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Menstruation: Myths, mechanisms, models and malfunctions

Edited by
Fiona L. Cousins and Philippa T. Saunders

Published in
Frontiers in Reproductive Health

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ISSN 1664-8714
ISBN 978-2-8325-4935-3
DOI 10.3389/978-2-8325-4935-3

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Menstruation: Myths, mechanisms, models and malfunctions

Topic editors

Fiona L. Cousins — Hudson Institute of Medical Research, Australia

Philippa T. Saunders — University of Edinburgh, United Kingdom

Citation

Cousins, F. L., Saunders, P. T., eds. (2024). *Menstruation: Myths, mechanisms, models and malfunctions*. Lausanne: Frontiers Media SA.

doi: 10.3389/978-2-8325-4935-3

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frontiers | Frontiers in Reproductive Health

TYPE Editorial
PUBLISHED 03 April 2023
DOI 10.3389/frph.2023.1158317

Check for updates

OPEN ACCESS

EDITED AND REVIEWED BY
Pamela Stratton,
National Institutes of Health (NIH), United States

*CORRESPONDENCE
Philippa T. K. Saunders
✉ p.saunders@ed.ac.uk

SPECIALTY SECTION
This article was submitted to Gynecology, a section of the journal Frontiers in Reproductive Health

RECEIVED 03 February 2023
ACCEPTED 15 March 2023
PUBLISHED 03 April 2023

CITATION
Cousins FL and Saunders PTK (2023) Editorial: Menstruation: Myths, mechanisms, models and malfunctions.
Front. Reprod. Health 5:1158317.
doi: 10.3389/frph.2023.1158317

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Editorial: Menstruation: Myths, mechanisms, models and malfunctions

Fiona L. Cousins[1] and Philippa T. K. Saunders[2]*

[1]The Ritchie Centre, Hudson Institute of Medical Research, Clayton, VIC, Australia, [2]Centre for Inflammation Research, Institute of Regeneration and Repair, the University of Edinburgh, Edinburgh, United Kingdom

KEYWORDS

endometrium, menstruation, endometriosis, fibroids, scarless repair, abnormal uterine bleeding, heavy menstrual bleeding

Editorial on the Research Topic
Menstruation: Myths, mechanisms, models and malfunctions

Introduction

The endometrium is a remarkable, resilient, hormone-dependent tissue that prepares each month for the arrival of a blastocyst and a pregnancy. If no pregnancy occurs, endometrial tissue surrounding the uterine cavity breaks down during menstruation releasing tissue fragments, blood, and fluid into the lumen. The appearance of "blood" in the vagina is the hallmark of menstruation, and in a modern society with low birth rate, may occur 400 times during a woman's fertile, reproductive life. During menstruation the endometrium resembles a bloody wound (1) with a strong inflammatory response (2).

The appearance of blood in vaginal fluids has been linked to many societal and religious taboos: there are still large gaps in our knowledge about the mechanisms that regulate menstruation and how their dysregulation contributes to pathologies that have a huge impact on the quality of life of women.

The aim of this research topic was to bring together a diverse range of contributions spanning a wide range of topics from models to societal impacts. The topic contains thirteen papers which fall into three broad categories: Societal Attitudes, Mechanisms and Models, and Disorders (abnormal bleeding and endometriosis).

Societal attitudes to menstruation

In their fascinating original study entitled "*Menstrual Hygiene Management—Knowledge, Attitudes, and Practices Among Female College Students in Bhutan*" Tshomo et al. have focused on the challenges of menstrual hygiene management faced by young people in countries classified as having a 'lower middle income economy' by the World Bank (https://www.worldbank.org/en/country/bhutan/overview). They analysed the data from a self-administered questionnaire completed by just over 1,000 participants. Half of the participants reported their daily activities were affected by menstruation and a quarter missed time at college due to painful periods. It was striking that challenges included lack

