, difficulty interpreting populations included in published literature, and lost opportunities for multi-site research collaboration for clinical research on treatments for AUB.

In 2005, the Menstrual Disorders Workgroup/Committee of the International Federation of Gynecology and Obstetrics embarked on a world-wide consensus-building process to generate and disseminate a simple symptom description system and a classification system for the etiologies associated with AUB.[8, 9] The first system, “Terminologies and Definitions”, includes standard definitions for bleeding symptoms domains which include regularity, frequency, duration, and volume.[3] The second system, “Classification of Causes of AUB in the Reproductive Years”, commonly referred to as the PALM-COEIN system, includes a list of etiologies that can be associated with AUB (polyps, adenomyosis, leiomyoma,

malignancy and hyperplasia, coagulopathy, ovulatory, endometrial, iatrogenic, and not otherwise classified). Results of this work were published in peer-reviewed publications and were used by the American College of Obstetricians and Gynecologists (ACOG) during their process standardizing terminologies used across gynecologic specialities.[4, 5] ACOG and the members of the Women's Health Registry alliance convened the revitalize GYN Data Definitions initiative in December 2013 to develop standardized data elements and definitions in gynecology. Throughout this process, ACOG engaged a broad range of stakeholders to identify priority topics and definitions and then worked with a core group of contributors to generate a total of 119 data elements, including 7 pain-related and 7 bleeding-related definitions.[4, 5] Although there is more work to be done in terms of evaluating these definitions across diverse populations of women, these standardized terminologies represent positive first steps to facilitate research data collection, collaboration for study participant recruitment, and identification of study cohorts with similar etiologies when investigating the prevalent symptom of AUB.

Another area of progress has been increased emphasis on the patient experience of bleeding, quality of life, and related symptoms associated with the menstrual cycle in both research and national guidelines. The National Institute for Health and Clinical Excellence (NICE) Clinical Guidelines on Heavy Menstrual Bleeding published a patient-centric definition of the symptom of heavy menstrual bleeding, which they define as "*excessive menstrual blood loss that interferes with a woman's physical, social, emotional, and/or material quality of life*".[10] Qualitative and quantitative research with women to learn about their experiences with and knowledge of menstrual bleeding, fibroid-related symptoms, pain and other associated symptoms has begun to inform research priorities, educational tools and the need for outcome measures for AUB and uterine fibroids.[11-14] Several studies have suggested that patient-reported outcome measures (PROMs), which include standardized interviews, questionnaires, charts, and surveys that assess the patient's own evaluation of her health and symptoms, are the key to assessing impact of illness and symptomatology among women with reproductive health issues including AUB.[2, 15-19]

Progress in menstrual health literacy and communication

There has been recent progress in the areas of menstrual health literacy, advocacy, and communication, in part facilitated by the rapid acceptance of mobile health (mHealth) applications, the use of mobile technologies to provide health-related services (tracking information and/or providing information or education to support an individual's achievement of health objectives).[20, 21] At present, there are over 300 reproductive health mHealth products in the IOS and Google stores, with the majority of the apps focused on women of child-bearing age. The apps vary in their depth of pelvic health information, with the majority including cycle and fertility trackers. Two of the larger apps as far as global reach, CLUE and FLO, have expanded both their tracking (CLUE) or daily notifications and information (FLO) to include facts or daily tips, which though brief, may be useful to assist in identifying women with the symptom of AUB. Additional data are needed to better understand the demographics of and reasons why women utilize these applications.

III Significant conceptual, practical, or technical challenges in the field of menstrual health research and menstrual health literacy

The progress in the field of menstrual health outlined above is remarkable given the multiple challenges and obstacles in the field of menstruation science. Menstruation is a physiologic process that is experienced almost universally across cultures from the ages of menarche to menopause. What makes menstrual health and menstrual health literacy challenging to study is that for many, it is a normal process that is not associated with any distress or disability, but for some it can be associated with significant negative impact on quality of life. Collecting data on a nearly universal process will require collaboration across the spectrum of disciplines and careful consideration of "who" to collect data from, "what" data elements to collect, and "how" to best collect data. Also, the normalization of women's pain and stigma surrounding menstrual bleeding and reproductive health represent significant barriers to women's care seeking, diagnosis, and ability to conduct research in this area.[22] Menstrual health and menstrual health literacy research is further complicated by a lack of standardization of tools and access to those tools, the

multiple different etiologies of heavy menstrual bleeding, the multi-dimensional symptom complex surrounding bleeding, lack of clear diagnostic tests for reproductive health disorders that affect menstrual health, suboptimal norms for menstrual health and bleeding across the lifespan, and insufficient information related to cultural perceptions related to menstrual bleeding and health.

Although awareness of the importance of patient experience with menstrual bleeding and menstrual symptoms has increased in research and clinical care, sustained reliance on "objective" laboratory measures for outcomes related to menstrual health represents an additional conceptual barrier to progress in this area. To provide an AUB-specific example, traditionally in research, bleeding was measured by volume of menstrual blood lost (>80 ml) as measured by the collection of used sanitary products and quantified using the alkaline hematin method.[23] However, research has highlighted that the majority of women who seek treatment for heavy menstrual bleeding do not meet objective mean blood loss criteria for heavy bleeding and clinical care objectively measured blood loss is not feasible.[19] As a result of these studies and others, The National Institute of Clinical excellence of the National Health Services in the UK stated in 2018 that "From the woman's point of view objective reduction in mean blood loss are poor indicators of treatment effectiveness for heavy menstrual bleeding".[10] This lack of consistency between what has been prioritized as a measurement for research and what women prioritize in terms of desired outcomes represents a current obstacle for high-quality research and synergy between research on heavy menstrual bleeding, clinical care, and patient-centered care delivery.

IV Critical gaps in menstrual health literacy/advocacy/communication and how they can be addressed to optimize women's menstrual health

Conceptual, practical, and technical challenges related to research on menstrual health and menstrual health literacy and communication have led to several critical gaps in the evidence base in this area. During the meeting, several gaps in the evidence base and opportunities to improve women's health by addressing these gaps with high-quality research were discussed.

Data to inform "norms" that hold across populations and span from menarche to menopause. Generation of standard terminologies related to norms for uterine bleeding among adult women represents significant progress in the field of menstrual health, and there has been significant progress especially in describing symptom expectations in the later reproductive life stages and during the menopausal transition.[24-27] To ensure that menstrual bleeding norms represent bleeding patterns and other menstrual health symptoms across a racially and ethnically diverse and contemporary population (relative to comorbid conditions and body mass index) of women of all ages, further research is needed.[28] Prospective longitudinal cohort data on menstrual bleeding, menstrual symptoms, and reproductive health diagnoses could fill this critical gap.

Developments in mHealth could also be used to inform norms and measure the personal impact menstruation and menstrual symptoms have on women across the lifecycle. For population based data outside of clinical care, data collected from mHealth and mobile device applications are starting to enable the analyses of population-level longitudinal menstrual symptom and cycle data.[29] Additionally, these m-Health data could facilitate investigation into cultural differences, knowledge, attitudes, and behaviors. By partnering with mHealth and app platforms, researchers, clinicians, and industry could generate data collection mechanisms and assist in generating research programs and interventions that could aid women with identifying when they are having a problem, address stigma and perceptions related to menstrual disorders, delays in diagnosis of reproductive health disorders, and delays in care-seeking.

Standardizing data collection in research, clinical care, and mobile health technologies to promote consistency and optimize comparative effectiveness research

A shift in research to focus on measuring patient experiences with symptoms and chronic health problems, including reproductive health and menstrual health issues represents significant progress in the arena of women's health. However, although there are several validated patient reported outcome

measures for AUB, however there is no single high-quality PROM that is considered "standard of care" or "standard for use across studies".[17, 30, 31] This translates into hundreds of outcome measures, of varying quality, used across studies and an inability to combine data across studies to summarize patient experience. In a systematic review of patient-reported outcomes used across studies of AUB, authors found 80 studies has used at least one PROM and 77 different PROMs were used across studies.[31] The Society of Gynecologic Surgeons, in a systematic review comparing treatments for heavy menstrual bleeding, identified that 114 different outcomes were collected and reported across 79 distinct clinical trials.[17] The end result was that, because the method of assessing outcomes differed from study to study, data could not be combined or summarized for these outcomes (such as quality of life and bleeding-related quality of life), which prohibited the group from generating consensus on treatment effectiveness relative to patient reported outcomes.[17] Researchers across disciplines of menstrual health research have expressed challenges describing the menstrual symptom phenotype of patients involved in clinical research because of a lack of standardized structured menstrual history data elements. Lastly, discussions at this meeting also highlighted the importance of a broader view of menstrual health that goes beyond bleeding to include other associated symptoms which will need additional research and standardized data elements.

The research community can collaborate to address this challenge and standardize outcomes and data elements for research and quality assessments. For example, The Core Outcomes in Women's and Newborn Health (CROWN), an international initiative led by journal editors and is endorsed by over 80 peer-reviewed journals in women's health, is working to stimulate the development of outcome sets that can be used across studies to ensure consistent outcome reporting thereby improving the interpretability of study results and the feasibility of combining data across studies.[32, 33] Efforts to standardize data elements from a structured menstrual history describing frequency, regularity, duration, and patient-quantified volume of bleeding along with other associated menstrual symptoms are needed to facilitate

consistent descriptions of populations in studies on menstrual health, AUB, uterine fibroids, and other reproductive health issues.[34]

V Additional future directions in menstrual health research

Transforming comparative effectiveness research by incorporating patient-reported outcome measures into electronic health records

Looking to the future, standardizing and harnessing the potential of patient-based outcomes assessment could transform comparative effectiveness research. Emerging technology developments may be paving the way to have PROM collection integrated into electronic health records which would promote patient-centered comparative effectiveness research.[35, 36] Researchers, policy makers, and professional societies are currently working out best practices for integrating PROMS and electronic health records.[35, 36] This integration could mean substantially greater capabilities for patient-relevant comparative effectiveness research and health services research, which often relies on electronic health record or administrative datasets that rarely incorporated patient reported data elements, particularly on reproductive health problems that impact quality of life.

Incorporating patient-reported outcome measure collection into clinical care encounters may represent major opportunities to evaluate processes of healthcare delivery. Future research opportunities include assessing whether or not incorporating patient reported outcome measures into electronic health records and clinical encounters for menstrual health disorders can improve physician-patient interactions and be used to monitor patient symptoms or progress over time. On the population level, incorporating PROMs into clinical care can assist with clinical care quality assessment and population surveillance. For example, in the UK's National Health Services, PROs are collected before and after certain surgical interventions to determine the quality of care delivery and to facilitate counseling patients on what to anticipate in terms of the personal impact of the surgery.[37]

Partnerships across diverse disciplines and stakeholder groups

Innovative solutions to address comprehensive menstrual health across the lifespan will require collaboration across scientific disciplines, social science disciplines and involvement of patient and person-facing organization to ensure the relevance and success of these solutions for addressing the needs of the population. Menstrual health research in the future could be enhanced by developing collaborative interdisciplinary teams to investigate comprehensive menstrual health pre-menarche to menopause. Additionally, including patient-facing groups in study design and beta testing of programs from the beginning and partnering with patient groups and advocacy groups to create and disseminate communication platforms and menstrual health educational initiatives could enhance the fields of menstrual health, menstrual health literacy, and menstrual health communication.

VI Conclusion

Each year, 4.5 million women in the United States experience at least one gynecologic health problem, and many of these problems are related to menstrual health.[6] Although significant progress has been made in menstrual health research in terms of emphasizing patient experience, standardizing terminologies related to menstrual bleeding, and use of patient-reported outcome measures for menstrual disorders, more work and research are needed to standardize data collection, generate longitudinal data on contemporary norms of menstrual bleeding and related symptoms, and optimize use of new technologies and educational interventions. Health communication strategies that are accessible to low literate groups and address potential stigma associated with menstruation also will help to address barriers. Increasing the evidence base on menstrual health and menstrual health literacy will aid in the evolution of contemporary clinical care that meets the unique needs of women. Bringing women and advocacy groups to the table and bringing data collection and information directly to women through innovative technologies, smartphone applications and mHealth has the potential to move the field of menstruation science away from treating problems and toward optimizing women's overall health, and more specifically menstrual health. Continuing the recent momentum on patient-focused menstrual health

research to sustain progress in the field of menstrual health, literacy, and communication has the potential to have substantial impact on the lives of women.

2. The Evolutionary History of Menstruation

Günter P. WAGNER, PhD

Menstruation and its associated diseases like heavy menstrual bleeding and abnormal uterine bleeding are a significant burden on women of reproductive age (see section *Menstruation and abnormal bleeding* below) which raises the question why women menstruate at all. This question is particularly pertinent given the fact that menstruation is dispensable for mammalian reproduction (see below). Answers require a review of the evolutionary history of mammalian reproduction, given that humans and great apes, i.e. species that menstruate, evolved from ancestors that did not menstruate. What are the advantages menstruation affords humans and other primates that, from a biological point of view, could make the origin and biological role of menstruation understandable?

I Menstruation is rare among animals

Menstruation is defined as the shedding of the upper (the so-called "*functionalis*") layer of the uterine lining after the luteal phase of the ovarian cycle. While menstruation is a normal part of the life of a woman during her fertile years, it is only found in a small minority of animals. As menstruation is a function of the female reproductive organs one would expect to find menstruation in animals with a similar mode of reproduction as humans, that is, the so-called placental mammals (technically called "eutherian mammals"). Eutherian mammals are all the species that descended from the most recent common ancestor of humans and elephants, meaning all the mammals that we are most familiar with: apes, monkeys, farm animals, cats, dogs, seals, hedgehogs and others more (Figure 2). All of these animals have a placenta and a gestational period that is longer than their ovarian cycle, so-called trans-cyclic gestation;[38] except animals that have pseudopregnancy in the absence of fertilization such as the dog.[39]

In spite of the substantial similarities, with respect to female reproductive biology, between humans and all other eutherian mammals (as compared to reptiles and birds, for example), menstruation only happens in a small minority of eutherian species. The largest cluster of menstruating species is found among our closest relatives, the primates. In particular apes, old world monkeys and most but not all of new world monkeys have menstruation. More basally diverging primate lineages do not (lemurs and tarsiers, where in the latter conflicting evidence has been reported, summarized in Emera et al.[40]). Outside the primates, menstruating species are rare. Among the rodents, only one species has been described as menstruating: the spiny mouse, *Acomys cahirinus*.[41] This is surprising, given the large number of rodent species (2,277 species). Then there is a small hand full of bat species belonging to two groups of bats, one molossid bats and three phyllostomid bats.[42] Most distantly related menstruating species to humans is the elephant shrew (*Elephantulus myurus*[43, 44]) related to elephants and other afrotherian mammals. These menstruating species add up to 84 species, or about 1.6% of the 5,149 recognized extant eutherian species. This estimate could be a slight undercount, since it is not easy to diagnose menstruation in species that have not been kept in laboratories or zoos and have been closely monitored.

If we put the menstruating species on the phylogeny of mammals (Figure 2), we see a rather dispersed distribution. Clearly all the primate species that menstruate are relatively closely related, but the spiny mouse, bats and the elephant shrew are not. The conclusion that follows from these facts is that menstruation must have evolved at least four times independently during the evolutionary history of mammals. This conclusion is also supported, for instance, by differences in the exact location and nature of the endometrial changes in the elephant shrew (summarized in [45]). The rarity and repeated evolution of menstruation raises the question about its biological role. Menstruation is clearly not necessary for a mammal, since it is rare, but it might have a specific role, rather being there accidentally, since it originated at least four times independently.

Before we turn our attention to the question of why some mammals menstruate and others not, we should mention that not every case of vaginal bleeding by a healthy animal is menstruation. The best known example is the vaginal bleeding of the dog, which is not a sign of menstruation.[39] The main difference between what is happening in dogs and in menstruating species is that the vaginal bleeding in dogs happens in pro-estrus, i.e. in preparation for mating, rather than after the fertile phase is over, as it is the case in women. The bleeding in dogs is due to extravasation during the growth of the uterine lining, which can break through the epithelium leading to a vaginal efflux.

II Why did menstruation evolve?

Given the fact that menstruation plays a major role in the life of a woman and given that it is rare among animals, has inspired many scientists, anthropologists and medical researchers to speculate about its biological role.[46-52] Here is not the place to review all the ideas that have been proposed to explain the evolution of menstruation but note that the most honest and shortest answer to this question is: *we do not know*.[53] Nevertheless, there has been some progress in reframing the question that points to two plausible answers.

An important breakthrough in understanding the evolution of menstruation was the realization that menstruation itself may not be the direct biological trait that was shaped by natural selection, but rather that menstruation could be a secondary consequence of an underlying biological trait: *spontaneous decidualization*.[52]

Decidualization is the process by which the uterine lining prepares for pregnancy. This is a complex process including proliferation of the endometrial stroma, the traffic of various kinds of white blood cells into the endometrium, and the differentiation of the endometrial fibroblasts (ESF) into so-called decidual stromal cells (DSC).[54] Decidualization in the narrow sense refers to the differentiation of DSC, rather than to the whole organ level process. In most animals decidualization occurs in the

estrogen/progesterone primed uterus in response to the presence of the embryo. This is *induced decidualization*. In humans, however, decidualization occurs even in the absence of an embryo and is therefore called *spontaneous decidualization*. It turns out that all menstruating species undergo spontaneous decidualization,[40, 41, 52] suggesting that the evolved trait is not menstruation per se, but spontaneous decidualization. In humans it has been shown that the proximate cause for menstruation (see section to follow on "Menstruation and abnormal uterine bleeding") is the decrease in progesterone levels due to the degeneration of the *corpus luteum*. An experimental model of artificial decidualization in a non-menstruating species, the mouse, *Mus musculus*, shows that in fact progesterone withdrawal after decidualization is sufficient to cause menstruation like symptoms, i.e. degeneration of part of the endometrium and vaginal bleeding.[55-58]

There are several versions of this experiment but the cleanest model is the one published by Rudolph and collaborators in 2012[56]: intact female mice were mated with sterile, vasectomized, males which in mice causes pseudopregnancy, meaning that the female maintains a high level of progesterone even though no fetus is developing in her uterus. After copulation the pseudopregnant mice were injected with a small droplet of oil into the lumen of the uterus. It is known that this treatment causes the uterine lining of the mouse to decidualize, leading to a so-called "deciduoma", i.e. a condition that, in many respects, mimics fetus induced decidualization. The key observation of this experiment then was that as progesterone levels were decreasing towards the end of the pseudopregnancy menstruation ensued. This result supports a model according to which menstruation is an inevitable consequence of spontaneous decidualization if fertilization and pregnancy do not occur (Figure 3).

Both, the comparative evidence, namely the association between menstruation and spontaneous decidualization among mammals, and the experimental evidence with artificial decidualization lead to the conclusion that the real question thus is not: "*why some species menstruate?*" but: "*why do some species*

show spontaneous decidualization, and menstruate as a consequence?" There are two plausible answers, but no definite consensus on this issue has been reached.

One model assumes that spontaneous decidualization is a protective device for the mother against an aggressive fetus.[40, 47, 52] This model is based on the observation that the degree of invasiveness of the placenta varies between species. This is even the case among species with so-called hemochorial placentation, i.e. where the fetus is destroying not only the uterine luminal epithelium but also some of the uterine blood vessels so that the placenta is in direct contact with maternal blood. For instance, great apes have extravillous trophoblast cells, which invade the maternal blood vessels (spiral arterioles), the stroma and even the muscular layer of the uterus (myometrium).[59] On the other hand, clinical observations show that a placenta that embeds too deeply into the uterus, a condition called "*placenta accreta*" or "*placenta percreta*" depending on the depth of invasion. These conditions can threaten the life of the mother after birth due to massive uterine bleeding.[60] Finally, one of the roles of the decidual cells is to both enable and limit the invasion of the placenta and thus regulate the depth of implantation even though the mechanisms are still unclear. Hence, it seems plausible that spontaneous decidualization is ensuring that a conceptus finds an environment which is prepared to allow and at the same time limit the degree of placental invasion. To our knowledge no formal test of this model has been attempted. In particular one would need a way to measure invasiveness of the conceptus in various animals, many of which are not laboratory models and thus hard to work with. Secondly, we have no information whether and which close relatives of menstruating species are also menstruating to test for a correlation between menstruation and depth of placental invasion.

The second model to explain the evolutionary origin of menstruation assumes that spontaneous decidualization is an adaptation to allow the female to "test" the viability of the conceptus before definite pregnancy ensues .[61-64] This model is inspired by the observation that decidual cells have the ability to sense the vitality of the embryo and react with a stress reaction when the embryo is of inferior quality.

The idea is that this ability of DSC helps the mother avoid investing resources in an ultimately unsuccessful pregnancy and thus increases the reproductive fitness of the female by allowing her to achieve pregnancy sooner. This idea is supported by the fact humans have a rate of pregnancy loss of 10 to 25% ([65]; higher estimates found in the literature seem to be spurious) and that spontaneous decidualization is primarily found in animals with small litter size, i.e. one or two neonates per pregnancy and thus with correspondingly higher investment into each offspring. The recently described, yet not fully evaluated, spiny mouse is somewhat an exception as the litter size is usually two or three but can be as high as six.[41] Again there is little comparative data to fully test this idea, given that we do not know the rate of pregnancy loss in most animals, and whether it is different between closely related species that differ in the presence or absence of spontaneous decidualization.

III An evolutionary argument for the validity of menstruation as a diagnostic tool

In a later section the utility of menstrual efflux as a diagnostic tool will be discussed in detail. Here we review an evolutionary argument that supports the idea that menstruation may be predictive of pregnancy complications in the future.

In the evolution of spontaneous decidualization the decidualization process becomes independent of the actual initiation of pregnancy. Nevertheless it is uncontroversial that the process of spontaneous decidualization is homologous to the process of embryo induced decidualization as the previous evolved from the latter.[40] The only difference is the mode in which the decidualization is triggered, either by maternal hormones as in spontaneous decidualization (as in women) or by the embryo as in induced decidualization (as in the mice/ rodents). This is the reason why experimental work on mice are a valid approach towards understanding human decidualization even though the mode of decidualization is different between these two species.

At the end of the ovarian cycle menstruation is caused by the withdrawal of the supportive function of progesterone for the decidua. As a consequence, menstruation has substantial mechanistic similarities with the processes that initiate labor.[66] Birth is also associated with either a systemic progesterone withdrawal either through the degeneration of the *corpus luteum* (*luteolysis*) or by functional progesterone withdrawal caused by inhibition of progesterone signaling.[67, 68] Hence, it is likely that the mechanisms deployed in the uterus during menstruation are homologous to those during parturition.[66] If in fact menstruation and the uterine manifestations of parturition are homologous it is likely that defects that affect the maintenance pregnancy and/or the initiation of parturition could also manifest themselves as aberrations in menstruation. Pavličev and Norwitz[66] therefore suggest that substantial research effort should be dedicated towards testing whether biomarkers expressed during menstruation are associated with pregnancy complications that could be useful as preconception diagnosis of likely pregnancy complications.

3. Menstruation in Humans

3A Menstruation and abnormal uterine bleeding

Hilary O. D. CRITCHLEY, MD, Jacqueline A. MAYBIN, PhD

I The impact of menstrual bleeding complaints

Understanding the mechanisms underpinning the pivotal human event of menstruation is critical to our understanding of abnormal uterine bleeding (AUB). AUB, which includes the symptom of heavy menstrual bleeding (HMB),[3] is a chronic complaint that impacts the quality of life and wellbeing of one in four women of reproductive age (Figure 4).[69] Previously, women experienced menstruation approximately 40 times due to pregnancy and lactation amenorrhea, whereas in developed economies today women can expect up to 400 menses in their lifetime.[70] Therefore, AUB is becoming more common and problematic for women and society. In contemporary society women are delaying having children for a variety of reasons including personal choice, prioritization of career and other factors that impose a delay in childbearing. Therefore, these women wish to preserve their uterus alongside their fertility. As a consequence, surgical options are not always appropriate as these end fertility and may also involve higher risks than medical management alternatives. In a recent systematic review relevant to the United States it was conservatively estimated that annual direct and indirect economic costs of menstrual bleeding complaints were in the order of one billion and twelve billion US dollars respectively.[71] Leiomyoma (uterine fibroids) are common, present in 70-80% of women by the age of 50,[72] and associated with AUB/HMB. Amongst women in their thirties and forties leiomyomas are often the underlying cause of abnormal uterine bleeding, anemia, and iron deficiency anemia. When the presence of uterine fibroids is considered along with complaints of AUB the annual estimated direct costs of this complaint in the USA, when surgery, hospital attendances, outpatient visits, and prescribed medications

are taken into account, are as high as 4.1-9.4 billion. Furthermore, lost work hours resulted in costs ranging from 1.55 to 17.2 billion.[71]

II A classification system for abnormal uterine bleeding (AUB)

In order to provide diagnostic precision and specific treatment of AUB, classification of causes of uterine bleeding is crucial. The FIGO Menstrual Disorders Committee has led on the classification systems for causes of chronic abnormal uterine bleeding in the reproductive years[3, 73]. As already mentioned there are two systems: the first system focuses on terminology with an encouragement for the removal of ill-defined terminologies such as "menorrhagia" and "dysfunctional uterine bleeding". The second system focuses on the underlying causes of AUB, utilizing the acronym PALM-COEIN[3, 73] for structural and non-structural causes respectively (PALM ployps/adenomyosis/leiomyoma/malignancy, COEIN coagulopathy/ovulatory/endometrial/iatrogenic/not otherwise classified) (Figure 5). It is hoped that these two FIGO systems will be utilized globally to improve management of women with AUB.

In the absence of any other features, for example leiomyoma or a coagulopathy[3] then bleeding from the endometrium may represent a "primary endometrial disorder" (AUB-E). In the presence of structural features such as leiomyoma, polyp, adenomyosis[3] then it is not known whether the presence of myometrial structural entities such as AUB-L (leiomyoma) or AUB-A (adenomyosis) actually result in a "secondary endometrial disorder"(Figure 6). There remains a true lack of knowledge about the phenotype of the endometrium when adenomyosis and/or leiomyomas are present.

This exciting area merits substantial research and many questions remain. What is the aberration in women with AUB-E? Do leiomyomas/adenomyosis contribute to the genesis of AUB/HMB? If so, is it because they directly impact the molecular mechanisms of endometrial haemostasis? Do leiomyomas actually need to be adjacent to the endometrium to cause AUB?[74] To answer these important questions, we need to fully understand endometrial physiology and pathology.

III Methods for the study of menstruation

Identification of aberrations in endometrial function necessitates study of human endometrial tissue. Women must have a detailed clinical history, examination and undergo investigation to determine if structural disorders are present. For research purposes, women should have measurement of their menstrual blood loss to enable categorisation as having heavy or normal menstrual bleeding. An objective measurement of blood loss may be obtained using the alkaline haematin method and total menstrual volume by using a menstrual cup.[23, 75] Alternatively, a pictorial menstrual blood loss assessment chart has been validated to assess menstrual blood loss volume and duration[76]). In addition, tissue must be carefully classified to determine correct stage of the menstrual cycle.

Studies in women are often limited to generation of observational data. For more incisive functional studies animal models of simulated menstruation have been developed.[55, 77-79] The non-human primate (rhesus macaque) has been studied extensively and provides an excellent model of the human menstrual cycle.[80, 81] More recently attention has focused on refinement of the mouse model of simulated menstruation.[55, 57, 77, 79]

A detailed study of the cellular and histological events occurring in the mouse endometrium during simulated menstruation have been reported to recapitulate several of the local events that occur in the human endometrium at the time of menstruation, these being, apoptosis preceding cytokine/chemokine expression and extensive neutrophil influx into the endometrium.[82] There is an interesting recent discovery of a previously unrecognized menstruating rodent, the spiny mouse, which may provide another tool in the study of menstruation.[41]

The combination of observational data generated from well categorized human endometrial tissue and mechanistic studies in validated animal models will facilitate definitive experiments to determine menstrual physiology and pathology.

IV Initiation of menstruation

The human endometrium is a highly dynamic multicellular structure. Its physiological functions are preparation for implantation and, in the absence of pregnancy, menstruation. The regulation of normal menstruation is governed by sequential exposure to circulating sex steroids, estrogen and then estrogen and progesterone followed by corpus luteum demise causing a fall in both circulating estrogen and progesterone. Progesterone withdrawal is the trigger for menstruation.[53, 83]

Menstruation involves a remarkable sequence of endometrial cell proliferation, differentiation, shedding, and regeneration that may occur as many as 400 times across the reproductive life course.[70] The mechanisms underpinning menstruation still remain poorly understood. There are crucial interactions between the endocrine and the immune system.[53, 83] These cellular interactions, which are menstrual cycle phase dependent, involve epithelial and stromal cells along with an influx of innate immune cells and differentiation of the endometrial vasculature (spiral arterioles). The local endometrial events at the time of menses resemble those of an inflammatory event. There is increase in endometrial blood vessel permeability and fragility, tissue breakdown, and an influx of innate immune cells into the endometrium, particularly neutrophils and macrophages.[82, 84, 85]

V Cessation of menstruation

The cessation of menstrual bleeding and endometrial repair requires three closely related events: these being vasoconstriction of the highly specialised spiral arterioles; local endometrial haemostasis; and re-epithelialization of the injured endometrial mucosa (Figure 6). Following menstruation, the restoration of the injured mucosal surface is a rapid event and the role of endometrial stem cells is addressed in the

following contribution concerning endometrial regeneration. Endometrial repair of the denuded epithelial surface following menstruation has been described by Garry and colleagues,[86] using hysteroscopy, histology, and scanning electron microscopy. These various imaging techniques detail the temporal repair of the epithelial surface, which occurs in a piecemeal fashion adjacent to actively menstruating tissue.[86] The regulation of this endometrial repair process is not fully defined. There is a recent interesting interpretation of the link between human menstruation and separation of the placenta following delivery. Both are underpinned by progesterone withdrawal and critically involve uterine spiral arterial function.[87]

In women with complaint of HMB impaired vascular differentiation due to impaired spiral arteriole maturation has been described[88-90] along with exposure to an imbalance of locally generated vasoconstrictors and vasodilators.[91-93] An increase in blood vessel radius will impact upon resistance to blood flow (Poiseuille's Equation).[91] The pivotal role for vasoconstriction following progesterone withdrawal was described nearly eighty years ago.[94] Study of autologous transplants of rhesus macaque endometrium into the anterior chamber of the eye and visualization of the events of menstruation through a slit-lamp ophthalmoscope revealed transient and intense vasoconstriction 4-24 hours prior to menstruation in response to steroid withdrawal. Authors proposed that this vasoconstriction was consistent with local tissue hypoxia. The presence and role of endometrial hypoxia in the process of menstruation has been debated. There is now experimental support for a pivotal role for transient physiological hypoxia as it has been demonstrated to occur in the menstruating endometrium.[58] The stabilization of hypoxia inducible factor 1 (HIF-1; a marker for hypoxia), results in generation of local repair factors to "heal" the injured mucosal surface (menstruating endometrium).[58]

Women with HMB demonstrate decreased endometrial HIF-1α at the time of menstruation and these women also experience prolonged menstrual bleeding episodes. These observational data have been

recapitulated in a mouse model of simulated menstruation where physiological endometrial hypoxia is also reported to occur at the time of endometrial bleeding.[58]

Fibrinolysis is an important component of regulation of normal endometrial bleeding. The human endometrium contains tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA), along with PAI (inhibits fibrinolytic activity) and the uPA receptor. Women complaining of HMB have raised levels of t-PA activity on the second day of bleeding when compared to those with normal menstrual blood loss, consistent with an overactive fibrinolytic system.[95, 96] Tranexamic acid, a popular non-hormonal treatment for HMB in many countries, targets the over activation of the fibrinolytic system with a reported 58% reduction in menstrual blood loss.[97]

VI Endometrial pathology in structural AUB

In the presence of structural pathologies (AUB-P/A/L), a secondary endometrial disorder has been proposed (Figure 6). The current literature presents lines of evidence to support the concept that there may be an element of resistance to normal progesterone regulated events.[98, 99] The latter is based on descriptive data and may certainly be implicated given the often reported poor response to many progestin based therapies, for example the levonorgestrel-releasing intrauterine system (LNG-IUS) and, oral, implant or injectable progestins.[69]

VII Therapies targeting the progesterone receptor

Progestins have long been used to modulate endometrial bleeding either through their action on ovarian function/abolition of ovulation along with a direct effect on the endometrium to reduce bleeding. All progestins, when delivered orally, systemically, or via an intrauterine route, improve menstrual experience in many women however there remains a consistent 20% who experience unscheduled endometrial bleeding and spotting. This is often a reason for discontinuation of use of progestin therapies. The mechanisms underpinning this unscheduled bleeding still remain elusive despite studies

focusing on many candidate pathways.[100-104] To date there has been no reliable preventative intervention albeit there are strategies to stop or reduce a heavy bleeding episode in users of progestin only preparations.[105]

Selective progesterone receptor modulators (SPRMs) reduced endometrial bleeding in women with uterine leiomyomas.[106, 107] SPRMs inhibit ovulation in 90% of women and also impact on the endometrium and many women experience amenorrhea.[108] The mechanisms of action of SPRMs on the endometrium in women with abnormal bleeding and uterine leiomyoma still remains poorly understood. SPRMs have an interesting anti-proliferative effect and study of cell to cell interactions within the endometrium in women exposed to this class of drug are a current topic of investigation.[109] SPRM administration is associated with an unusual morphological effect on the endometrium known as progesterone receptor associated endometrial changes (PAEC). These morphological features are associated with alterations in expression and localization of sex steroid receptors.[109-112] The fact that circulating estradiol levels remain consistently in mid follicular range has raised concerns amongst clinicians about risks of hyperplasia and endometrial cancer. However, no studies to date that have explored in detail the endometrial impact have reported increases in either hyperplasia or endometrial cancer.[113, 114] Indeed a recent systematic review reporting the endometrial effects of SPRM (ulipristal acetate;UPA) use in 10 studies involving 1450 women supports the current view that PAEC is essentially a benign endometrial morphology that is reversible on discontinuation of UPA use. [115]

VIII Summary comment

Understanding the pathology underlying AUB is essential to improve treatments for this common symptom that has a significant negative impact on women and society. Progesterone and progesterone receptor interactions play essential roles in uterine physiology and reproduction. Progesterone withdrawal remains the major trigger for the onset of endometrial bleeding. Menstruation itself involves repeated episodes of physiological "injury and repair" and a detailed knowledge of endometrial function

is essential for understanding of how disturbances of endometrial function play a role in abnormal uterine bleeding. A particular gap is the understanding of endometrial function in women with myometrial structural features such as leiomyoma or adenomyosis and whether this represents a “primary or secondary endometrial disorder". There is without doubt utility and validity of mouse models of simulated menstruation, particularly when used alongside human studies. Ligands for the progesterone receptor, i.e. progestins and SPRMs may reduce endometrial bleeding and modulate endometrial form and function. Identification of novel targets for the treatment of AUB is vital to address the significant personal and societal burden of this common disorder.

3B Regeneration after menstruation- the role of stem cells

Hugh S TAYLOR, MD

I Introduction

In each monthly menstrual cycle the endometrium is renewed from the basalis layer.[116-118] This regenerative process recapitulates some features of development and includes production of all components of the endometrium, including glands, stroma, vasculature and an influx of immune cells. The ability to rapidly and repetitively regenerate this tissue is fundamental to reproduction. It is therefore not surprising that there exists a population of cells that serve to replace and maintain the endometrium despite repetitive loss with menstruation.[116-118] These stem cells maintain a reservoir of regenerative cells while simultaneously giving rise to more differentiated cells.

II Endometrial stem cells

Early research in stem cells centered on the hematopoietic system, as experimental transplants to repopulate bone marrow could be performed using tissue ablation.[119] These studies gave rise to the concept that stem cells divides asymmetrically, reproducing the stem cell and giving rise to a more differentiated cell, in contrast to the symmetric division observed in somatic cells.[119] However, translation of this asymmetric division concept to other tissues and organs has recently become controversial, as tremendous plasticity in the fates of epithelial cells in the intestine, liver, and other organs is being uncovered.[119] While specific mechanisms remain debated, stem cells throughout the body maintain the pool of regenerative stem cells for populating each tissue and organ. Most tissues contain a collection of stem and progenitor cells that replace adult cells lost to age or damage. In many organs stem cells divide only under unusual conditions such as in response to injury. In other organs characterized by rapid turnover, such as the gastrointestinal tract, stem cells regularly divide in order to replace worn or damaged cells as part of normal tissue homeostasis.[119] In the endometrium the vast majority of cells are lost every month, making the need for frequent stem cell division more acute and essential.

Whereas totipotent, pluripotent and multipotent stem cells give rise to many different tissues, tissue-specific stem and progenitor cells give rise to a limited set of differentiated cells in a local environment. Tissue-specific stem / progenitor cells may give rise to a single cell type or several types of cells that make up an individual organ. In the endometrium, multiple lines of evidence in mice and humans support the presence of a population of stem / progenitors that give rise to stromal fibroblasts and another population that gives rise to epithelia. Much of the current knowledge on endometrial stem cells comes from studies in mice, where cell lineages can be traced using molecular tags and reporters, but understanding of the human endometrium is accelerating as more signatures of stem / progenitor cells in other organs are identified, investigated, and validated in the endometrium.[116-118, 120, 121] While early and even more recent studies in humans suggest that endometrial stem / progenitor cells are localized to the basalis layer,[122-124] more recent evidence of stem / progenitor cell markers in the luminal region suggests a more complex picture of wider dispersal,[125] as they are in non-menstruating species such as the mouse.[126] Indeed, recent studies using tracers in the mouse endometrium have identified stem / progenitor cells that give rise to both epithelial glandular and luminal epithelial cells[126 127, 128] while other tissue-resident stem / progenitor cells give rise to stromal cells in the mouse endometrium.[128] It is possible that there is a common stem cell that gives rise first to stromal cells and can also differentiate into a distinct bipotential epithelial progenitor cell, as suggested by studies in mice showing gene expression evidence of mesenchymal-epithelial transitions (MET)[57] as well as morphological evidence of such MET in humans.[129] These tissue-resident stem and progenitor cells regenerate the endometrium after menstruation in each menstrual cycle.