of access to proper handwashing facilities (soap/water; 80%). The authors hope their findings will inform government initiatives to improve the lives of women and girls. This study was nicely complemented by one conducted in college students in the United States, a rich first world country. Gruer et al. set out to see what strategies were useful in addressing issues of menstrual equity and period poverty in their study entitled "*Menstrual Equity Initiatives at USA Universities: A Multiple Case Study of Common Obstacles and Enabling Factors*". They focused on Universities undertaking free menstrual product initiatives to conduct a qualitative study finding that, although all had been successful, they varied in terms of implementation strategy with limitations imposed by resources. There are clearly opportunities to share ideas from different countries and initiatives with regard to scale and funding—it is to be hoped that more countries take the same step as the Scottish government who passed a law in 2021 requiring local authorities and education providers to make period products obtainable free of charge for anyone who needs to use them (https://www.gov.scot/publications/period-products-free-provision-scotland-act-2021-equality-impact-assessment/).

Mechanisms and models

The endometrium is a dynamic and multicellular tissue and in their review, entitled "*Genetic Regulation of Transcription in the Endometrium in Health and Disease*", Mortlock et al. reviewed the similarities and differences in endometrial gene expression with other body tissues and the role of specific genes in the risk of developing endometrial diseases. Their paper is an excellent primer for anyone interested in improving their understanding of complex genetic methods and concludes with a plea that researchers and clinicians must consider an individual's genetic background when investigating and managing fertility and disease.

Menstruating mammals share a number of different features including spontaneous ovulation and fertility that is associated with an endometrium which has been transformed into a "receptive" state (3). The review by Muter et al. focusses on the importance of the spontaneous differentiation (decidualization) of the endometrial stromal cells that occurs in response to high concentrations of progesterone produced by the ovary following ovulation. This advance in our understanding of the existence of different subpopulations of decidual cells has highlighted mechanisms that may explain risk of miscarriage and offer new therapeutic targets to improve pregnancy outcomes.

The endometrium is almost unique amongst adult tissues in its ability to heal without forming a scar or fibrotic tissue in response to the endometrial "wound": several of the papers in this special issue focused on the latest evidence related to the mechanisms that are implicated in endometrial repair during the menstrual cycle (Salamonsen Cousins et al. Bellofiore et al.).

Salamonsen reminds the reader that during the shedding of the inner (luminal) surface of the endometrium the tissue is bathed in menstrual fluid which contains live cells, as well as activated leukocytes, soluble cellular components and extracellular vesicles. She highlights the evidence from cell culture and skin and pig wound models that "*Menstrual Fluid Factors Mediate Endometrial Repair*". Notably she argues that the analysis of this fluid may provide much needed new insights that could be applied to the treatment of poorly repairing skin wounds which are an increasing problem in old age (4).

In two complementary reviews Kirkwood et al. and Bellofiore et al., and their colleagues in Scotland and Australia respectively, review the data on "menstruation" generated using laboratory and Spiny mice and highlight how they have informed our understanding of the basic mechanisms responsible for endometrial shedding and repair. Kirkwood et al. remind the reader that the mouse endometrium does not normally experience shedding and review the refinement of methods that have been applied to laboratory mice to recapitulate the main features of human menstruation including rapid breakdown, hypoxia, shedding and repair as well as the advantage of using genetically manipulated mice for these studies. They have recently followed up on these studies with new data showing transformation of mesenchyme cells into epithelium may complement other mechanisms including epithelial cell proliferation (5). The discovery of naturally occurring menstruation in the Egyptian spiny mouse (*Acomys cahirinus*), a species that also exhibits scarless healing of skin (6), has led to intense interest in the potential of this model species "to Build a Bridge from Bench to Bedside" and improve translation of laboratory studies into clinical therapies for endometrial disorders including heavy menstrual bleeding. The authors discuss insights from studying cycle variation between individual animals and how this might assist in better understanding of vascular remodelling successful implantation.