III Bone-marrow derived stem cells

There also exist multipotent stem cells in several tissues that can divide and differentiate into multiple types of cells and found in many tissue types. Most notably, bone marrow hosts both hematopoietic stem

cells, which give rise to circulating white cells, red cells, and platelets, and also mesenchymal stem cells, which give rise to bone, cartilage, and fat.[130] Bone marrow hematopoetic and mesenchymal stem cells are found in the circulation, where they can be recruited to sites of injury and contribute to tissue repair in ways that are still incompletely understood in humans.[131-133] In human patients who received bone marrow transplants, allowing donor cells to be tracked via sex chromosomes or HLA type, early studies reporting that bone marrow cells differentiated into hepatocytes or other epithelial tissue types are now mostly attributed to cell fusion or artefactual protocols.[133] However, bone marrow fusion to endometrial stromal cells has been characterized in mice and is rare in comparison to bone marrow cells directly contributing to endometrial cell fates.[134] Although the ability of mesenchymal stem cells to transdifferentiate broadly into cells in other tissue types remains controversial,[135] convincing evidence from human studies using single cell sequencing indicates that bone marrow derived donor cells differentiate into mature adipocytes – a known cell fate for mesenchymal stem cells.[136] Studies in mice and humans support the idea that bone marrow-derived stem and progenitor cells also contribute to the reproductive tract, supplementing the resident stem and progenitor cells. In both the mouse model and in humans bone marrow-derived cells are incorporated into the endometrium where they differentiate into endometrial stromal cells, epithelial cells and endothelial cells.[137, 138] The vast majority of bone marrow derived endometrial cells are stromal cells with epithelial cells differentiating slowly and in smaller numbers. Other groups have subsequently confirmed a bone marrow origin for endothelial cells in the human[139] and for stromal and epithelial cells in mouse[140, 141] establishing a potential role of bone marrow in endometrial repair in humans and prompting human clinical studies aimed at treating endometrial disorders.[118] Perhaps due to the depletion following menstruation, exogenous stem cells may be even more essential in the uterus than in other organs. Further, increased recruitment and engraftment of these cells to the uterus occurs in response to injury such as hypoxia or inflammation to aid in repair and regeneration.[142]

IV Consequences of stem/progenitor cell loss

Infection and iatrogenic trauma can lead to endometrial destruction and loss of progenitor cells, causing failure to regenerate lost tissue and resulting in permanent damage. Multipotent stem cells circulate to the endometrium and engraft, contributing to regeneration of damaged endometrium and mitigating endometrial atrophy, thin endometrium and Asherman's syndrome.[143, 144] However, these circulating bone marrow-derived stem cells are found in only very limited numbers in the circulation. In the setting of severe injury, the number of stem cells may prove insufficient to repair the damage. We have shown that augmented numbers of bone marrow cells in the circulation can prevent injury to damaged tissue including the endometrium. Transfer of bone marrow cells to mice after endometrial injury led to subsequently normal fertility while those receiving placebo had severe infertility due to Asherman's syndrome. Several case reports and non-randomized trials have explored delivery of endometrial stem cells to women with inadequate endometrial development or Asherman's syndrome with promising results for this potential novel therapy.[145, 146] Understanding normal menstruation and endometrial repair may provide insight to several endometrial pathologies.

We have also demonstrated that the chemokine CXCL12 attracts bone marrow mesenchymal stem cells to the endometrium.[147] In a mouse model we demonstrated that administration of CXCL12 to the damaged uterus can mobilize and recruit stem cells from the bone marrow to the uterus. In a mouse model of Asherman's syndrome intrauterine administration of CXCL12 led to restoration of normal fertility.[148, 149] Similarly, in a mouse model of thin endometrium treatment with either bone marrow supplementation or CXCL12 administration restores normal endometrial architecture and fertility.[144] Future therapy for Asherman's syndrome may make use of chemokines that mobilize and attract bone marrow cells without the need for bone marrow stem cell transplantation.

V Menstruation and potential role of endometrial stem cells in endometriosis.

While rapid endometrial regeneration is essential for reproduction in menstruating species, one of the adverse consequences of menstruation and a rapidly regenerating endometrium is endometriosis. Menstruation allows for retrograde menstruation and the possibility of ectopic implantation of endometrial tissue. Continued menstrual flow regularly feeds the endometriosis and allows for lesion expansion. Retrograde menstruation of stem cells in particular contributes to the lesions [150]. Further, bone marrow stem cells contribute to the continued growth of endometriosis lesions [138, 151]. Bone marrow derived stem cells may be responsible for those rare endometriosis cases outside of the peritoneal cavity such as endometriosis occasionally seen in the lungs or brain. The very processes designed to regenerate and repair the endometrium after menstruation can lead to disease. Here the circulating stem cells can even lead to endometriosis in areas where endometrial cells cannot reach even through retrograde menstruation.

While retrograde menstruation is a well-established cause of endometriosis, in reality endometrial cell trafficking is common; we have previously shown that stem cells from endometriosis can be found in the circulation in a mouse endometriosis model.[152] Similarly, we have shown that endometrial cells can be identified in very small numbers in multiple organs not typically associated with endometriosis including the brain, lung, spleen and liver.[153] This vast cell migration may explain many of the systemic effects of endometriosis. Women with this disease are more likely to suffer from depression, anxiety, autoimmune disease and have a lower average body mass index.[154] The regenerative ability of endometrium and use of circulating stem cells may allow for regeneration after menstruation and enhance fertility however also may predispose menstruating animals to endometriosis and associated disease. Endometriosis can be considered a systemic disease where widespread cell trafficking contributes to the pathophysiology.[154]

VI Endometrium, stem cells and pregnancy

Finally, endometrium has an essential role in the establishment of pregnancy. Indeed, many complications throughout pregnancy have their origin at the time of implantation.[155] It is not surprising that stem cells are an important part of endometrial/decidual function in pregnancy. We have recently shown that there is a major flux of bone marrow derived stem cells to the uterus in pregnancy.[156] These cells differentiate into endothelial cells as well as decidual cells that have a functional role in pregnancy. In a mouse model of infertility based on an endometrial receptivity defect, administration of normal bone marrow can restore fertility and successful pregnancy in otherwise infertile animals. This leads to the fascinating conclusion that some instances of infertility or pregnancy loss may be due to inadequate bone marrow rather than defects in reproductive organs or gametes. Indeed, one can now include the bone marrow as a key reproductive organ!

VII Beyond the Uterus: Menstrual-derived Stem Cells in the Context of Regenerative Medicine

The fast, scarless regenerative power of the endometrium, along with the relatively easy access to endometrial stem cells from menstrual effluent (see Section 4), has spurred efforts to use menstrual-derived endometrial stem / progenitor cells therapeutically for a range of regenerative medicine applications beyond those in the uterus mentioned above [157, 158]. Endometrial mesenchymal stem and progenitor cells[120, 124] share many properties with mesenchymal stem cells derived from bone marrow, adipose tissue, and other sources.[159] Like mesenchymal stem cells from these other sources, they can be readily expanded in culture, show features of differentiation into the canonical mesenchymal stem cell connective tissues (bone, cartilage, and fat) and produce a range of immune-modulating cytokines, chemokines and growth factors.[157, 159] Reports that mesenchymal stem cells from endometrium and other sources can transdifferentiate into a variety of non-connective tissues, including liver, pancreatic beta cells, and hepatocytes, both *in vitro* and in animal models or in studies of human bone marrow transplant patients, have been attributed to experimental artifacts, as described above and in Section 4.

Over 1000 clinical trials involving human mesenchymal stem cells or their products are currently listed on *Clinicaltrials.gov* as of May, 2020, with many in advanced Phase III stages of testing. Of these trials, about 400 are listed as involving autologous cells and about 300 as involving allogeneic cells (others do not specify in a searchable term; they may involve cell products such as matrix or exosomes). Clinical applications listed on *Clinicaltrials.gov* may broadly be divided into direct, permanent regeneration of connective tissues (bone, cartilage, fat, or related tissues), where autologous cells are required, and immunomodulatory applications that tilt healing toward regeneration rather than fibrotic repair through transient action of the therapeutic cells. For example, a phase III clinical trial is underway at the Cleveland Clinic to treat Crohn's disease fistulas with allogeneic bone marrow-derived mesenchymal stem cells following a successful Phase III trial outside the United States using adipose-derived mesenchymal stem cells.[160] Similarly, advanced clinical trials using allogeneic or autologous bone marrow-derived mesenchymal stem cells to modulate inflammation are underway for aplastic anemia,[161, 162] liver,[163] lung,[164, 165] and many other acute or chronic inflammation pathologies.

Of the mesenchymal stem cell clinical trials listed on Clinicaltrials.gov, only two employ menstrual-derived cells, both taking place Zhejiang University in Hangzhou, China: one for chronic liver disease;[166] and one for Type 1 diabetes. However, because menstrual blood-derived mesenchymal stem cells are an attractive source for autologous transplant in regeneration of connective tissues in women,[157] especially considering that connective tissue cells exhibit strong sex-based phenotypic differences, several regenerative applications are advancing through large animal studies. Particularly promising is the potential for endometrial mesenchymal stem cells to repair pelvic organ prolapse by seeding cells onto degradable scaffolds.[158] These and other connective tissue applications are moving toward human trials. Whether the ease of collection of human menstrual effluent-derived stem cells, or performance factors of these cells, will overcome the established infrastructure that relies on bone marrow, adipose tissue, and other sources for regenerative medicine or other purposes is difficult to predict. But they are in the running.

VIII Conclusions

In summary, menstruation in humans requires rapid regeneration of endometrium that is facilitated by stem cells. Stem and progenitor cells in the basalis layer are the major source of new endometrium each cycle. Bone marrow stem cells are engaged to repair endometrium after damage and are now known to be functionally important for pregnancy in mice.[156] Stem cells play a crucial role in reproduction. With this stem cells flux comes the possibility of disease related to aberrant endometrial growth- namely endometriosis. Endometriosis is a systemic disease of inappropriate stem cell differentiation. Menstruation is far more complex than simple loss of endometrium and regrowth- it requires contributions from stem cells both within the uterus and bone marrow. Menstruation also predisposes to endometriosis, which also involves far more than just the immediate surroundings of the uterus where most endometriosis settles.

3C What does fibroid (leiomyoma) research teach us about endometrial function?

Elnur BABAYEV, MD, Serdar E. BULUN, MD

I Pathophysiology of uterine fibroid (leiomyoma) formation and growth

Uterine fibroids are extremely common. More than half of women will develop uterine fibroids by 50 years of age[72] Patients present with abnormal uterine bleeding and/or pressure symptoms such as pelvic discomfort and pain, constipation or changes in urinary habits. Submucosal fibroids are also associated with infertility and early pregnancy loss.[167-169]

Fibroids are benign uterine tumors characterized by disordered monoclonal proliferation of uterine smooth muscle cells embedded in an abundant extracellular matrix. One proposed mechanism of fibroid formation involves genetic and/or epigenetic changes in multipotent stem cells in the myometrium that lead to abnormal proliferation and differentiation. Physiological fluctuations in sex steroid levels with subsequent growth and involution of myometrial cells during the menstrual cycle make these stem cells vulnerable to mutations or epigenetic changes and fibroid formation. The genetic, epigenetic, molecular and paracrine mechanisms underlying fibroid pathophysiology are highly diverse, which explains the observed variations in individual tumors' clinical behavior (growing, stable or regressing) and response to medications.[167, 168]

Fibroid growth is hormone-dependent and the sex steroids estrogen and progesterone are important regulators of fibroid growth. Most fibroids decrease in size after menopause, whereas pregnancy can lead to an increase in fibroid size. Both systemic and local estrogen can stimulate fibroid growth, but local estrogen production via aromatase activity in fibroid tissue appears to play an important role in fibroid pathophysiology.[170-172] Estrogen induces progesterone receptor expression and progesterone responsiveness of the tumor. Progesterone, on the other hand, has been shown to be essential for fibroid

growth in animal studies[173]. Progesterone may regulate fibroid growth indirectly via its action on differentiated smooth muscle cells, which in turn secrete paracrine molecules that stimulate proliferation of multipotent stem cells.[174, 175]

II The role of vasoactive substances and inflammatory molecules in the pathogenesis of abnormal uterine bleeding secondary to fibroids

Fibroids may interfere with normal endometrial function. In fact, heavy menstrual and/or irregular bleeding is the most common clinical presentation of fibroids and can affect the physical, social and emotional well-being of women. The degree of endometrial dysfunction appears to be related to the size and location of the fibroids. Submucosal fibroids located immediately beneath the endometrium are more likely to disrupt endometrial integrity and cause abnormal uterine bleeding. Subserosal fibroids are less likely to do so. Intramural fibroids represent an intermediate pathology, though large intramural fibroids that distort the endometrial cavity will likely lead to abnormal menstruation.[168, 169] Dissecting the mechanisms of interaction between fibroids and endometrium can help us understand menstrual biology and may lead to the development of novel therapeutic modalities for abnormal uterine bleeding.

Fibroids are space-occupying lesions that, depending on their size, can place significant mechanical stress on uterine architecture. Fibroids can increase the amount of bleeding by simply increasing the surface area of the endometrium. In addition, changes in cell shape and stretch can affect gene expression in the myometrium and endometrium.[176-178] Large intramural and submucosal fibroids may interfere with the myometrial contractions that occur during menstruation. These contractions help to evacuate menstruation material from the uterus and decrease blood loss from endometrial vessels under physiological conditions; thus, even a small submucosal fibroid can lead to significant blood loss in these patients.[167]

Endothelin-1 (ET-1) is a vasoconstrictor that affects spiral arterioles in the endometrium,[53] and plays an important role in myometrial contractility.[179] Altered expression levels of ET-1 and endothelin receptors (ET_A-R and ET_B-R) in uterine fibroids may interfere with the normal physiological function of the myometrium during menstruation. Fibroids have higher levels of ET_A-R and lower levels of ET_B-R compared to normal myometrium.[180, 181] Thus, it may be envisioned that altered endothelin biology induced by a uterine fibroid may affect the vascular function of the adjacent endometrium, giving rise to its irregular development or shedding.

Menstruating endometrium is rich in cytokines and prostaglandins. How this inflammatory milieu affects fibroids and vice versa is an active area of investigation. The composition of inflammatory cells is different in areas of the endometrium that overlay fibroids compared to distant sites. Perifibroid endometrium has increased numbers of macrophages in all phases of menstrual cycle; however, the number of uterine natural killer (uNK) cells is decreased in the secretory phase.[182] $PGF_{2\alpha}$ levels are increased in fibroid uteri, which may explain the disordered contractility and increased blood loss observed in these patients. Moreover, PGE2, which is produced in the normal menstruating uterus, affects leiomyoma (fibroid) cells. Normal myometrial cells do not show any changes in gene expression in response to PGE2, whereas leiomyoma (fibroid) cells demonstrate increased expression of anti-apoptotic micro-RNAs.[183] Celecoxib, a cyclooxygenase 2 (COX-2) inhibitor, reduces the proliferation rate of leiomyomas fibroids) via a nuclear factor κB (NFκB)-mediated decrease in expression of cytokines and growth factors.[184] Our understanding of the role of prostaglandins in fibroid pathogenesis may lead to new therapeutic approaches. Gene expression analysis of endometrial biopsies from women with heavy menstrual bleeding have also demonstrated differential expression of antigen processing pathway genes in women with and without fibroids.[185] Thus, specific molecular pathways might be responsible for abnormal bleeding associated with fibroids.

III Growth factors as primary mediators of endometrial dysfunction in fibroid uteri

Fibroid uteri demonstrate rich vascularity and increased venous plexus.[168, 169] There also appears to be defective vasoconstriction as evidenced by dilated venous spaces and vasocongestion. Increased angiogenesis is also apparent in patients with fibroids,[181, 186] with altered expression of angiogenic growth factors and their receptors. Variations in the number and type of inflammatory cells, which produce angiogenic factors, in the endometrium of fibroid uteri may contribute to the differences in the expression of these factors. Moreover, angiogenic genes are differentially expressed in fibroids, myometrium immediately adjacent to fibroids and distant myometrium.[168] Fibroids express increased levels of the important angiogenic molecule basic fibroblast growth factor (bFGF) and endometrium associated with fibroids demonstrates increased expression of the bFGF receptor, basic fibroblast growth factor receptor 1 (FGFR1).[186] Increased activity of bFGF through its receptor represents a possible pathophysiological mechanism underlying increased angiogenesis in fibroids, which may ultimately contribute to heavy menstrual bleeding.

A paracrine interaction between fibroids and the endometrium exists that is not just localized to the endometrium overlying the fibroid; this interaction has global effects on endometrial function.[187] For example, the WNT/β catenin pathway plays an important role in fibroid growth. Activation of this pathway leads to increased expression of transforming growth factor β3 (TGF-β3) and fibroids demonstrate increased expression of TGF-β3 compared to normal myometrium. TGF-β3 stimulates smooth muscle cell proliferation and fibronectin expression.[188] Moreover, TGF-β3 affects endometrial receptivity and decidualization by altering the expression of bone morphogenetic protein-2 (BMP-2 receptors).[189] This effect is likely secondary to decreased expression of homeobox A10 (HOXA10). Removal of intramural, but not submucosal, fibroids seems to reverse the changes observed in HOXA10 levels.[190, 191] Interestingly, endometrium obtained from patients with fibroids demonstrate decreased levels of plasminogen activator inhibitor 1 (PAI-1) and thrombomodulin. Endometrial stromal cells exposed to TGF-β3 *in vitro* show decreased levels of PAI-1, antithrombin III and thrombomodulin.[74]

These experiments suggest that fibroids affect menstruation by altering homeostasis in the endometrium. Changes in clotting factor levels may tip the balance towards anti-coagulation, which may at least partially account for the increased bleeding seen in patients with fibroids.

Taken together, fibroids appear to cause abnormal menstruation by interfering with myometrial contractility, paracrine signaling (growth factors, prostaglandins, endothelin, angiogenic factors) and hemostatic regulation (alteration in the expression of clotting factors) in the endometrium. Understanding the mechanisms of abnormal uterine bleeding secondary to fibroids will shed light on these endometrial functions in normal menstruation physiology and may lead to the development of new therapeutics for women with fibroids.

3D Microbiome of the Endometrium

Inmaculada MORENO, PhD, Iolanda GARCIA-GRAU, Carlos SIMON, MD, PhD

I Introduction

Humans have always lived in a microorganism-colonized world, in which microbes, especially bacteria, exist in clear symbiosis with humans. The concept of a human microbiota refers to the sum of microorganisms that inhabit the human body, and the number of commensal microbes is estimated to be the same as the number of human cells.[192] Human physiology is influenced by the presence of such microorganisms through expression of microbial genes (of which there are several million in total in contrast to only 23,000 human genes).[193] Thus, the human microbiome is considered our second genome and interacts with the host genome creating what is called a hologenome defining a whole complex organism and contributing to genetic diversity.[194] The balance between host and bacterial cells has been shaped through evolution, and the microbiota in each body niche has adapted in response to intrinsic (e.g., host genetics) and extrinsic or environmental factors (e.g., diet). This individual microbiota constitutes a critical component of immunity, and thus, colonization by different bacteria may turn this mutualistic or commensal interaction into a parasitic relationship predisposing the host to pathological conditions with variable severity of symptoms.