Following endometrial repair, which occurs in a hormone-depleted environment, the inner layer of the endometrium (the 'functionalis") grows rapidly from the basal (unshed) portion of endometrium in response to rising concentrations of oestrogens in the blood. This regenerative capacity of the endometrium is attributed to the "Endometrial Stem/Progenitor Cells" which occur in both the epithelial and stromal compartments. Cousins et. al. provide a comprehensive review of the markers used to identify putative progenitors, their identity, location and hierarchy across the menstrual cycle (Cousins et al.).

Endometrial disorders

The last group of papers in this special issue consider different aspects of endometrial function that might contribute to its malfunction and how these changes can be used to better understand and treat disorders that have an impact on the quality of life of millions of individuals. Three of these papers are focused on abnormal uterine bleeding (AUB) which can include abnormal frequency as well as prolonged and heavy bleeding (HMB) (Chodankar et al. Watters et al. Uimari et al.) whilst two are on endometriosis (Kuan et al. Mbuguiro et al.).

One of the barriers to improving the management of menstrual symptoms has been inconsistency in terminology which has created considerable confusion. In their article "*Historical*

Perspectives and Evolution of Menstrual Terminology" Chodankar et al. give a comprehensive overview of the history and evolution of terminology. The paper has a useful figure showing the timeline of the relevant publications and meetings which have resulted in two internationally accepted classifications under the banner of the Federation of Gynecology and Obstetrics (FIGO). The paper by Watters et al. considers current understanding of endometrial physiology at menstruation highlighting the contribution of the specialised endometrial vasculature and coagulation system. They use these insights as a platform for better understanding of gaps in knowledge and what is known about aberrations in endometrial physiology that can cause symptoms of AUB, concluding with an ideal model for management of AUB that includes consideration of patient preferences. One of the causes of AUB identified in the FIGO classification system is the presence of uterine fibroids (Leiomyomata): in their review, Uimari et al. remind the reader that in more than half of patients these benign growths cause HMB, pelvic pain or infertility (Uimari et al.). They consider the treatment options available for fibroids (and symptom relief) and the current theories about the link between disordered vasculature architecture and/or vasoactive growth factors and the increased incidence of HMB in this patient group.

Endometriosis is estimated to occur in ∼10% of women of reproductive age: symptoms can begin early in adolescence and can be debilitating (7). In their review Kuan et al. consider how "*Menstrual Dysregulation*" can contribute to the pathogenesis of endometriosis which is associated with the occurrence of tissue "lesions" resembling endometrium in sites outside the uterus, most often in the peritoneal cavity. Their review highlights some parallels with other endometrial disorders such as AUB including dysregulation of inflammatory factors and the potential use of menstrual fluid as a source of biomarkers complementing the information in the review by Salamonsen. One of the challenges faced by patients with endometriosis is the time taken for those experiencing symptoms to get a diagnosis which is ∼7 years on average. This is in part reflects the lack of robust and reproducible diagnostics that do not depend on imaging or surgery. Mbuguiro et al. make the case for the application of "*Computational Models for Diagnosing and Treating Endometriosis*" by considering three computational modelling approaches that have been used and how each approach (regression, pharmaco- kinetics/dynamics and quantitative systems pharmacology) can answer different questions about endometriosis. This paper is particularly useful for the non-expert as they summarise the mathematics involved, the benefits and limitations of each model and how we might combine these approaches in the future.

Conclusions and prospects for future studies

These papers highlight how different approaches and resources have shaped and informed our understanding of menstruation and endometrial disorders. They offer a unique resource to people wishing to learn more about access to resources, endometrial function and malfunction. In addition, the potential of this information to inform improved diagnostics and therapies for disorders such as AUB and endometriosis is considerable and a better understanding of menstruation may offer unique insights into mechanisms of repair without fibrosis.