II Role of the microbiota in human health and disease

The first evidence that microbes contribute to health and disease comes from the 17th century when it was shown that bacteria from different body niches in the same individual are different, and there are different bacterial communities in the same body site in healthy versus diseased subjects.[195] It is now apparent that microorganisms, specifically bacteria, exert functional roles in our body and communicate with host cells by influencing metabolic function, training of the host immune system, and modulating drug interactions.[196] It is also known from studies in mice and humans that the profile of microorganisms inhabiting an individual early in life contributes to post-natal development and adulthood by influencing

metabolism, respiratory function, bone-growth, and immunomodulation.[197-200] Several studies have revealed that the gut microbiota also impacts neurodevelopment affecting an individual's behavior and cognition as well as susceptibility to mental disorders through the gut-brain axis.[201, 202] More recently, the gut microbiome has been linked to the secretion of circulating estrogens, leading to the estrobolome concept (Figure 7). Because estrogens are implicated in numerous biological processes, disequilibrium of the microbiota may subsequently contribute to a large variety of estrogen-modulated conditions, including metabolic disorders (e.g., metabolic syndrome, obesity), alterations of female reproductive function, and diseases in women (e.g., polycystic ovary syndrome, endometriosis, endometrial hyperplasia).[203]

III The existence of an endometrial microbiota

Molecular detection of bacterial communities via 16S rRNA gene sequencing has helped show that the human microbiota is related to human health and welfare, as symbiotic microorganisms colonize every human organ, including the reproductive tissues.[204] In adult women, the vaginal microbiota contributes to 9% of the total bacterial load and is characterized by a high stability with low richness and diversity indexes.[205, 206]

Highly sensitive detection techniques, for example the latest sequencing technology applied to microbiology, permits the study of microbial communities at the molecular level, providing ecological information about the microbiota of low biomass samples that have traditionally been considered sterile due to the inefficiency of culture-dependent methods for isolating some types of bacteria under standard laboratory conditions.[207] It must be noted that low biomass samples are susceptible to being masked by background bacterial DNA contained in laboratory reagents and equipment.[208] For this reason, the analysis of such samples requires extra caution during handling and manipulation as well as the simultaneous analysis of blank controls for monitoring potential contamination.[209] The endometrial microbiota is considered a low biomass microbiota as the total amount of bacteria colonizing the uterine cavity is 10^2-10^4 times lower than the total bacterial load in the vagina.[210, 211]

The microbiota of the upper reproductive tract was identified by studies applying molecular techniques, such as quantitative polymerase chain reaction or parallel sequencing, to endometrial, fallopian, and peritoneal samples (for a review Koedooder et al, 2019).[212] Recently, the existence of a microbiota continuum throughout the reproductive tract has also been described. *Lactobacillus* spp. are the most frequently identified bacteria in the lower reproductive tract of asymptomatic reproductive-age women, but the abundance and structure of the microbiota changes progressively towards the upper tract.[211] To date, several studies have analyzed the composition of the endometrial microbiome of reproductive age women using culture-independent methods. Comparative studies have shown that the endometrial and vaginal microbiota are similar but not identical in every woman.[210, 213, 214] Routes of endometrial seeding have been proposed,[212] with the most likely route being the ascent of bacteria from the vagina, as supported by the resemblance of the microbiota in consecutive spatial niches and the identification of *G. vaginalis* biofilms in the endometrial walls of women with bacterial vaginosis (BV).[215] The majority of studies analyzing the endometrial microbiota agree on reporting *Lactobacillus* as the most common bacteria detected in studies using culture-independent techniques, while other genera, from the Bifidobacteriaceae, Comamonadaceae and Streptococcaceae families, are also commonly found in the uterine cavity of healthy and fertile women.[211-213, 216-220] However, a recently published paper shows that, although 60% of the analyzed endometrial samples present a detectable microbiota compared to background controls and different from that in the vagina, rectum and oral cavity, *Lactobacillus* was rarely abundant in this type of sample.[221] Because each study used different designs, types of samples, and sequencing platforms, defining the core endometrial microbiota is challenging, no consensus has been reached so far regarding the molecular signature of the uterine cavity. In addition to the investigation of the core endometrial microbiota under physiological conditions, the role of endometrial microbiota in the origin and/or maintenance of several gynecological diseases, including pelvic inflammatory disease, endometriosis, and cancer, is currently under study.[211, 222-225]

IV. The impact of the endometrial microbiome on reproductive health outcomes, fertility and pregnancy

Fertility problems can be related to microbial disbalance in the reproductive tract. The cervico-vaginal microbiota of infertile women is more diverse and has lower levels of Lactobacilli (specifically *L. iners*) and higher levels of BV-associated bacteria (*A. vaginae, G. vaginalis, Ureaplasma* spp., *Leptotrichia, Sneathia*) than the microbiota of fertile women.[226-230] Moreover, the abundance of *Lactobacillus* spp. in vaginal and endometrial samples of infertile patients undergoing assisted reproductive technology (ART) is significantly lower than in samples from fertile volunteers.[217]

Pregnancy success is impacted by the endometrial microbiota as demonstrated by conventional culture techniques showing that isolation of bacterial pathogens from the tip of catheters used for embryo transfers associates with poor reproductive outcomes,[231-235] but the effect of bacteria on human reproduction is not restricted to the uterine cavity. Microbial culture of ovarian and follicular fluid showed that isolation of dysbiotic bacteria correlates with higher embryo discard rates and adverse ART success after IVF, while isolation of *Lactobacillus* spp. associates with better pregnancy outcomes.[236]

Our research group has used 16S rRNA sequencing to prospectively investigate the microbiota of endometrial fluid samples collected from IVF patients with repeated implantation failure in relation to their clinical results after embryo transfer.[213] *Lactobacillus* was more abundant in patients with successful pregnancy compared to those with cycle failure. Interestingly, high *Lactobacillus* abundance in endometrial samples was a significant variable for predicting the reproductive success of the patients. In contrast, low abundance of Lactobacilli together with specific pathogens associated with poor reproductive outcomes resulting in implantation failure, biochemical pregnancy, or clinical miscarriage.[213]

Also, as an incidental finding, we have been able to compare, at the taxonomical and functional level, the human endometrial microbiota present in a successful 4th-week pregnancy to that of a previous 8th-week

spontaneous clinical miscarriage in the same patient with euploid embryos. Bacterial diversity was lower and *Lactobacillus* abundance higher (*Lactobacillus iners* was the only bacterium found) during the healthy pregnancy.[237] These novel observations may profoundly impact our understanding and possible clinical translation of the microbiome in relation to healthy or pathological human pregnancy.

V Reproductive tract microbiome before, during, and after reproductive age

Understanding endometrial microbiota fluctuations during the lifecycle and in response to different stimuli will help to anticipate dysbiotic shifts from the *Lactobacillus*-dominated physiological state and will allow the design of novel interventional strategies to restore the endometrial microbial profile. However, because sampling the endometrium is invasive, no longitudinal studies have as yet been published describing the stability of the endometrial microbiota in the lifecycle of healthy, diseased, and/or infertile subjects. In contrast, the microbial profile of the vagina, which is easily sampled, has been temporally analyzed. Estrogen levels are the most critical variable driving vaginal microbiota changes occurring over a lifespan. Estrogen modulates the availability of glycogen in the vaginal epithelium and the subsequent growth of Lactobacilli (Figure 8).[238] Since *Lactobacillus* spp. produce lactic acid, the dominance of the vaginal niche by Lactobacilli entails the acidification of the niche (where *Lactobacillus* have a growth advantage) creating a hostile environment that impedes the growth of pathogens.

In children, the vaginal microbiota is mainly colonized by common aerobic bacteria (i.e., Enterobacteria, *Streptococcus*, *Staphylococcus*) and other Gram positive (i.e., *Actinomyces*, *Peptostreptococcus*) and Gram negative (i.e., *Veillonella*, *Bacteroides*) anaerobes. Interestingly, *Lactobacillus*, *Gardnerella vaginalis*, and *Prevotella bivia*, some of the most representative reproductive tract bacteria in adults, are absent from the vagina during this period.[239, 240] Then, coinciding with the estrogen rise at the onset of puberty, the bacterial profile is reshaped to resemble that of adult women, with increased abundance of *Lactobacillus* detected in premenarcheal adolescents. After menarche, the vaginal microbiota is

definitively stabilized and obtains the reproductive-age microbiota profile with dominance of *Lactobacillus* clusters in the majority of studied subjects. Interestingly, *G. vaginalis* levels also rise during puberty in some subjects even before their first sexual contact.[241]

The vaginal microbiome of healthy women can be classified in different community state types (CST) based on the structure of bacteria identified. Four CSTs are characterized by the dominance of *Lactobacillus* spp., namely *Lactobacillus crispatus* (CST-I), *Lactobacillus gasseri* (CST-II), *Lactobacillus iners* (CST-III) and *Lactobacillus jensenii* (CST-V). These four clusters associate with vaginal health, while a non-*Lactobacilli* microbiota abundant in reproductive tract pathogens, such as *G. vaginalis, Atopobium vaginae*, *Dialister*, *Megasphaera*, *Prevotella*, and *Sneathia*, is classified as CST-IV and associates with bacterial vaginosis (BV).[242]

Colonization and maintenance of microbial populations in the vagina of premenopausal women may be affected by many factors: age, hormonal milieu, hygiene, menstruation, use of contraceptives, sexual activity, ethnicity, etc. leading to potential CST shifts over short periods of time or even within one menstrual cycle.[242-244] During pregnancy, the richness and diversity of the vaginal microbiota tends to decrease, accompanied by increased *Lactobacillus* which is consistent with higher levels of estrogens.[245-248] However, dominance of vaginal microbiota by *G. vaginalis*, *Ureaplasma*, *Prevotella* or other pathogenic taxa during pregnancy associates with complications, mainly preterm birth.[249-251] After delivery, vaginal bacterial diversity increases and may generally shift to CST-IV for up to one-year post-partum even for women with high *Lactobacillus* abundance during pregnancy.[248]

During menopause, estrogen levels drop, and *Lactobacillus* spp. levels fall to become 10 to 100-fold less than in premenopausal women. This occurs with a concomitant increase in *Prevotella*, *Gardnerella*, *Atopobium*, *Ureaplasma* and anaerobic bacteria belonging to CST-IV. Interestingly, postmenopausal

women receiving hormone replacement therapy have levels of *Lactobacillus* similar to those observed before the menopause.[252-254]

VI Influence of menstruation on the reproductive tract microbiota

Hormonal changes within the menstrual cycle are proposed major regulators of the reproductive tract microbiota. During the menstrual cycle, circulating estrogens and progesterone positively correlate with community constancy, while during menses, the microbiota is more prone to bacterial changes.[243] There are different community trends during the menstrual cycle with some communities remaining stable across the whole cycle, while others suffer CST shifts in response to menses[243] and shift back after menstruation. A stable pattern is observed in some women colonized by *L. crispatus,*[255] while the majority of women undergo microbial population changes with menses, entailing transitions from microbiota dominated by *Lactobacillus* to microbiota with *L. iners, G. vaginalis,* Gram-positive cocci or other dysbiotic bacteria.[256, 257]

L. iners and *G. vaginalis* levels may rise in the vagina during menses because of their capacity to grow under adverse conditions. For example, *G. vaginalis* cannot grow in iron-limiting conditions but is able to secrete vaginolysin to lyse host cells (i.e., erythrocytes) to gather iron. Also, some *Lactobacillus* strains have protective mechanisms enabling them to grow in the presence of iron. For example, *L. crispatus* encodes an iron transport system. Similarly, *L. iners* synthesizes a unique iron-sulphur protein cluster that confers the ability to sequester iron from menstrual blood, providing *L. iners* with an advantage over *L. gasseri, L. jensenii,* etc. within the vaginal niche during menses.[255, 258, 259]

At the functional level, fluctuations of the cervicovaginal microbiota have been associated with innate immunity, HIV acquisition, inflammatory status and epithelial barrier function.[260] For example, women with *G. vaginalis* showed a sharpest decrease of the epithelial barrier protein Repetin (RPTN) from the ovulatory to the luteal phase than women with a *Lactobacillus*-dominated microbiota.[260] However, no

studies have identified yet the relevance of the microbiota for menstrual function, neither if the observed changes are driven by the cycling sex-hormone levels, the microbiota profile, other causing agents, or a combination. To shed some light on the role of bacterial taxa on menstruation, a recent study has characterized the endometrial and cervical microbiota of women with abnormal uterine bleeding at different phases of the menstrual cycle.[261] This study has revealed significant differences in the endometrial microbiota between women presenting with heavy menstrual bleeding and dysmenorrhea. While the endometrial samples of women with dysmenorrhea presented an increased abundance of *Acinetobacter* spp., facultative anaerobic genera were increased in endometrial samples of patients with dysmenorrhea, suggesting a potential contribution of microbial communities to these menstrual symptoms, although the cause-consequence analysis has yet to be undertaken.[261]

VII Mechanisms for bacterial-host interaction

How bacterial cells communicate with their hosts is still under investigation, but several mechanisms have been proposed. Bacteria can synthesize small molecules (e.g., short-chain fatty acids, proteins, oligosaccharides, vitamins, short non-coding RNAs, neurotransmitters, etc.) that may interact with host cells in several ways, including regulation of the physicochemical conditions of a niche, epigenetic regulation through proteins interacting with the host transcriptional machinery, or binding to host receptors (see reviews) (Figure 9).[207, 262, 263] Of note, amines produced by gut bacteria can, due to their chemical and structural similarity to human endogenous ligands, effectively bind G protein-coupled receptors (GPCRs) demonstrating how microbial metabolites might regulate host functions.[264] GPCRs comprise the largest family of receptors in humans and are responsible for a wide variety of intracellular processes in response to extracellular signals mainly mediated by hormones, neurotransmitters, or other stimuli. During reproduction, GPCRs are responsible for responding to neuropeptides and essential hormones, such as gonadotropin-releasing hormone, luteinizing hormone, follicle-stimulating hormone, prostanoids, and others, in the hypothalamus-pituitary-gonadal axis.[265] This type of host-microbial interaction is of outstanding relevance in pathological processes, as GPCRs are pharmacological targets of

35% of approved drugs, and the composition of the microbiota and its derived products could interfere with drug efficacy. Conversely, up to 25% of non-antibiotic pharmaceutical drugs designed to target human cells, including antidiabetics, antidepressants, antipsychotics, and some anti-inflammatory drugs, present antimicrobial activity or alter the composition of the indigenous microbiota, leading to potential side effects and increasing resistance to antibiotics.[202]

VIII Conclusions

The reproductive tract microbiome is currently considered a pivotal player in women's health. Further investigation of the underlying mechanisms of host-bacterial interactions is needed to better understand both physiologic and pathologic conditions. Translational implementation of this knowledge might allow us to shape the microbiome to promote global health using alternative methods and thereby avoid antibiotic abuse.

4. Menstruation as an investigative tool and diagnostic resource

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I Introduction

The process of menstruation produces a natural tissue biopsy that is arguably under-appreciated as a potential source of rich information on the health status of the endometrium. Growing awareness among patient populations about menstrual disorders, combined with advances in mHealth applications, data science, and the ever-decreasing costs of sequencing, are driving new opportunities to characterize normal and pathological menstrual functions. Interest in the endometrium as a model of fast, scar-less healing for a variety of regenerative medicine applications further motivates the study of both normal and pathological menstrual shedding and regeneration, using both analysis of shed menses but also tissue engineering approaches to capture complex interactions among epithelia, stromal, immune, and other cell types present in the uterus. Complementing these approaches, insight into the behaviors of the endometrium in the context of the uterus in health and disease are achieved with recent advances in imaging technologies.

II Using Menstrual Effluent (ME) to aid diagnosis of menstruation-associated conditions

Menstrual discharge contains shed endometrium, comprising endometrial epithelial cells, stromal cells, endothelial cells, other non-immune and immune cells together with microbial species present in the uterus (see section 3D on the microbiome) and vaginal tract along with a vast array of proteins, RNA, DNA and metabolites. It is distinctly different from peripheral blood and its composition aligns closely with that of the endometrium.[266] Since it is considered a waste product discharged from the body, the term “menstrual effluent” has been used by some investigators (in 41 papers on Pubmed as of Jan, 2020), while others refer to the discharge as menses or menstrual blood.

Menstrual effluent offers many advantages for investigating uterine health compared to endometrial/uterine tissues collected via surgical biopsies, including: non-invasive collection methods; relatively large sample volumes; and opportunities for repeat collections (within and across cycles). Although these advantages have been recognized since menstrual cups first became clinically available in the 1950's,[267, 268] menstrual effluent remains surprisingly understudied and until recently, only a few sporadic attempts in small-scale studies have been made to relate properties of the menstrual effluent to disease states, or to use it for disease diagnostics.[269-280] Biochemically, in healthy women, menstrual blood has been shown to have comparable concentrations of steroid hormones but higher prolactin levels when compared to peripheral blood[269] and proteomics analysis has revealed over 300 different proteins in menstrual blood compared to peripheral blood,[275] including scores of proteins and molecules involved in wound healing and regeneration.[277] Small-scale investigations of platelets and coagulation proteins in menstrual blood compared to peripheral or uterine vein blood have thus far given little insight into potential causes of heavy menstrual bleeding, but revealed that menstrual blood platelets were largely degranulated.[270, 271] At the cellular level, in healthy women, small studies showed that the NK cell repertoire in menstrual blood is stable over many menstrual cycles and different than peripheral blood,[276] and menstrual blood has relatively fewer CD16+ monocytes and more NKT cells compared to peripheral blood.[266, 279] Extending the immune cell analysis to disease states, a small (38 patient) study reported alterations in immune cell populations in the menstrual blood of women with fertility disorders.[280] Viable shed endometrial tissue collected from menstrual cups has also been studied to investigate the role of matrix metalloproteinases (MMPs) in endometriosis, though again the studies were pilot in nature.[272-274]

Several current forces support using menstrual effluent for diagnostics. Women are more comfortable with various forms of hygiene products, including menstrual cups, which are becoming more mainstream for managing menstruation, potentially allowing for more reproducible collections. At the same time, women are gravitating toward consumer health and digital recruitment platforms, creating new

opportunities for research and diagnostic development.[281, 282] Greater awareness of menstrual disorders and gynecological diseases is promoted by widespread reporting of personal suffering by celebrities, along with an explosion of social media platforms engaging patients, thus priming women/adolescents to seek answers for debilitating gynecology problems. Finally, the growing awareness of the shortcomings of blood-based biomarkers in diagnosis of complex, chronic diseases is driving increased interest in proximal tissue-based approaches.[283]

Endometriosis affects 6-10% of reproductive age females and is estimated to cost more than $20B per year in the US.[284-287] Endometriosis offers a particularly compelling case for development of minimally-invasive menstrual effluent-based diagnostics. Endometriosis is characterized by lesions of endometrial-like glands and stromal cells growing outside of the uterus, which are often associated with debilitating pain and infertility.[284, 285, 288] Although several theories for its etiology involve developmental origins,[289, 290] Sampson's theory of reflux menstruation into the peritoneal cavity remains a plausible explanation in light of the clinical presentation observed in most cases of endometriosis.

Numerous factors likely contribute to a diagnostic delay for endometriosis of up to 7 to 10 years.[291-293] Some women/adolescents experience vague symptoms that overlap with other conditions, while other women/adolescents have few or no symptoms and are not diagnosed until they present with infertility. In addition, women often experience minimization or dismissal of pain symptoms, more frequent misdiagnoses related to pain, and gender-related disparities in the treatment of pain when compared to men.[294, 295] Whereas standard MRI or ultrasound imaging can suggest the presence of endometriomas and a limited number of lesions in other locations, most patients display no detectable lesions upon imaging, thus motivating more invasive investigation of symptoms. The definitive diagnosis of endometriosis requires invasive surgery, a procedure many women/adolescents delay, avoid, or cannot afford. None of the peripheral blood biomarkers proposed for diagnosing endometriosis exhibit the accuracy required for clinical use.[296]

Menstrual effluent also offers a particularly attractive non-invasive diagnostic for endometriosis as numerous differences between the eutopic endometrium in women with endometriosis compared to unaffected women have already been cataloged at the cellular and molecular level based on analysis of biopsies.[297-299] These characteristics prompted the launch of a large-scale study to use cells in menstrual effluent as a minimally-invasive diagnostic for endometriosis. To date, over 5400 women, with and without endometriosis, have been recruited and enrolled through Research OutSmarts Endometriosis (ROSE) (https://feinstein.northwell.edu/institutes-researchers/institute-molecular-medicine/robert-s-boas-center-for-genomics-and-human-genetics/rose-research-outsmarts-endometriosis) and the Genotype and Phenotype (GaP) Registry,[300] respectively. Women consented to provide samples of menstrual effluent and access to their medical records (including the pathology report(s) documenting their diagnosis), and completed health/lifestyle questionnaires. In early studies women provided menstrual effluent samples using a re-usable menstrual cup (provided by Diva International). Once menstrual effluent collections and processing methods were standardized, the cellular composition of menstrual effluent cells were profiled and the menstrual effluent-derived stromal fibroblast cells (ME-SFCs) were characterized to develop a non-invasive diagnostic for endometriosis.[301]

As observed in previous studies, the menstrual effluent in the ROSE study was observed to be a complex, heterogeneous mixture of numerous cell populations, with a predominance of hematopoietic/immune cells.[266, 279, 301] Although the sample size in the initial published study is relatively small (n = 14 controls and 6-8 endometriosis subjects), menstrual effluent samples from endometriosis subjects were characterized by lower numbers of uterine natural killer (uNK) cells when compared to healthy control subjects.[301] Within the menstrual effluent, stromal cells (comprising <1% of cells) show numerous phenotypic and functional differences between controls and endometriosis patients,[301] similar to those previously described for stromal cells isolated from endometrial biopsies.[297, 302-304] Additional results

from examining the genetic and functional characteristics of menstrual-derived stromal cells support a dysregulated retinoic acid pathway associated with endometriosis vs. controls.[301]

One of the major barriers of this study was the inability to reliably collect menstrual effluent from women with pelvic pain using the menstrual cup. In response to this challenge, a novel diagnostic menstrual collection sponge is being developed for external use. Although this external collection sponge is still in development to maximize cell yield and collection of non-cellular content, the early experience has considerably simplified menstrual effluent collections from all populations, including adolescents. Ongoing studies are focusing on 1) refining assay methods to quickly and non-invasively diagnose endometriosis with reasonable sensitivity and specificity; 2) implementing a prospective study of women who provide menstrual effluent samples prior to diagnostic surgery, and then subsequently undergo laparoscopic surgery to definitively diagnose endometriosis as validation of the predictive power of this diagnostic test; and 3) enrolling adolescents (>9 years old) with symptoms of endometriosis, as this patient group may greatly benefit from an early diagnostic. If large enough samples sizes are evaluated, results may identify diagnostic phenotypes or stratify endometriosis subtypes for treatment. Repeated sampling of menstrual effluent may allow treatment responses to be assessed, if correlates with treatment response can be identified.

Collection and analysis of live cells in menstrual effluent offer potential for discrimination of patient subgroups based on analysis of cell identities, as well as phenotype responses to various stimulation ex vivo that promote decidualization, proliferation, or progesterone responsiveness, that can potentially provide insight into patient responses to therapies. However, cell isolation, characterization, and culture is a resource-intensive approach – similar to that employed for amniocentesis or chronic villus sampling. This method is feasible for research studies, but may be challenging to translate into routine clinical practice. By contrast, stabilization of the molecular constituents in menstrual effluent to allow sample storage and batch processing offers the possibility of lower cost, high information content data regarding

cell types present via highly standardized sequencing approaches. Genomic sequencing data on well-controlled patient populations are rapidly becoming available not only for the microbiome (see Section 3D), but also at single-cell resolution of the endometrium characterization for better disease genotyping.[305, 306]

To both take advantage of and contribute to the increasing availability of genomics data focus on the endometrial microenvironment, the company NextGen Jane developed a Smart Tampon system to provide facile access to menstrual effluent for diagnostic assessment of women's reproductive health using granular genomic analysis and bioinformatic deconvolution. The Smart Tampon may also be used on non-bleeding days for sampling the vaginal tract, allowing for a natural enrichment of the various cell types found in the reproductive tract, depending on day of cycle (ovarian/fallopian tube cells, cervical or endometrial cells and vaginal microbiome).

NextGen Jane studies show that transcriptional analysis of menstrual fluid has specific genomic characteristics that are unique from cervico-vaginal and venous blood samples. Analysis of menstrual fluid has shown that the genomic profile of menstrual blood varies greatly by day of cycle, with nearly 800 genes that are differentially expressed in menstrual blood on heavy flow day (day 2) compared to venous blood. Day 1 of the menstrual cycle shows little variability to venous blood, compared to day 2 where the greatest differential expression may be observed. In the future it is hoped that the optimal Smart Tampon will allow analysis of the genetics, epigenetics, microbiome, and transcriptome at scale. Methylation sequencing, transcriptomics, small RNA sequencing, microbiome analysis, and exome sequencing can produce up to 35 gigabytes of data. This platform has the potential to help fulfill the promise of machine learning and precision medicine for malignant, and non-malignant conditions in women's health.

Finally, analysis of menstrual effluent at either cell or genomic levels offers potential to improve clinical therapies by pointing to new mechanisms that might stratify patients into subgroups for different therapies. In many cancers, patients are stratified according to molecular markers that are related to the disease mechanism, prognosis and response to therapy. For diseases as common as endometriosis, adenomyosis, and others, it is likely that there are subtypes of patients with different molecular features that might respond to different therapies.[307, 308] Compared to cancer, where somatic mutations guide targeted therapies, the molecular features in endometriosis and adenomyosis are harder to identify as the presence of somatic mutations is still not well-established.[309] Menstrual effluent provides both molecular and cellular materials and hence, may improve diagnosis, as well as patient stratification towards a particular therapy.

III Tissue Engineering and Microfluidic Approaches to Study Menstruation Phenomena

Paradoxically, one of the most well-studied potential applications of menstrual effluent over the past 30 years is as a source of mesenchymal stem and progenitor cells (MSCs) for various non-reproductive tract tissue engineering applications. Although early reports that endometrial MSCs could transdifferentiate into insulin-producing islets,[310] cardiac tissue,[311] and other differentiated tissue have not borne out, applications in reconstructing connective tissues in the reproductive tract still hold promise.[312, 313] Tissue engineering of the endometrium as a target of learning about menstruation – defined as growing 3D models with at least stromal and epithelial cells present - has percolated at a low level for decades, hindered in part by the incredible difficulty in expanding and cryopreserving human primary endometrial epithelial cells compared to the relative ease of growing human primary endometrial stromal cells (even from menstrual effluent). The landscape changed dramatically in 2017 with publication of two papers reporting robust expansion of human primary endometrial epithelial cells as organoids in basement membrane Matrigel,[314, 315] using modifications of protocols established by the Clevers group for expansion of human intestinal epithelial cells[316] Recently, scaffold-free endometrial organoids comprised of both epithelial and stromal cells from endometrial tissue, were established providing yet another three

dimensional model of the endometrium to study important paracrine actions between two important cell types in response to menstrual cycle hormones.[317, 318] These protocols enable creation of tissue banks comprising all the major endometrial cell types, and lay the foundation for an explosion of activity in building models of the menstrual cycle.

Efforts to grow the endometrium and cells from the endometrium as a means to investigate its pathophysiology date back almost 100 years, with the earliest efforts targeted at trying to understand whether Sampson's hypothesis for retrograde menstruation as a cause for endometriosis could be substantiated.[319] The difficulty of growing epithelial cells – they reportedly grew poorly unless stroma was abundant, and epithelial grew as sheet to cover the explant – was noted in these early explant cultures.[319] The first 3D co-culture of primary human endometrial epithelial and stromal cells, comprising stroma embedded in a collagen gel, overcoated with basement membrane Matrigel seeded with epithelia, resulted in a well-differentiated confluent epithelial monolayer with a basement membrane with ciliated (luminal) and secretory epithelia and was tailored to study blastocyst implantation.[320] This model, which recapitulates hormone receptor expression and morphology, also revealed the changes in uterine receptivity that occurred with mifepristone compared to levonorgestrel.[321] An alternate model employing de-cellularized human endometrium re-seeded with stromal cells and epithelial glands showed hormone responsiveness over a 28-day cycle by secreting prolactin and IGFBP1, but it was unclear whether a monolayer with endometrial epithelial morphology was achieved.[322] The creation of multicellular endometrial organoids with polarized epithelial cells surrounding stromal cells provided a model to study paracrine interactions between two important cell types of the endometrium in response to hormones.[317, 318] Although several implantation and cell cross-talk models have been developed with endometrial cell lines,[323-327] the profound differences in production of cytokines and growth factors by cell lines and primary cells call into question the utility of such models.[328] However, a cell-line based model comprised of stromal cells embedded in a hormone cues: degradation and breakdown of tissue was observed in response to withdrawal of decidual-levels of progesterone.[329] The intricate crosstalk between

endometrial stromal and epithelial cells in driving hormone responses during menstruation has prompted efforts to create synthetic extracellular matrices for co-culture of endometrial stromal and epithelial cells in 3D, in a manner affording gentle dissolution of the ECM to release local cytokines and growth factors in the local pericellular environment and formation of confluent, stable epithelial monolayer in co-culture with an underlying stroma.[326, 328] While these approaches have not yet directly been applied to menstrual tissues, they are poised for this application.

A crucial missing element in 3D culture models of the endometrium needed for menstruation is microvasculature, which provides initial signals for decidualization[330] and regulates oxygenation cues important for tissue breakdown and repair.[58] Such models are on the horizon, as several microfluidic culture models of microvascular networks have been developed for studies of immune cell-microvascular interactions, tumor cell extravasation and growth, and blood-brain barrier.[331-335] Recently, approaches to using these models as foundations for mucosal barriers have been described. Aside from the intrinsic interest in menstruation, the interest in endometrium as a model of fast scar-less healing/tissue repair[277] has created momentum for applying these types of models to menstruation, in hopes of gaining broader insights into regenerative processes.

Finally, microfluidic approaches allow integration of multiple so-called “microphysiological systems” (MPSs), or 3D models representing part of a tissue or organ on a microscale. Integrated systems allow investigation of systemic effects, including hormonal and other factors that might influence menstruation. An enabling technology for such integration is a now-commercialized on-board microfluidic pump, first used to drive long-term culture of 3D liver tissue[336, 337] and adapted to study gut-liver interactions[338, 339] and ultimately an integrated platform supporting 10 different interconnected MPSs communicating in a common culture medium for a month,[340] including a 3D endometrium.[328] This platform pumping technology was also adapted to build a model of interconnected 3D units of ovarian, fallopian, uterine, cervical and liver tissues integrated into a single communicating fluidic system,[341] allowing the

assessment of up to five different types of tissues at a time over a menstrual cycle mimic. These cultured MPSs are responsive to ovarian hormones and when combined with other tissues, hormones responses were amplified.[341] As observed in other interacting-MPS studies, paracrine actions between tissues allowed the use of one universal medium without compromising the viability of the tissues during this study. Microfluidic technologies quickly are evolving as the need for user-friendly and affordable systems become evident for the research community. Microfluidics will change the way *in vitro* studies are done and will allow for new discoveries that will deepen our understanding of uterine biology and menstruation in a systematic way.

IV Next Generation Uterine Imaging

Uterine imaging has been employed to allow for non-invasive methods for diagnosing women's health symptoms. Imaging may be used to non-invasively assess conditions of pregnancy, as well as aspects of uterine health and pelvic health, including endometriosis.[342-344]

The indication for pelvic imaging varies by patient age and clinical presentation. Common indications in premenopausal patients include evaluation for focal endometrial or myometrial lesions (for example, uterine leiomyoma [fibroids]) in patients with symptoms of abnormal uterine bleeding and pelvic pain. In post-menopausal patients endometrial imaging is often performed to evaluate the endometrium in patients with post-menopausal bleeding.

Currently, diagnosis relies primarily on anatomical imaging using both ultrasound and magnetic resonance imaging (MRI) allows for direct visualization of the endometrium, which is complementary to the above described techniques, similarly noninvasive, but allowing for direct visualization of the endometrium in situ, rather than sloughed endometrial tissue. Ultrasound and MRI may evaluate for the presence of endometrial thickening, as well as the presence of focal endometrial lesions or polyps. In the evaluation of suspected endometriosis transvaginal ultrasound may be employed to assess for deeply

infiltrating endometrial implants.[345] MRI is useful to map endometrial implants throughout the pelvis, including extra-uterine locations, and confers advantages in terms of the larger field of view, multi-planar capabilities and excellent contrast resolution.[346, 347] A systematic review and meta-analysis (based on the results of six studies) compared the accuracy of transvaginal ultrasound vs. MRI for diagnosing deep infiltrating endometriosis.[343] The detection of deep infiltrating endometriosis by both MRI and transvaginal ultrasound methods demonstrated similar sensitivities - between 0.59-0.85 depending on the site, with greater sensitivity for detection in the rectosigmoid segment over rectovaginal, uterosacral and rectovaginal septum locations.[343] The specificities of MRI and transvaginal ultrasound were similar and like sensitivities, showed a wide range depending on the location.[343] It is expected that imaging methods will continue to improve and are likely to be employed in the diagnostic work-up for women suffering with symptoms of endometriosis.

Other potential future clinical applications of uterine imaging techniques include early endometrial cancer detection, distinguishing between leiomyoma and leiomyosarcoma, and assessing cancer response to treatment. Uterine and/or pelvic imaging may be combined with the cellular/molecular assessment of menstrual effluent to help aid in the diagnosis of uterine pathology and for improving the diagnosis of endometriosis via non-invasive methods.

MRI and USS are modalities currently used for monitoring and predicting response to therapies offered to reduce menstrual bleeding/ achieve amenorrhea prior to surgical interventions for management of AUB. For example, gonadotrophin releasing hormone (GnRH) analogues, a treatment expected to reduce leiomyoma volume and perfusion. Contrast-enhanced MRI is used clinically to assess suitability of patients with uterine leiomyoma for uterine artery embolization and to demonstrate reductions in perfusion post treatment.[348] Applications of T2-weighted (T2W) MRI for estimation of uterine and fibroid volume, may be augmented with dynamic contrast-enhanced MRI (DCE-MRI) for assessment of tissue perfusion and permeability, and magnetization transfer MRI (MT-MRI) to assess changes in

fibrosis and macromolecular content. Such approaches have been explored extensively in other organs.[349, 350] There has been limited application of DCE-MRI and MT-MRI in the assessment of uterine leiomyoma. DCE-MRI has been reported to be sensitive to vascular changes considered to accompany successful GnRH analogue treatment of leiomyoma.[351] Future development of MRI capabilities may offer complementary non-invasive modes to assess treatment responses for menstrual complaints. Furthermore, evolving MR imaging techniques during pregnancy that can track fetal motion and evaluate glucose and oxygen transport across the placenta may provide anatomical and functional information regarding placental health and fetal well-being.[352, 353]

V Conclusions

The analysis of menstrual effluent in combination with other new modalities for the understanding of uterine biology are in the very early stages of development. It is highly likely that the application of new technologies of genomic and cellular analysis of menstrual effluent and uterine tissues, including single cell approaches, will yield a deeper understanding of uterine pathophysiology, as well as new and less invasive methods of diagnosis, including, developments for body imaging. These new technologies may be applied to a variety of uterine health and female reproductive disorders, including endometriosis, uterine leiomyoma, as well as adenomyosis and, uterine-factor infertility, and will thereby aid management strategies for the symptom of abnormal uterine bleeding. We hope that these exciting scientific opportunities will catalyze a new era of collaborative investigation that will correct the past deficit of attention to female reproductive health and biology.

5. Addressing Menstruation Globally: Progress and Gaps

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I Introduction

The global agenda to address menstruation, and specifically menstrual health and hygiene, has gained significant momentum in recent years, ranging from increasing investment in addressing the menstruation-related barriers facing girls in schools in low- and middle-income countries, to the more recent "menstrual equity" and "period poverty" movements spreading across high-income countries. While there is growing recognition of menstruation as a relevant issue within public health globally,[354] there still exist many gaps in the evidence for informing program and policy. Reviewing how the menstruation agenda has shifted in the last fifteen years provides useful insights into how efforts have evolved, and what remains to be done.

II Shift in Menstrual Agenda Over the last 15 years

In reviewing how the global menstruation agenda has evolved, we explore shifts in the population of interest, the research and programs underway, the variation in activities by country income status, and the milestones achieved. There emerge from the analysis five periods of time during which distinct efforts were underway.

Earlier than 2004-2005

Prior to 2005, multiple efforts were underway exploring or addressing menstruation within global health. The population of interest included adult women of reproductive age, and in high-income countries, an interest in the declining age of menarche among girls. Interventions addressing adult women's menstruation-related needs were primarily within the clinical realm, such as a focus on reproductive health and disorders[355, 356] and the promotion of family planning.[357] Although the latter did not address

menstruation as a life course issue, there was attention to the challenges of unscheduled, breakthrough bleeding amongst other contributors to contraceptive discontinuation.[358]. There also existed a rich literature on menstruation within the social sciences, primarily derived from anthropologists documenting menstrual traditions and rituals, and its relationship to girls' and women's roles within society. In the 1980s and 1990s, in high-income countries in particular, researchers explored girls' maturation experiences,[359-361] examining the psychological effects of menarche, and the associations of early menarche with girls' engagement in risky behaviors, such as increased vulnerability to early sexual initiation,[362, 363] depression[364, 365] and substance use.[366] Overall, the focus in high-income countries remained on the individual and the clinical aspects of menstruation.

In contrast, in low- and middle-income countries there began to emerge a public health lens on menstruation. Alongside of the family planning agenda, there were burgeoning efforts within the water, sanitation and hygiene (WASH) field to address menstruation as a challenge facing girls in school.[354] UNICEF hosted a roundtable event in Oxford aimed at bringing attention to "menstrual hygiene management" (MHM), a newly coined concept focused on addressing menstrual management within WASH ,[367] and the Rockefeller Foundation supported a series of case studies on sexual maturation in schools in Africa.[368, 369] In humanitarian contexts, UNHCR recognized the provision of sanitary pads to refugees as part of one of its core mandates,[370, 371] providing important recognition of menstruation as a key response aspect.

2005-2011

This window of time brought an increased focus on girls as a population of interest, with a growing public health approach to menstruation in low- and middle-income countries. More specifically, important formative research was conducted with girls in and out of school, exploring their first menstrual experiences, their levels and sources of knowledge about menstruation, and how the onset of menstruation and puberty might be influencing girls' education.[372-374] The studies, conducted primarily in Africa and

Asia, suggested that many girls were experiencing their first menstrual period with no prior information or support, thus feeling confusion, shame and embarrassment, and for some, a significant fear that they were ill or dying.[375, 376] Multiple studies highlighted on-going taboos, restrictions and stigma around menstruation, and how menstrual onset and its management negatively impacted girls' abilities to engage and participate in school.[375, 377, 378] Social and physical barriers included, for example, inadequate toilets, water and disposal within school grounds, insufficient guidance and support around managing their menstrual periods, and for some, a lack of effective menstrual products and underwear.[379, 380] In response, a number of interventions emerged, such as the WASH in Schools (Wins) agenda that focused on addressing MHM in schools,[381] puberty books developed for girls in low-income countries that included content on MHM[382] new social entrepreneurs developing improved locally produced menstrual products for girls,[383, 384] and public-private partnerships by global sanitary pad companies focused on improving access to products.[385]

2012-2015

Over the next few years, menstruation gained traction as a public health issue for girls in particular. While in high-income countries it remained within the clinical realm for girls and women, in low- and middle-income countries, research documentation of the MHM barriers facing girls continued, and pilot trials began to be funded, primarily by the U.K. Government Medical Research Council (MRC), exploring MHM interventions for adolescent girls in school.[386, 387] A pilot trial in Kenya included, for example, the provision of sanitary pads, menstrual cups and reproductive health information, examining the impact on rates of sexually transmitted infections (STIs) and on reproductive tract infections (bacterial vaginosis), unintended pregnancy, school attendance and performance.[386] A case-control study in India examined women's vulnerability to reproductive tract infections in relation to the menstrual cloths or products they used; the sample drawn from hospitals.[388] Systematic reviews analyzed, for example, the psychosocial and educational impacts of addressing menstruation,[389] and a small number of studies explored the impact of early menarche on rates of infection with Herpes Simplex Virus (HSV) and HIV

and AIDS.[390, 391] These growing efforts, particularly those emerging from the water and sanitation arena, contributed to a decision to include MHM in the lobbying related to the new Sustainable Development Goals (SDGs), with the aim of having targets and indicators addressing MHM included in the SDGs. This led to the development of a formal definition for MHM (see Box 1).[367]

Box 1: Definition of MHM (JMP, 2012)

Women and adolescent girls are using a clean menstrual management material to absorb or collect menstrual blood, that can be changed in privacy as often as necessary for the duration of a menstrual period, using soap and water for washing the body as required, and have access to facilities to dispose of used menstrual management materials. They understand the basic facts linked to the menstrual cycle and how to manage it with dignity and without discomfort or fear.