Author contributions

The authors edited the special issue and wrote the editorial. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Reproductive Health

ORIGINAL RESEARCH
published: 27 August 2021
doi: 10.3389/frph.2021.703978

Menstrual Hygiene Management—Knowledge, Attitudes, and Practices Among Female College Students in Bhutan

Tashi Tshomo[1], Mongal Singh Gurung[2], Safieh Shah[3], Julita Gil-Cuesta[3], Peter Maes[3], Rinchen Wangdi[1] and Jamba Tobden[4]*

[1] Department of Public Health, Ministry of Health, Thimphu, Bhutan, [2] Policy and Planning Division, Ministry of Health, Thimphu, Bhutan, [3] The Medical Department, Médecins Sans Frontiéres, Operational Center Brussels, Brussels, Belgium, [4] The Institute for Gross National Happiness Studies, Royal University of Bhutan, Thimphu, Bhutan

Background: Girls and women face substantial menstrual hygiene management (MHM) challenges in low- and middle-income countries. These challenges are related to inadequate knowledge and insufficient water, sanitation, and hygiene (WASH) facilities. Currently, the literature on MHM among college-attending women in Bhutan is scarce. We aimed to explore the knowledge, attitudes, and practices (KAP) of female college students from all the 10 government colleges of Bhutan, documenting the conditions of available MHM facilities, from August to September 2018.

Methods: A cross-sectional KAP survey was conducted with a random sample of female students from all years and a random sample of MHM facilities at each college and hostel. A questionnaire was adapted from a similar study conducted with school students in Bhutan. Socio-demographics, overall KAP findings, and differences in KAP between first and final year students were analyzed; college and hostel toilets were self-reported and directly observed.

Results: In the survey, 1,010 participants completed the self-administered questionnaire. The comprehensive knowledge of menstruation was found to be low (35.5%) among participants. Half of the participants (50.3%) reported their mother as the source of information, and 35.1% of the participants agreed that women should not enter a shrine during menstruation. It was also reported that approximately 4% of median monthly pocket money was spent on the absorbents, and 96.9% of absorbents were wrapped before disposal. Half of the participants (55.1%) reported that their daily activities were affected due to menstruation, and 24.2% of the female students missed college due to dysmenorrhea. One-fifth of the participants (21.3%) reported unavailability of water in college, 80.1% of the participants reported absence of soap for hand washing, and 24.1% described no bins for disposal. The participants also reported that in 33.7% of hostel toilets, the door locks were missing. The direct observations also had similar findings.

Conclusions: Female students living in hostels during college years lose considerable resources during their formative years of learning, such as time, energy, and money,

OPEN ACCESS

Edited by:
Philippa Saunders, University of Edinburgh, United Kingdom

Reviewed by:
Silvia Vannuccini, University of Florence, Italy
Alexandra Alvergne, UMR5554 Institut des Sciences de l'Evolution de Montpellier (ISEM), France

****Correspondence:***
Tashi Tshomo
tashitshomo@health.gov.bt

Specialty section:
This article was submitted to Gynecology, a section of the journal Frontiers in Reproductive Health

Received: *01 May 2021*
Accepted: *19 July 2021*
Published: *27 August 2021*

Citation:
Tshomo T, Gurung MS, Shah S, Gil-Cuesta J, Maes P, Wangdi R and Tobden J (2021) Menstrual Hygiene Management—Knowledge, Attitudes, and Practices Among Female College Students in Bhutan. Front. Reprod. Health 3:703978. doi: 10.3389/frph.2021.703978

due to issues of menstruation management. Although the overall understanding of menstruation was low, the MHM practices of our participants scored highly, and the vast majority of them asked for a platform to discuss menstruation. Despite some agreement with menstrual taboos (e.g., visiting shrine), only 5.1% of the participants were uncomfortable conversing about MHM. Improved public health knowledge, psychosocial/medical support, and WASH infrastructure with freely available menstrual products could lead to more effective MHM practices among female college students.

Keywords: knowledge, attitudes, practice, menstruation, menstrual cycle, dysmenorrhea, taboo

INTRODUCTION

Menstrual hygiene management (MHM) refers to the specific hygiene and health requirements of girls and women during menstruation, such as the knowledge, information, materials, and facilities needed to manage menstruation effectively and privately (1). Inadequate water, sanitation, and hygiene (WASH) facilities, particularly in public places such as college campuses and hostels, can pose a major challenge to women and girls regarding the safe disposal of the used menstrual materials and the ability to wash their hands (2, 3).

Multiple systemic reviews and meta-analyses of studies of MHM behavior and practices in low and middle-income countries show that women and school girls report substantial health, as well as social challenges, when it comes to managing their menstruation (4–7).

Menstruation has been surrounded by misperceptions and taboos in society causing reluctance to talk about it (5, 8, 9). Studies show that beliefs regarding menstruation are deep-rooted, and girls describe the onset of menarche as a shocking experience, a curse from God, or even as punishment for the sins of their ancestors (1, 10–12). One such study in Bhutan demonstrated this even in schools (13).

Studies conducted in low- and middle-income countries such as Bhutan, India, Saudi Arabia, and Iran found that girls received information on menstruation mainly from their mothers, (11, 12, 14–16) who tended to focus on activities to be avoided due to traditional taboos (17, 18). Taboos lead to socially imposed restrictions, such as exclusion from daily prayers, avoiding certain foods, performing fasting ceremonies, avoiding touching holy books or flowers, and even preventing them from entering a kitchen or a temple (8, 18–21) as the blood of menstruation is considered "dirty" (14). The failure to fully acknowledge the physical reality of women has a range of serious impacts alongside with experiences of shame (22).

Studies have found a lack of safe and clean hygiene facilities, which leads to unsatisfactory opportunities to clean external genitalia and to change stained absorbents (8, 23). The existing evidence highlights either a lack of disposal facilities for absorbents or inadequate and poorly maintained means of disposal (24, 25). This affects the education of girls: They miss their classes during menstruation due to fear of staining, shame, ridicule by their peers, menstrual cramps, or the lack of facilities to manage their menstrual hygiene privately (10–12, 26). This has led many girls and women to dispose of absorbents and pads with routine waste in toilets or in open spaces (5, 8, 19).

In countries near Bhutan, China, and Bangladesh, tailored education sessions have been found to improve knowledge on menstruation and practices among adolescent girls (27, 28).

In Bhutan, research studies with school-going adolescent girls and rural women (9, 11, 17) have identified gaps in knowledge, inadequate facilities, and socio-cultural barriers for practicing hygienic MHM. However, little is known about this among women who attend college. Although college-going girls or women have not been studied, there is a societal assumption that they must be educated enough to have a good understanding of MHM. This may not be true. As the main source of menstrual information seems to be mothers in Bhutan, not checking the true knowledge, attitudes, and practices (KAP) of college women could have far-reaching implications if they continue to transmit incorrect information and stigma to their daughters (10, 19).

This study offered a unique opportunity to identify existing gaps in MHM among government colleges that need to be addressed throughout Bhutan. The findings should guide the Ministry of Health (MoH) in Bhutan to make recommendations for improving MHM in these colleges.

Thus, our objective was to describe the KAP of female college students on MHM and evaluate the physical conditions of college WASH facilities for basic MHM.

MATERIALS AND METHODS

Design

A cross-sectional KAP survey was conducted, and physical observation studies of MHM facilities were carried out in all 10 government colleges of Bhutan from August 8 to September 13, 2018.

Setting

There are two universities that provide modern education in Bhutan: the Royal University of Bhutan (RUB) and the Khesar Gyalpo University of Medical Sciences of Bhutan (KGUMSB).

Abbreviations: KAP, Knowledge Attitude and Practice; KGUMSB, Khesar Gyalpo University of Medical Sciences of Bhutan; MHM, Menstrual Hygiene Management; MSF, Medécins Sans Frontières; Nu, Ngultrum (Bhutanese Currency); REBH, Research Ethics Board of Health; RUB, Royal University of Bhutan; SD, Standard Deviation; SORT IT, Structured Operational Research and Training Initiative; STROBE, Strengthening the Reporting of Observational Studies in Epidemiology; WASH, Water and Sanitation; WHO, World Health Organization.