During this period, additional donors began to support projects related to MHM. The Canadian Government provided funding to UNICEF and the UN Girls Education Initiative (UNGEI) to partner with Emory University on a 14 country WASH in Schools for Girls (WINS4Girls) project, which focused on conducting formative MHM research and developing intervention packages addressing MHM in schools.[381] The UK Government supported research on MHM in emergencies, providing funds to the International Federation of the Red Cross (IFRC) to assess beneficiary preferences around the types of menstrual products (disposable vs. reusable) in differing emergency contexts.[392] A new platform arose for sharing learning with the launching of an annual virtual conference co-organized by UNICEF and Columbia University showcasing research, practice and policy on MHM in schools.[393] Funding from the Canadian Government also enabled the creation of the "MHM in Ten" agenda led by UNICEF and Columbia University, which brought together WASH, education, sexual and reproductive health, gender and adolescent health experts to develop a ten-year agenda (2014-2024) aimed at transforming schools for menstruating girls.[394] Additional social entrepreneurs focused on developing affordable menstrual

products, and advocacy campaigns grew around "breaking the silence" on menstruation, including support for WASH United from the Bill and Melinda Gates Foundation and other donors to launch an annual global Menstrual Hygiene Day on May 28th.[395]

In addition, new publications began to call attention to the overdue need to explore additional ways in which menstruation impacts girls' lives, such as the need for data on the average age of menarche in countries,[396] the potential for menarche to be a window of opportunity for engaging girls, their parents/caregivers and teachers on health as a step towards subsequent conversations on sexual and reproductive health, including family planning,[397] and for women, their MHM experiences in the workplace.[398] A study conducted in India explored associations between the use and management of menstrual cloths and disposal pads and reproductive tract infections.[388, 399] The first resource guidance on MHM, *Menstrual Hygiene Matters*, was published with support from the U.K. Government (DFID), recommending approaches for addressing MHM in development and emergency contexts,[400] and UNESCO, with support from Proctor & Gamble, published a puberty policy document including attention to menstruation and MHM as a key component of puberty and comprehensive sexuality education.[401] There also emerged a stronger articulation of menstruation as an issue of health and human rights.[402]

2016-2018

During these years, there has been an exponential growth in attention to the menstruation agenda in global health. This included increasing resources and attention focused on research and interventions in low- and middle-income countries, along with a growing awareness that high-income countries were overdue to address the menstruation-related needs of girls in particular. The population of interest expanded around the world, with an on-going focus on girls in and out of school (ages 10-19) but growing recognition that menstruation presents challenges for women and all individuals who menstruate, such as those with differing gendered identities. The Bill & Melinda Gates Foundation funded FSG to conduct a global landscape in 2016, *An Opportunity to Address Menstrual Health and Gender Equity*, which examined the

existing research links between MH and broader health outcomes, social norms, and education.[403] The UK Government (Enhanced Learning and Research for Humanitarian Assistance (Elrha) funding /DFID and Wellcome Trust) supported the International Rescue Committee (IRC) and Columbia University to build the evidence on MHM in humanitarian contexts[404] and develop the *MHM in Emergencies Toolkit*; the latter was launched in 2017, with 27 co-publishing humanitarian response organizations. In 2018, USAID/OFDA provided additional funding to the joint team to focus on the menstrual product disposal, waste management and laundering needs of displaced populations with the aim of developing additional evidence and a compendium of practice. There also emerged a growing social and mainstream media attention. *Newsweek* and other major outlets published significant stories on menstruation, and the Period Poverty and Menstrual Equity campaigns emerged, focusing on removing taxes on sanitary products.[405] This growing global movement also introduced new conceptualizations and terminology in relation to menstruation which sought to broaden the issue beyond that of the focus on water and sanitation, such as menstrual health, menstrual health and hygiene and others. Medical Research Council, DfID, Wellcome Trust Joint Global Health Trials have also supported both feasibility pilot and full-scale trial evaluating potential effect of menstrual support on schoolgirls' sexual and reproductive health (SRH) and schooling outcomes.[386, 387]

Research, programming and policy all expanded during this period. A small number of pilot and full-scale quantitative studies continued or were initiated in Africa evaluating MHM interventions in schools,[376, 386, 406] with findings generated on new measures for addressing menstruation. Menstrual health policies were drafted in multiple countries, such as India, Zambia and Kenya, [386, 387, 406, 407] and in high-income countries, new legislation began to emerge, such as the dignity acts in the US which improve access to menstrual products for incarcerated individuals, and policies focused on improving access to products in homeless shelters and public schools.[408-410] Despite these important legislative efforts, limited evidence exists from the US and other high-income countries on the actual experiences, including barriers faced, of managing menstruation among girls, the incarcerated and homeless individuals. However, a

small body of evidence is emerging, particularly around the menstrual management needs of low-income populations in the US.[411] In addition, this window of time brought an explosion of attention to the provision of menstrual products, with Grand Challenges Canada, the Case for Her, and other donors supporting the scaling of social entrepreneurial efforts in this arena such as AfriPads, BeGirl, and others [412, 413] the launching of new global advocacy and networking organizations, such as the Menstrual Health Hub, the Menstrual Health Alliance, and the UNFPA supported African Coalition on Menstrual Health Management; and new regional research capacity building initiatives, such as the U.K. Government Global Challenges Research Fund supporting an East African research group.[386, 387]

Two challenges that remained included the lack of support from sectors beyond WASH, including limited attention to menstruation and its relevancy within sexual and reproductive health, education, gender, and other key sectors; and the limited funding available for furthering the measurement aspects of the menstruation-related agenda that would enable demonstration of the range of impacts of addressing menstruation.

2019-onward

Already in 2019, the evidence base and action is growing, with new publications examining what is known about MHM among populations with disabilities,[414] proposed revisions to the MHM definition to broaden the concept and its measurement beyond the original WASH origins,[415] additional systematic reviews,[416, 417] and on-going menstrual equity campaigns, global advocacy, and intervention trials. In an effort to move forward the existing menstruation measurement-related challenges, including the lack of uptake amongst other key sectors, a “Monitoring Menstruation” meeting was hosted by Columbia University in March 2019 with support from the Water, Sanitation and Supply Collaborative Council (WSSCC) that brought together key global monitoring and measurement experts from WASH, gender, education and health (sexual and reproductive; psychosocial) to review and find areas of alignment between the priority outcome and impact measures of these sectoral areas with the progress being made

on menstruation.[415] Importantly, USAID provided new funding to explore and pilot interventions addressing menstruation and women's economic empowermen.t[418] However, overall, resources still remain limited globally to support systematic coverage of all menstruation components, including access to information, water and sanitation infrastructure, supplies, and related clinical aspects, such as engagement with healthcare workers well trained on regular and irregular bleeding.

III Evidence on Menstruation Globally

As described above, the early years of the menstruation agenda included the use of primarily qualitative research methods, given the need for formative research on a sensitive topic about which there was little documentation from a public health perspective. In recent years, there has been a shift towards intervention trials, which have brought a rigorous quantitative approach to examining the impact of select menstruation-related interventions for girls in school in development contexts. Research in emergency contexts has primarily also been qualitative in nature, including feasibility pilots of guidance and programmatic response approaches. Funding has remained limited for larger scale intervention trials that include attention to water and sanitation in schools, to longitudinal associations between inadequate and adequate attention to menstruation and sexual and reproductive health and education outcomes, and to the relationship between menstruation and women's economic productivity and empowerment in the workplace. In addition, there has been a growth in national level data, such as the PMA2020 national surveys incorporating questions around menstrual management,[419-421] and the inclusion of questions on MHM within UNICEF's Multiple Cluster Indicator Surveys (MICS) in select countries.[422] Lastly, there exists limited evidence on the menstruation-related needs and experiences of girls growing up today in high-income contexts, and the MHM challenges faced by low-income and other vulnerable populations in such contexts.

IV Status of Menstruation-Related Programming and Policy

There exists a broad range of menstrual-related programming around the world. This includes, for example, non-governmental organizations (NGOs) providing sanitary products, reproductive health or MHM information, and improvement of water and sanitation facilities in schools, both in development and emergency contexts. Many national governments, such as South Africa, India and Kenya, have also begun subsidizing the provision of sanitary pads (reusable and disposable) to girls in school. In addition, new innovations are emerging in humanitarian contexts, such as effort by Medicins Sans Frontieres (MSF) to build female-friendly washrooms with disposal mechanisms in the health clinics they run in the refugee camps in Bangladesh hosting Rohingya populations.[423] Social entrepreneurs, such as Days for Girls, Sustainable Health Enterprises, BeGirl and Afripads continue to develop and evaluate the production and distribution of menstrual products, ranging from reusable pads made by local populations, to period underwear.

As mentioned above, there has been a growth in menstrual policies around the world. The Uganda National Bureau of Standards (UNBS) passed one of the first national standards for reusable sanitary pads in Africa.[424] A recent analysis of the existing higher level education policies in low-income countries indicated that education sector plans and policies still lack inclusion of attention to menstruation and its proxies (such as the provision of gender segregated toilets), which has implications for the inclusion of budget line items to address the issue in schools;[425] WASH in Schools focused menstrual policies provide important guidance on what interventions are needed, but lack sectoral buy-in and financial support. The Philippines provides an important example with recent policy being incorporated into the country's Education Monitoring Information System (EMIS), providing local level incentive to include, for example, improved toilets in schools and the provision of supplies of sanitary pads for emergencies.[426] However, as the "Monitoring Menstruation" meeting held in Geneva in March 2019 indicated, menstruation has yet to be taken up by other key sectoral programming and policy, such as within sexual

and reproductive health, which could improve attention to anemia in adolescent girls, or the potential for the onset of menstruation to trigger child marriage

V Current and Future Pathways

Moving forward there is much left to be done, including addressing the menstruation-related issues faced by all menstruators, including transmasculine and other populations, menstrual barriers faced in workplace contexts, recognition that more evidence is needed on vaginal bleeding across the life course, including the implications for the provision of water, sanitation, supplies and access to health care and information, improved engagement of health care workers on the issue of menstruation in low- and middle-income countries in particular, the important intersection of menstruation and family planning and improved measures for monitoring and assessing the impact of menstruation-focused interventions along with cost-effectiveness studies. Funding support has thus far been limited for addressing this broad spectrum of issues, and for essential intervention and measures related work that are needed to demonstrate critical associations between menstruation and population health more broadly. There is an urgent need for a strong funding stream to assure this impactful work can be done.

Glossary of terms

16S rRNA gene: encodes a component of the 30S small subunit of a prokaryotic ribosome. 16S rRNA gene sequencing is used for phylogenetic studies, as its presence is highly conserved among bacteria, but its sequence is species-specific.

Aromatase: An enzyme that transforms androgens into estrogens

AUB: abnormal uterine bleeding

Biomass: amount of living biological organisms in a given niche or ecosystem at a given time. The upper genital tract has a significantly lower amount of bacterial DNA than other human microbiomes and is therefore considered a low biomass microbiota.

BMP-2: bone morphogenetic protein 2

COEIN: Coagulopathy, Ovulatory, Endometrial, Iatrogenic, Not otherwise classified

Community state types (CST): profile that defines the total bacterial community of a given body site based on the relative abundances of each bacteria. The human vaginal microbial communities were classified into five groups. Specifically, CSTs I, II, III, and V are dominated by *L. crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii* respectively, whereas CST IV has higher proportions of strictly anaerobic organisms.

DCE-MIR: Dynamic contrast enhanced-MRI

Diversity (Beta diversity): refers to the change in the number of taxa detected in two or more ecosystems. It is usually expressed as the total number of species that are unique to each of the ecosystems being compared.

Dysbiosis: shift in the physiologic microbiota resulting in an imbalance between commensal and pathogenic bacteria. Changes in microbial composition due to the gain or loss of the community members or changes in the relative abundance of microbes may contribute to the initiation and/or persistence of many diseases.

Epigenetics: Heritable phenotype changes without changes in genotype (DNA)

Estrobolome: represents the aggregate of enteric bacterial genes whose products are capable of metabolizing estrogens. Microbes in the estrobolome produce beta-glucuronidase, an enzyme that deconjugates estrogens into their active forms, which are capable of binding to estrogen receptors and influencing estrogen-dependent physiological processes.

FIGO: International Federation of Gynecology and Obstetrics

GaP: Genotype and Phenotype Registry (registry of normal/control research subjects)

GnRH: gonadotrophin releasing hormone

Growth factor: A substance capable of stimulating cell growth, proliferation and differentiation

Gut-brain axis: consists of bidirectional neural processing of information between the central nervous and digestive systems. Recent research indicates that gut microbiota is a crucial part of the gut-brain network and communicates with the brain via the microbiota–gut–brain axis.

HIF: hypoxia inducible factor

HMB: heavy menstrual bleeding

Hologenome: theory that maintains that the physiology of any macroscopic organism derives from the integrated activities of the individual genomes contributing to the organism (holobiont).

LNG-IUS: levonorgestrel-releasing intrauterine system

ME: menstrual effluent

ME-SFCs: menstrual effluent derived stromal fibroblast cells

mHealth: mobile health

Microbiota and Microbiome: the human microbiota encompasses the group of microorganisms that live in association with the human body. Conversely, the microbiome refers to the genes and genomes of this microbiota as well as their products within the host environment.

micro-RNA: small non-coding RNA molecule regulating post-transcriptional gene expression

MRI: magnetic resonance imaging

MT-MRI: magnetization transfer-MRI

Multipotent stem cell: A cell that can self-renew by division and can develop into multiple differentiated cell types

Natural killer (NK) cell: a type of lymphocyte that can bind to certain tumor cells and virus-infected cells without the stimulation of antigens, and kill them by the insertion of granules containing perforin.

PA: plasminogen activator

PAEC: progesterone receptor associated endometrial changes

PAI: plasminomgen activator inhibitor

PALM: Polyps, Adenomyosis, Leiomyoma, Malignancy

Paracrine signalling: signaling involving hormone which has effect only in the vicinity of the cell secreting it

PCOS: polycystic ovary syndrome

Richness (Alpha diversity): refers to the diversity within a particular area or ecosystem. It is usually expressed by the number of species (species richness) in a unique niche.

ROSE: Research OutSmarts Endometriosis (research program dedicated to studying endometriosis)

SPRM: selective progesterone receptor modulator

TGF-β3: transforming growth factor- beta 3

T2W: T-2 weighted

t-PA: tissue plasminogen activator

uNK cells: uterine natural killer cells

u-PA: urokinase plasminogen activator

Acknowledgments

We thank Sheila Milne for assistance with manuscript preparation and Ronnie Grant for assistance with figure preparation. We thank Helena Ward, Bachelor of Arts with Honours in Illustration & Animation (First Class) for preparation of Figure 5 in Section 3A, and Sheila M. Cherry, PhD, ELS, President and Senior Editor from Fresh Eyes Editing LLC, for editing Section 3D.

We also wish to acknowledge Diana Bianchi, Lisa Halvorson and Candace Tingen for recognizing the global importance of this topic and for convening and facilitating the meeting in Bethesda and for encouragement with preparation of this manuscript.

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Figure Legends

Figure 1: PubMed publications 1941-2018 **A:** search term "Menstruation". **B:** search term "Menstrual Blood"

Figure 2: Phylogenetic distribution of menstruating species among eutherian mammals. Lineages in black are from menstruating species and lineages in white from non-menstruating species. The lineages with red outline are the lineages where menstruation originated. Note that there are at least four independent originations of menstruation.

Figure 3: Schematic outline of the experiment by Rudolph and collaborators (2012)[56] testing the idea that menstruation is a secondary consequence of spontaneous decidualization. The experiment is conducted with the laboratory mouse which is a species that, under normal conditions, is neither decidualizing nor menstruating. In this species clitoral/vaginal stimulation during copulation leads to the maintenance of the corpus luteum even if no pregnancy ensued, leading to pseudopregnancy, as is the case by copulation with a vasectomized male. Further it is known that injection of a small droplet of oil into the uterine lumen causes decidualization. The experiments starts with mating a female to a vasectomized male to induce pseudopregnancy. At the morning of the following day the females are checked for a copulatory plug to verify that copulation has taken place. Then on day 4 post copulation, a small droplet of oil is injected into the uterus to induce decidualization. Day 4 is the normal day of implantation in mice. Then the mice are monitored for their level of progesterone and signs of vaginal bleeding. Progesterone is starting to decrease after day 7 and bleeding ensues at about day 9. This experiment shows that differentiation of the endometrium (decidualization) is sufficient to cause menstruation like symptoms in a species that normally does not menstruate.

Figure 4. The modern impact of menstruation. Previously women experienced menstruation approximately 40 times in their lifetime, due to pregnancy and lactational amenorrhea. Women may now expect to have over 400 episodes of menstruation, mainly as a result of fertility management. Therefore, abnormal uterine bleeding (AUB) is increasingly common. Women may experience significant anemia resulting in poor physical quality of life. A negative financial impact occurs due to the cost of managing their blood loss and an inability to work outside the home. These costs, alongside a loss of caring ability will have a negative impact on the wider family. The cost to society via loss of work days and healthcare costs is significant.

Figure 5: The PALM COEIN classification for abnormal uterine bleeding in the reproductive years illustrating the structural (PALM) and non-structural causes (COEIN) and as described in Munro et al. Int J Gynecol Obstet 2011 and 2018.[3, 73]

Figure acknowledgment: prepared by Helena Ward; Bachelor of Arts with Honours in Illustration & Animation (First Class).