As of 2018, there were 11,259 students pursuing various courses in these tertiary institutions and women made up 46% of the total enrollment (29). The students at these colleges (both male and female) are admitted based on their merit, independent of where they live. The majority of students do not live at home during their college years and are provided with a full government scholarship and accommodation in college hostels, independent of their socioeconomic status. Less than 20% stay outside the college campus either in rented private rooms or with their relatives.

Population

The target population for this study was female students pursuing undergraduate courses in 2018 at eight colleges of RUB and two colleges of KGUMSB. The colleges and institutes that do not offer undergraduate courses were excluded. All female students who provided informed consent and were above the age of 18 years were included in the study.

Sample Size

The sample size was estimated with an assumed percentage of absenteeism during menstruation to be 48% (11). At a 95% confidence level with a 5% acceptable margin of error in estimating the percentage of absenteeism during menstruation and a 90% response rate, estimates were calculated for three subgroups (first year, second year, and final year students). The final sample size was 1,280 students.

Data Collection

The data collection team was composed of the principal investigator (PI) and trained female research assistants from the MoH, Bhutan, and each college, respectively. Data were collected during working hours at the colleges for the period of 1 month, from August 8 to September 13, 2018.

The participants were randomly selected from the total list of eligible students enrolled in the 10 colleges (sampling frame = 4,194). The sampling was proportional to the size of the college. The data collection team visited the 10 colleges with the list of selected students based on this sampling. A common venue was arranged, and selected participants were requested to gather and learn about the study. Informed consent was sought from each individual before they completed the self-administered questionnaire. Enough distance was maintained at the venue between each of the participants to ensure the privacy of their responses.

A self-reported questionnaire with multiple-choice responses was used to assess the KAP of the participants. This questionnaire was adapted from the KAP survey on MHM conducted among school-going girls by the Ministry of Education in Bhutan. Socio-demographic variables, such as age, religion, year of study, current place of residence, and the educational level of the mother, were included. The questions related to physiology, female anatomy, and menstrual hygiene were asked under the knowledge domain. The participants were categorized as having "comprehensive knowledge" if they knew correct answers to all the five questions under the knowledge domain. "Attitudes" were assessed using a rating scale from strongly agree, agree, disagree, and strongly disagree for questions on social and cultural beliefs. To ascertain "practices," the participants were asked regarding the type of absorbents used, their cost, absenteeism, hygiene practices disposal, and whether there was a need for a platform to discuss MHM.

The study assessed MHM facilities through (i) responses to the KAP questionnaire by the participants about the toilet facilities in their hostels and at colleges and (ii) direct observation by our team that visited a cross-section of toilets at each of the 10 colleges. An observation checklist was formulated for the assessment. The observation was undertaken to corroborate the condition of the MHM facilities so that it was not based solely on the perception of the participants. For this, a checklist was used to assess MHM facilities in at least one toilet per college or hostel looking for soap, a dustbin, water, and a door with locks. The toilets nearest to the venue of data collection were chosen for observation.

A pilot study was conducted among the female postgraduate students at the Royal Institute of Management (RIM), an autonomous institute based in Thimphu, Bhutan, to trial the recruitment strategy and to evaluate the clarity of the questionnaire and informed consent prior to the study.

Data Analysis

Frequencies and percentages of the socio-demographic characteristics of the study population and their KAP were reported. Costs of absorbents in USD (US dollars), means, SD, median, and interquartile range were reported for MHM practices according to variable type such as avoiding some foods, taking a bath, and cleaning genitals. The subgroup analysis by year in college (first, second, and final year) was carried out for KAP. The differences in knowledge and practices between first and final year students were assessed using the chi-square test. A comparison was made between first and final year students to establish an association between MHM and educational level. MHM facilities, such as hand washing facilities, soap, pad disposal bins, and lockable doors reported by students through KAP and observed by data collectors, were analyzed and compared.