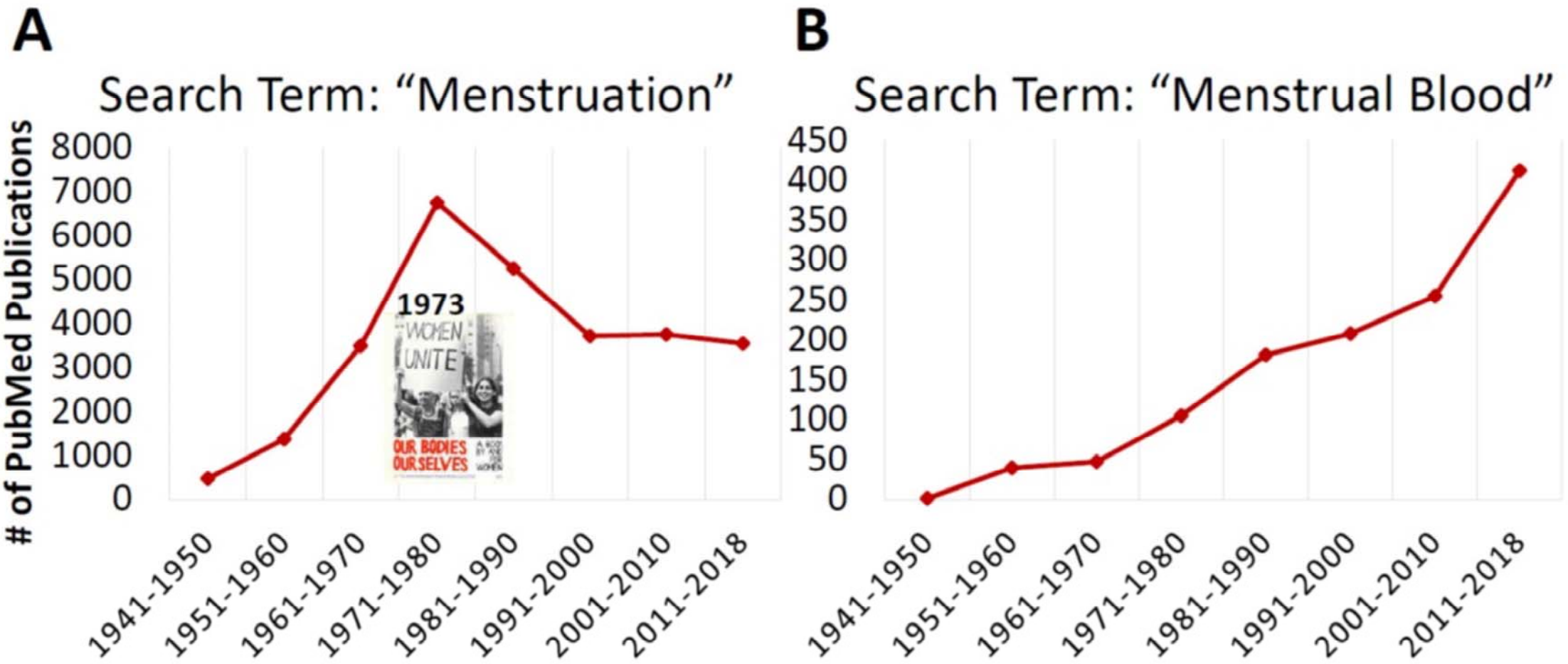
Figure 6. Potential mechanisms of 'primary' and 'secondary' endometrial abnormal uterine bleeding (AUB). As the corpus luteum regresses in the absence of pregnancy, progesterone levels fall. This occurs irregularly in those with ovulatory/iatrogenic AUB. Progesterone withdrawal causes a local inflammatory response in the endometrium, and may be increased in those with primary endometrial AUB. An increase in vasoactive factors results in intense vasoconstriction of spiral arterioles to limit blood loss, this may be decreased in primary endometrial AUB. Vasoconstriction may induce transient tissue hypoxia and stabilization of hypoxia inducible factor (HIF)-1, the master regulator of the cellular response to hypoxia, to coordinate endometrial repair. There is evidence that this is less intense in those with endometrial AUB. Efficient hemostasis limits menstrual blood loss at menstruation and this is defective in women with coagulopathy AUB. Structural and non-structural pathologies have the potential

to disrupt endometrial physiology at menstruation, leading to abnormal uterine bleeding, these mechanisms remain undefined.

Figure 7. The estrobolome plays a central role in health and disease through the gut microbiota-estrogen axis. Dysbiosis of gut microbiota may induce systemic inflammation and interferes with estrogen metabolism and receptor activation in estrogen-regulated organs influencing neurocognition, metabolism, and the onset of gynecological diseases and infertility (Baker et al., 2017).[203] "Reprinted from Maturitas, Vol. 103, Baker JM, Al-Nakkash L, Herbst-Kralovetz MM, Estrogen-gut microbiome axis: Physiological and clinical implications, Pages 45-53, Copyright (2017), with permission from Elsevier."

Figure 8. The vaginal microbiome during the lifecycle. Bacterial populations inhabiting the vagina change in response to estrogen levels, modulating glycogen availability in the vaginal epithelium and subsequently the growth of bacteria based on the physicochemical features of the niche at each phase of the lifecycle (Muhleisen and Herbst-Kralovetz, 2016).[254] "Reprinted from Menopause and the Vaginal Microbiome, Vol. 91, Muhleisen AL, Herbst-Kralovetz MM, Menopause and the vaginal microbiome, Pages 42-50, Copyright (2016), with permission from Elsevier."

Figure 9. Functional uterine microbiome may impact endometrial epithelial health through host-pathogen interactions, secretion of microbial molecules, and activation of the immune system (Baker et al., 2018).[207] "Reprinted from Frontiers in Immunology, Vol. 9, Baker JM, Chase DM and Herbst-Kralovetz MM, Uterine Microbiota: Residents, Tourists, or Invaders? Page 208, doi: 10.3389/fimmu.2018.00208, Copyright (2018), with permission from Creative Commons."


A
Search Term: "Menstruation"
# of PubMed Publications
8000
7000
6000
5000
4000
3000
2000
1000
0
1973
WOMEN UNITE
OUR BODIES OUR SELVES
1941-1950
1951-1960
1961-1970
1971-1980
1981-1990
1991-2000
2001-2010
2011-2018
B
Search Term: "Menstrual Blood"
450
400
350
300
250
200
150
100
50
0
1941-1950
1951-1960
1961-1970
1971-1980
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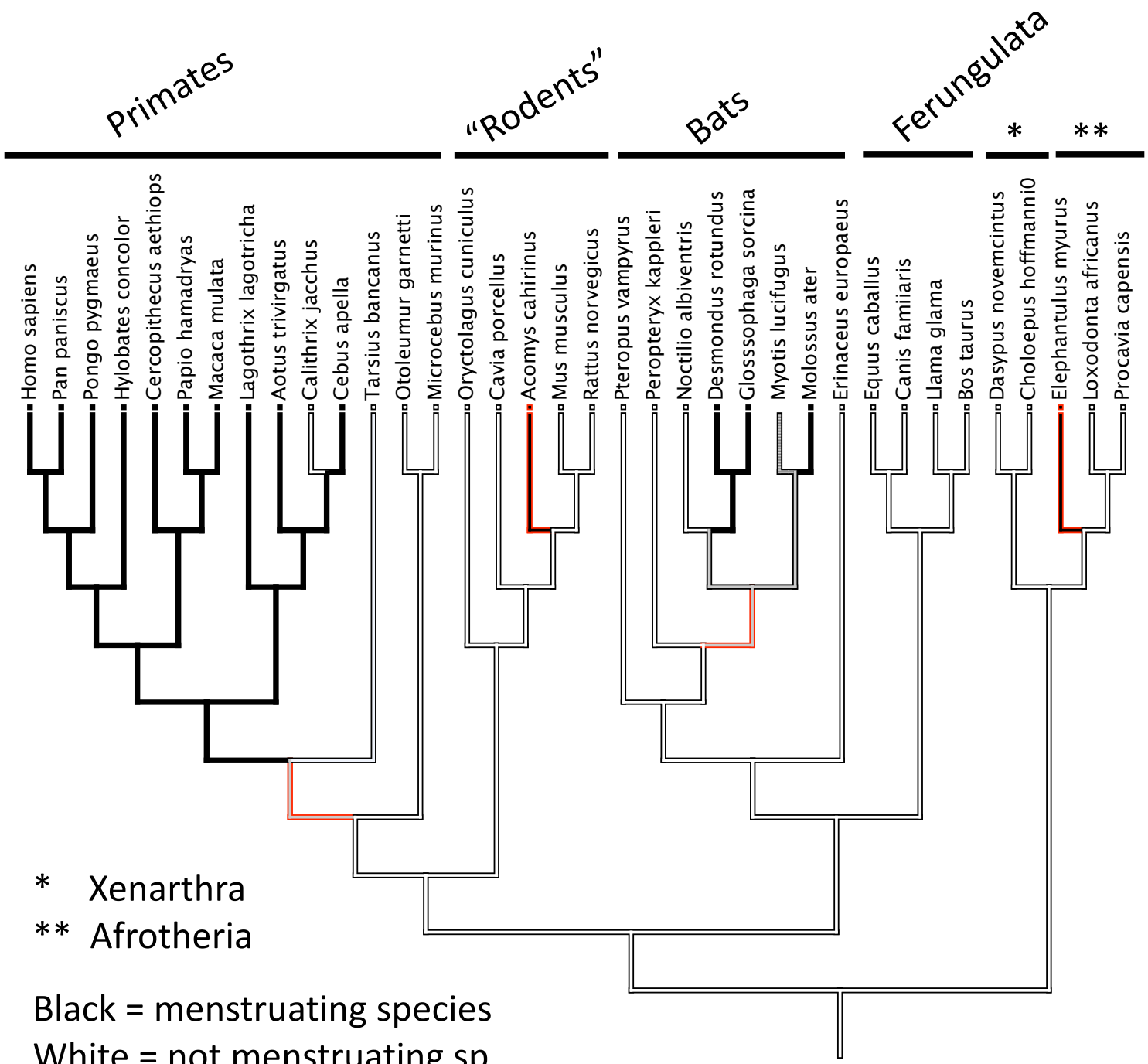
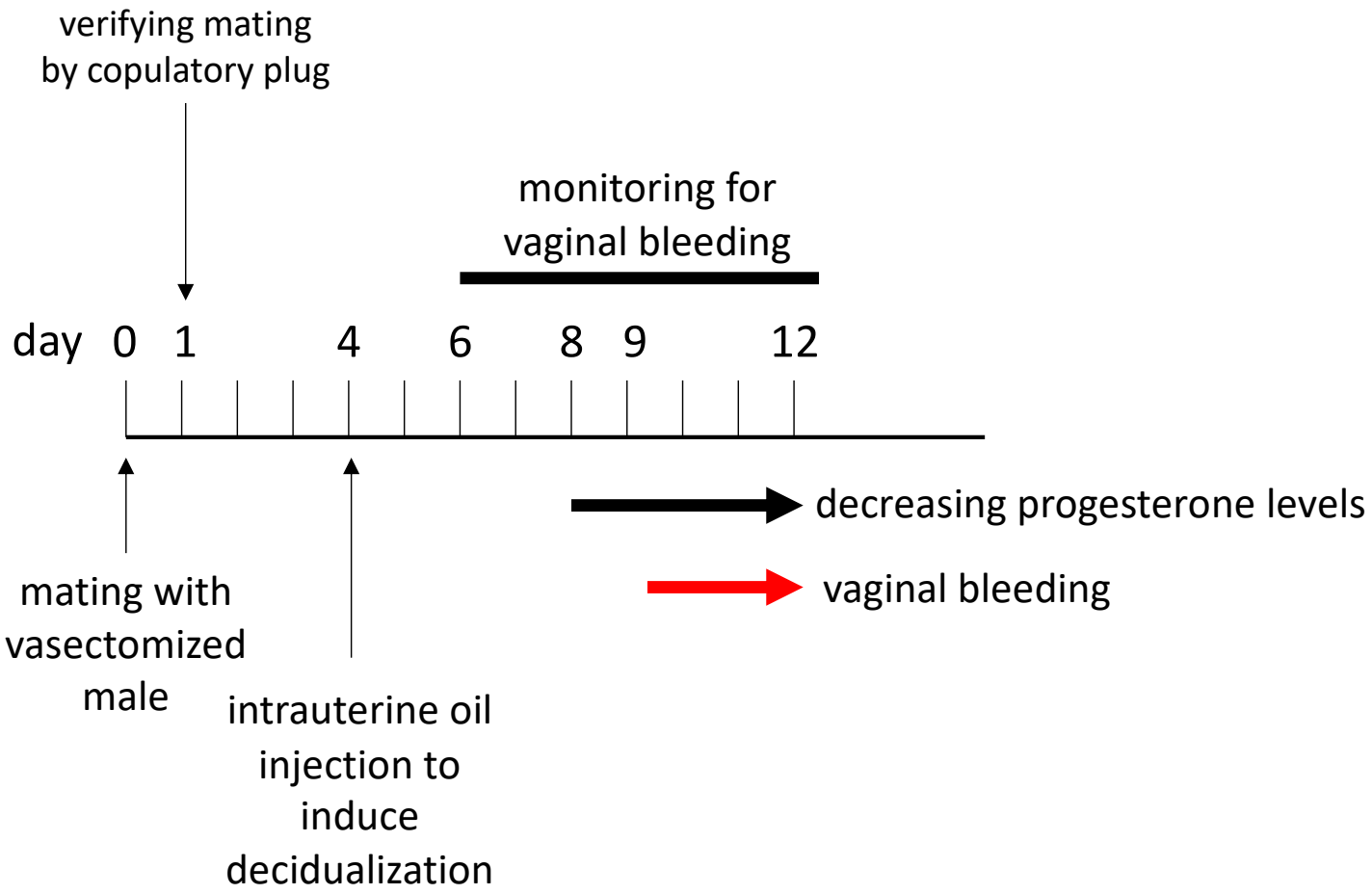
1991-2000
2001-2010
2011-2018


Primates
"Rodents"
Bats
Ferungulata
*
**
Homo sapiens
Pan paniscus
Pongo pygmaeus
Hylobates concolor
Cercopithecus aethiops
Papio hamadryas
Macaca mulata
Lagothrix lagotricha
Aotus trivirgatus
Calithrix jacchus
Cebus apella
Tarsius bancanus
Otoleumur garnetti
Microcebus murinus
Oryctolagus cuniculus
Cavia porcellus
Acomys cahirinus
Mus musculus
Rattus norvegicus
Pteropus vampyrus
Peropteryx kappleri
Noctilio albiventris
Desmondus rotundus
Glosssophaga sorcina
Myotis lucifugus
Molossus ater
Erinaceus europaeus
Equus caballus
Canis famiiaris
Llama glama
Bos taurus
Dasypus novemcintus
Choloepus hoffmanni0
Elephantulus myurus
Loxodonta africanus
Procavia capensis
* Xenarthra
** Afrotheria
Black = menstruating species
White = not menstruating sp.
Red outline: likely origins of menstruation


verifying mating
by copulatory plug
monitoring for
vaginal bleeding
day 0 1 4 6 8 9 12
decreasing progesterone levels
vaginal bleeding
mating with
vasectomized
male
intrauterine oil
injection to
induce
decidualization

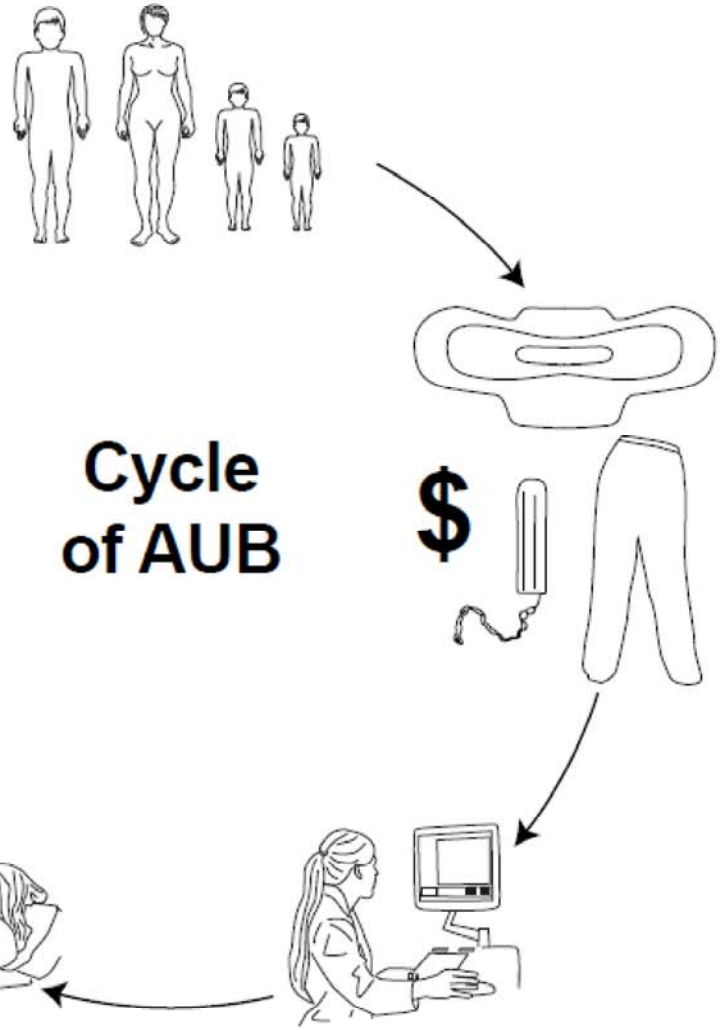
1919

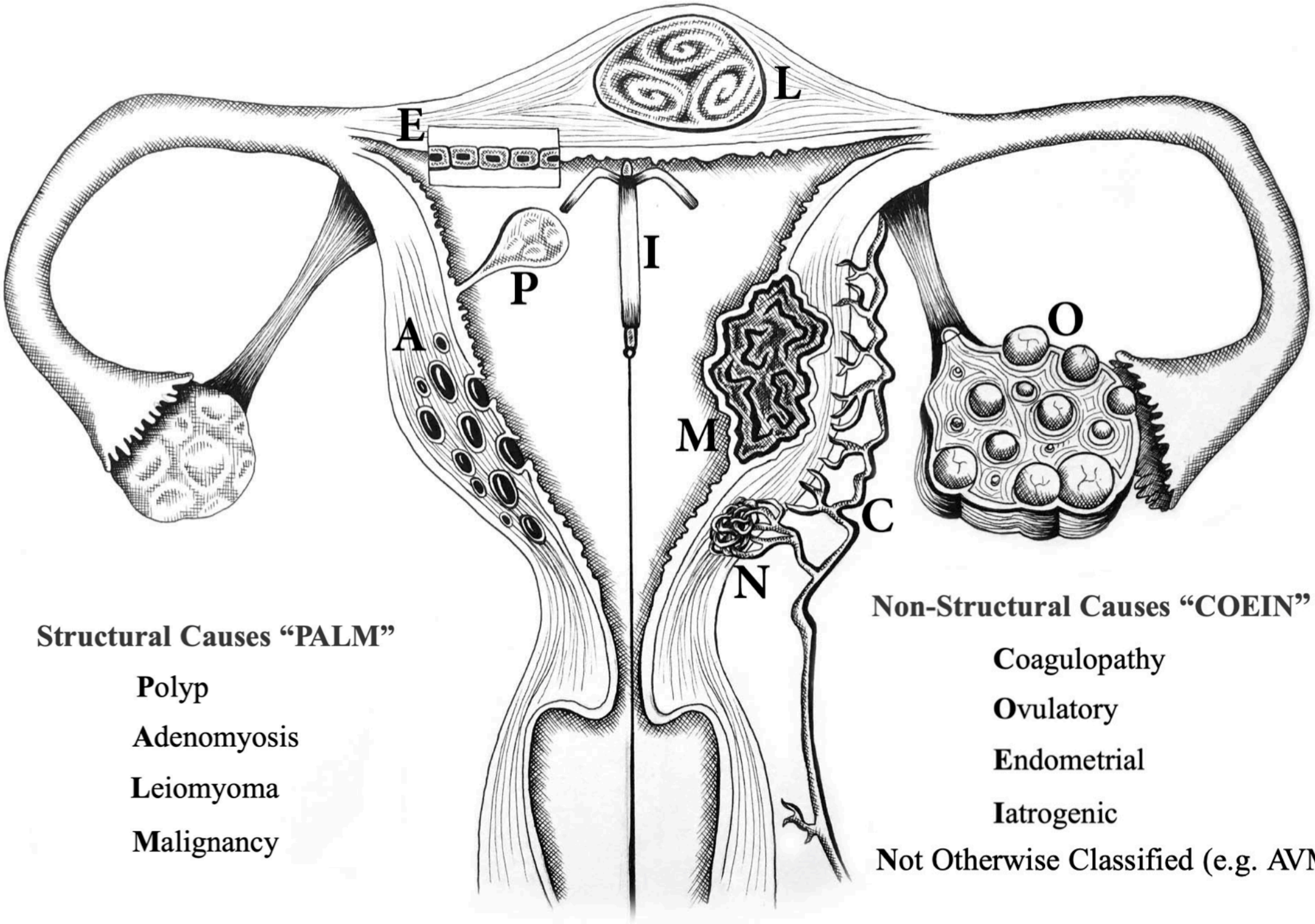
40

2019

400

Cycle of AUB





Structural Causes "PALM"

Polyp

Adenomyosis

Leiomyoma

Malignancy

Non-Structural Causes "COEIN"

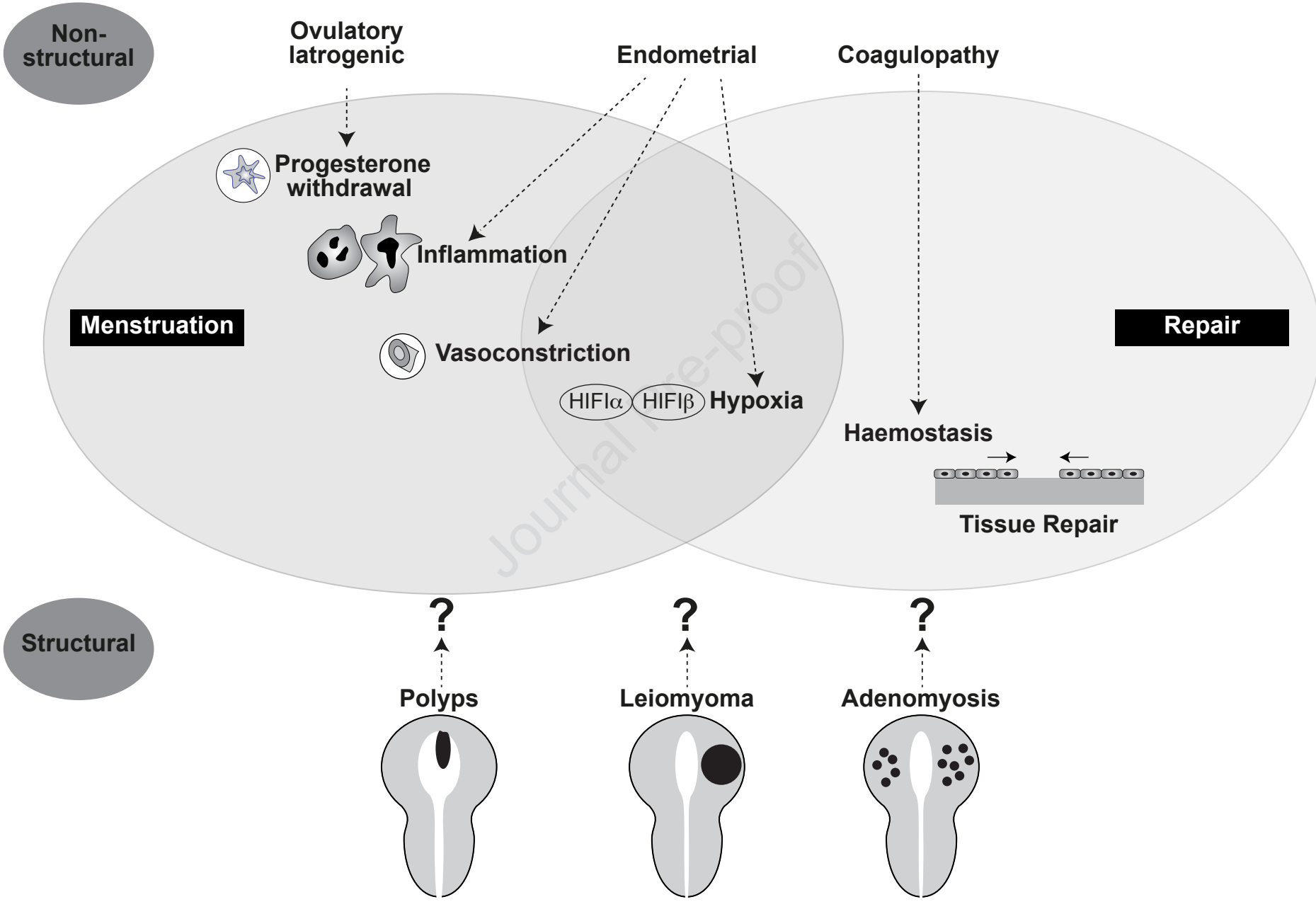
Coagulopathy

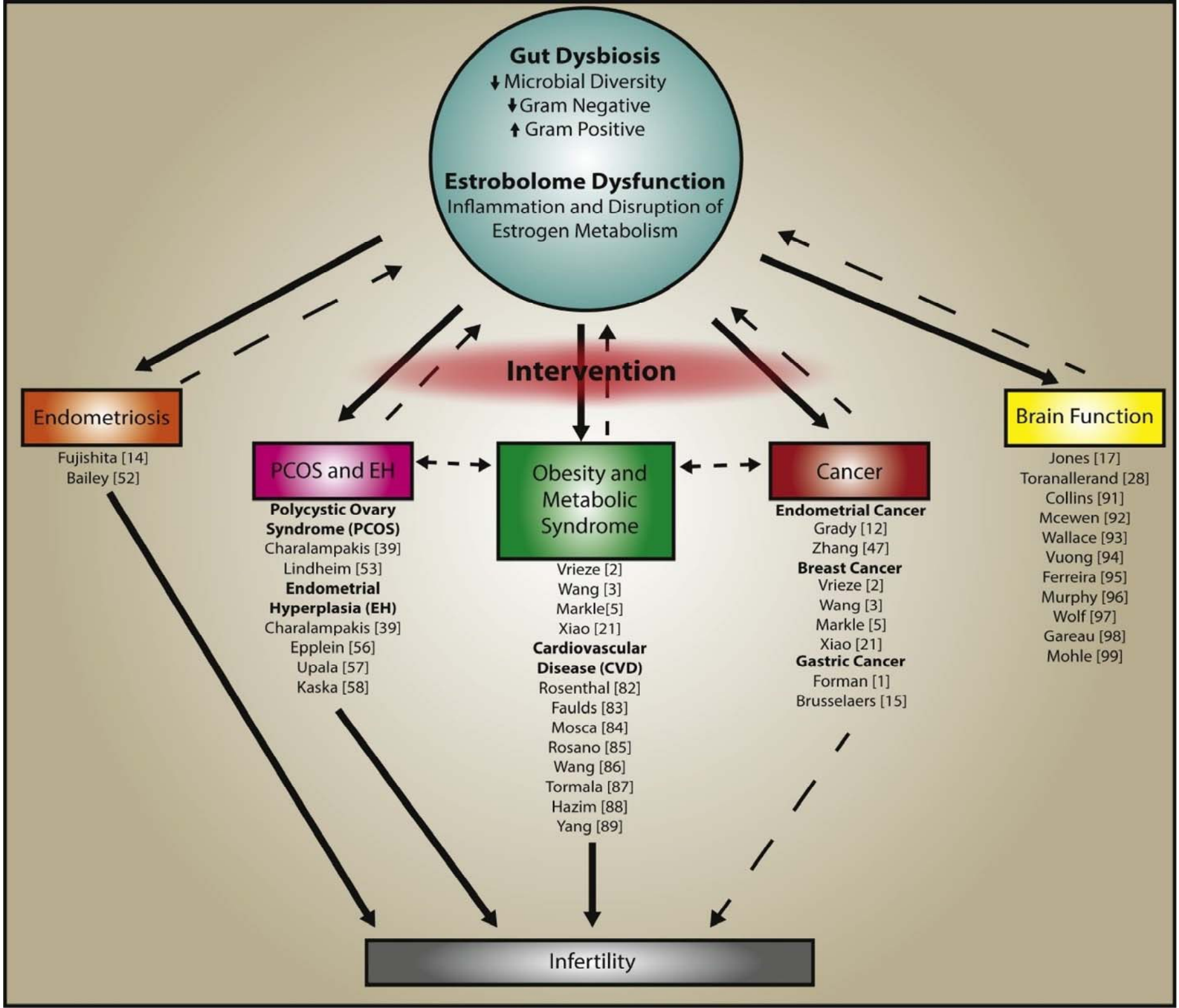
Ovulatory

Endometrial

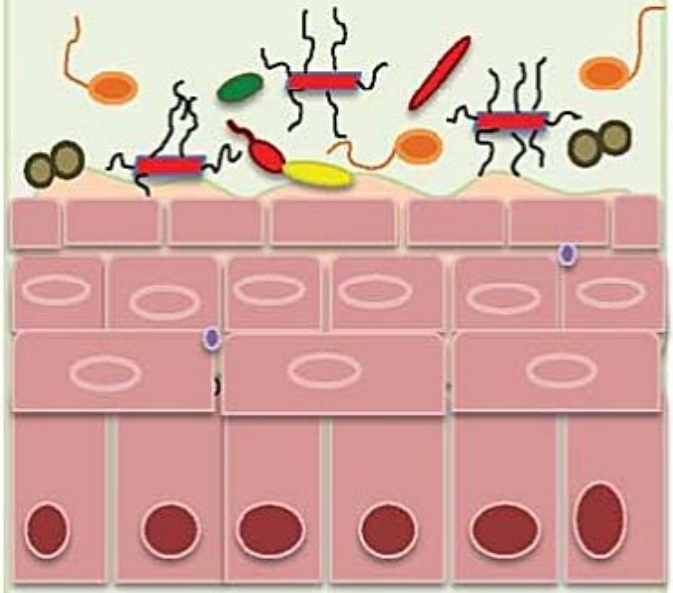
Iatrogenic

Not Otherwise Classified (e.g. AVM)



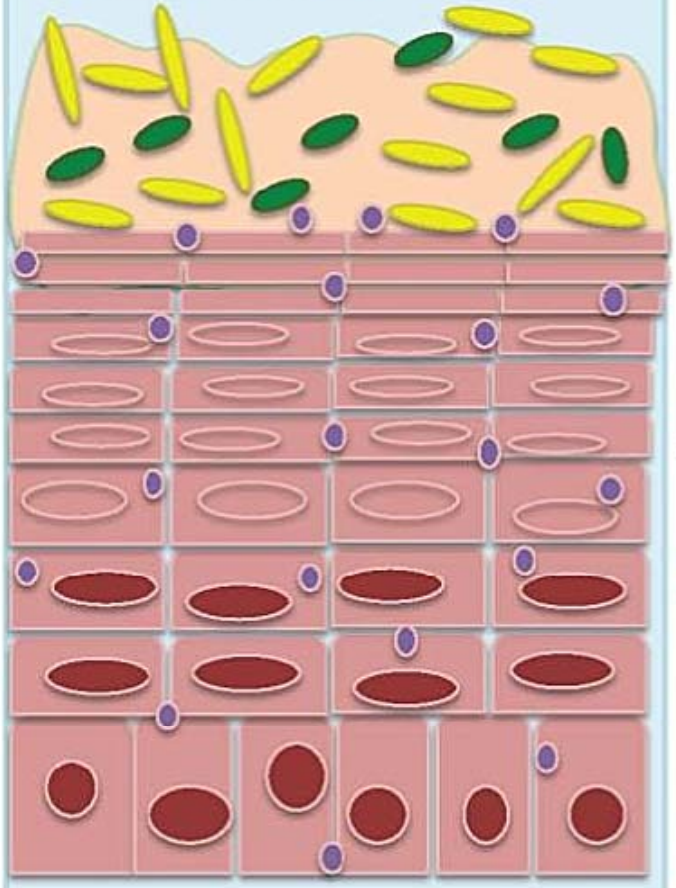

Gut Dysbiosis
Microbial Diversity
Gram Negative
Gram Positive
Estrobolome Dysfunction
Inflammation and Disruption of Estrogen Metabolism
Intervention
Endometriosis
Fujishita [14]
Bailey [52]
PCOS and EH
Polycystic Ovary Syndrome (PCOS)
Charalampakis [39]
Lindheim [53]
Endometrial Hyperplasia (EH)
Charalampakis [39]
Epplein [56]
Upala [57]
Kaska [58]
Obesity and Metabolic Syndrome
Vrieze [2]
Wang [3]
Markle[5]
Xiao [21]
Cardiovascular Disease (CVD)
Rosenthal [82]
Faulds [83]
Mosca [84]
Rosano [85]
Wang [86]
Tormala [87]
Hazim [88]
Yang [89]
Cancer
Endometrial Cancer
Grady [12]
Zhang [47]
Breast Cancer
Vrieze [2]
Wang [3]
Markle [5]
Xiao [21]
Gastric Cancer
Forman [1]
Brusselaers [15]
Brain Function
Jones [17]
Toranallerand [28]
Collins [91]
Mcewen [92]
Wallace [93]
Vuong [94]
Ferreira [95]
Murphy [96]
Wolf [97]
Gareau [98]
Mohle [99]
Infertility

Pre-Puberty



Thin vaginal epithelium
Thin layer of mucus
Low estrogen levels
Low glycogen =
Low *Lactobacillus* spp.
High microbial diversity
High vaginal pH

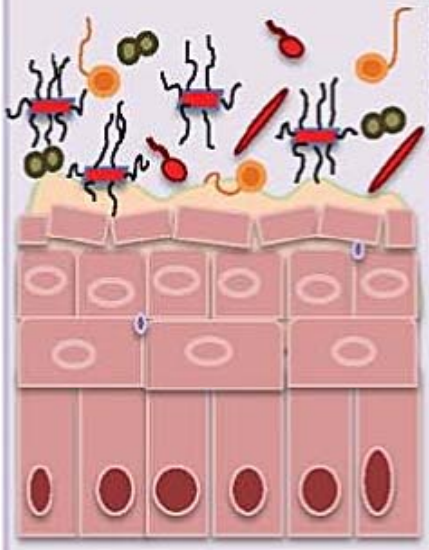
Pre-Menopausal (CST I, II, III and V)



Thick vaginal epithelium
Thick layer of mucus
High estrogen levels
High glycogen =
High *Lactobacillus* spp.
Low microbial diversity
Low vaginal pH

Post-Menopausal

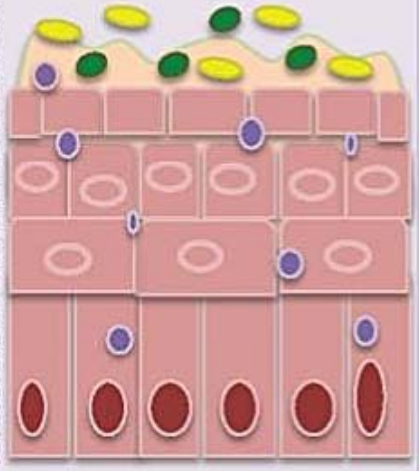
Symptomatic



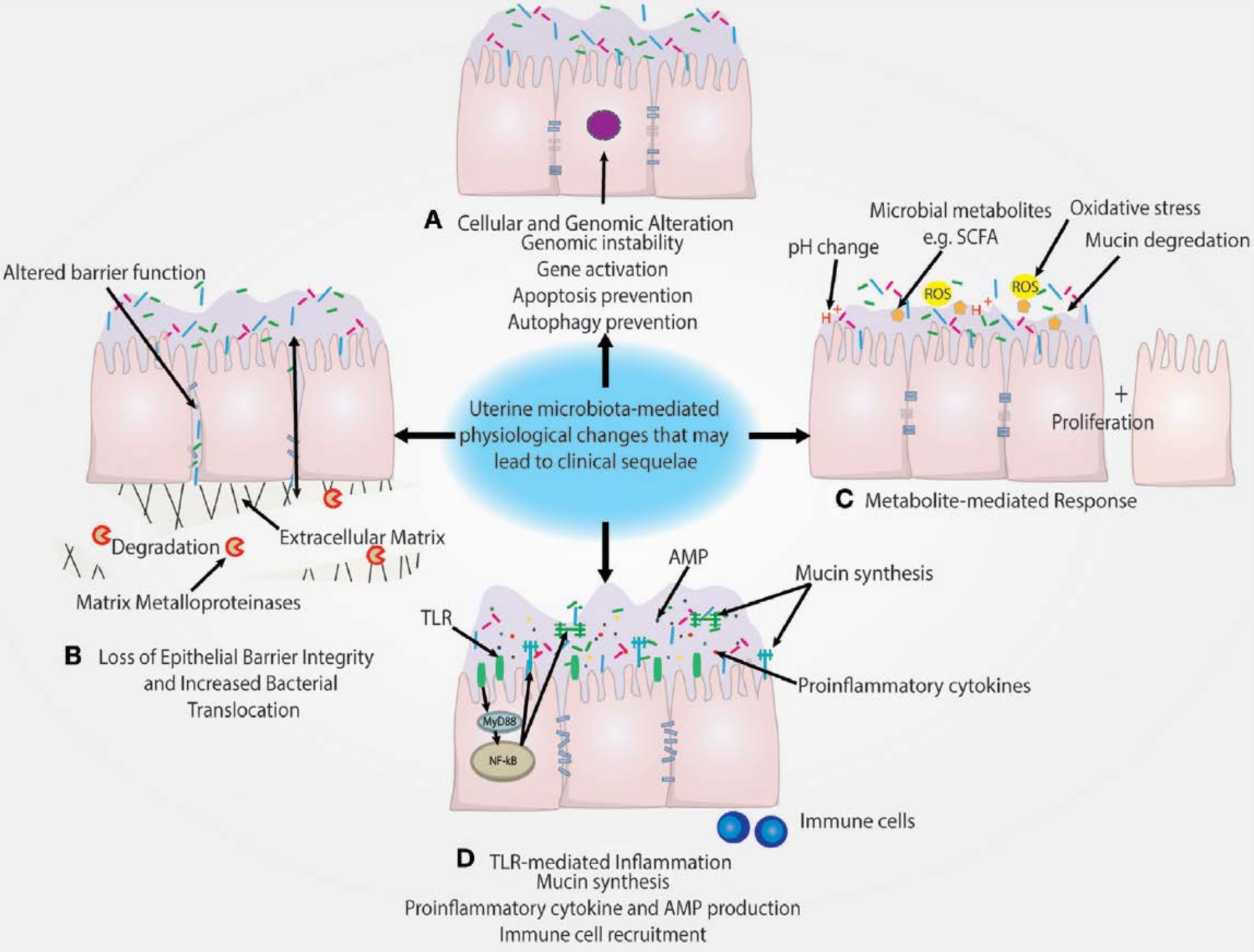
Thinner, disrupted vaginal epithelium
Thin layer of mucus
Low estrogen levels
Low glycogen =
Low *Lactobacillus* spp.
High microbial diversity
High vaginal pH

Asymptomatic

HRT Therapy



Thin vaginal epithelium
Thin layer of mucus
Mod estrogen levels
Mod glycogen =
Mod *Lactobacillus* spp
Lower microbial diversity
Low vaginal pH


A Cellular and Genomic Alteration
Genomic instability
Gene activation
Apoptosis prevention
Autophagy prevention
Uterine microbiota-mediated physiological changes that may lead to clinical sequelae
Altered barrier function
Degradation
Extracellular Matrix
Matrix Metalloproteinases
B Loss of Epithelial Barrier Integrity and Increased Bacterial Translocation
Microbial metabolites
e.g. SCFA
Oxidative stress
pH change
Mucin degredation
ROS
ROS
H+
H+
+
Proliferation
C Metabolite-mediated Response
AMP
Mucin synthesis
TLR
Proinflammatory cytokines
MyD88
NF-kB
Immune cells
D TLR-mediated Inflammation
Mucin synthesis
Proinflammatory cytokine and AMP production
Immune cell recruitment