The data were entered into EpiData Entry software version 3.1 and analyzed using Stata 15 (StataCorp. 2017. *Stata Statistical Software: Release 15*. College Station, TX: StataCorp LLC). Data were double-entered and validated by two data entry staff recruited and trained for this purpose.

Ethics Approval and Consent to Participate

Approval to conduct the study was obtained from the Research Ethics Board of Health (REBH) (Approval no: Ref. No. REBH/Approval/2017/077) under the MoH in Bhutan and Médecins Sans Frontières (MSF) Ethics Review Board (MSF approval no ID 1776). Further, administrative clearances were obtained from the RUB and KGUMSB college administrations. Individual written informed consent was obtained from each participant.

RESULTS

Socio-Demographic Characteristics

Out of 1,280 female students approached, 1,021 completed the questionnaires (response rate of 79.8%) and 1,010 were included (11 were minors, and informed consent from their parents or legal guardians could not be obtained). Their mean age calculated was 20.7 years (SD 1.71). The proportions by college year were roughly divided into three (**Table 1**). More than 90.7% of the participants were Buddhist. Other socio-demographic characteristics are reported in **Table 1**.

TABLE 1 | Socio-demographic characteristics of female college students in the study ($n = 1,010$).

Socio-demographic characteristics	Participants	
	Number	Percentage
Mean age of participants (Mean ± SD)	20.68 (±1.71)	
Years in College		
First	349	34.6
Second	340	33.7
Final (Third and Fourth)	321	31.8
College		
College of Language and Cultural Studies	166	16.4
College of Natural Resources	108	10.7
College of Science and Technology	61	6.0
Faculty of Nursing and Public Health	55	5.5
Faculty of Traditional Medicine	9	0.9
Gaeddu College of Business Studies	148	14.7
Jigme Namgyel Engineering College	67	6.6
Paro College of Education	89	8.8
Samtse College of Education	92	9.1
Sherubtse College	215	21.3
Accommodation[a]		
Hostel	905	89.8
Rented Rooms	75	7.4
Parents	22	2.18
Others (other relatives)	6	0.6
Religion[b]		
Buddhist	913	90.7
Hindu	74	7.4
Christian	15	1.5
Others (Kirat, Manav, etc)	5	0.5
Hometown by Region[c,e]		
Western	268	27.5
Central	292	30.0
Eastern	413	42.5
Mother's Educational Level[d]		
No education	537	53.4
Non-formal education (for adults)	196	19.5
Primary (PP to 6)	118	11.8
Secondary (7 to 12)	109	10.8
Degree and above	34	3.4
Monastic	7	0.7
Others (Secondary+Certificate/Diploma)	5	0.5

MHM, Menstrual Hygiene Management; SD, Standard Deviation [a]Missing, 2; [b]Missing, 3; [c]Missing, 37; [d]Missing, 4; [e]Bhutan has three regions. The eastern region has the highest number of registered voters.

Knowledge of Menstruation and Source of Information

Table 2 shows that 35.4% of the students knew the correct answer to all five questions and that 11 (1.1%) students did not know the correct answer to a single question. The proportion of students having comprehensive knowledge was higher among final year students compared to the first year (38.6% vs. 31.0%, *p*-value 0.037). The main source of information on menstruation was mothers for half (50.3%) of the students, with the rest reporting teachers (19.5%), friends (14.4%), sisters (14.3%), and others (1.9%).

Perceptions and Attitudes Toward Menstruation

Nearly half (44.9%) of the participants agreed that menstruation affects their daily activities, and 94.0% of the participants strongly agreed on the importance of talking about menstruation (**Figure 1**). More than 45% of the participants strongly agreed that men have the advantage of not having menstruation. Only 5.1% of the participants strongly agreed with the uncomfortable feeling to address MHM in a conversation. About one-third (35.1%) of the participants agreed that women should not enter a shrine when menstruating.

Practices During Menstruation

The median amount of money spent on absorbents by college students was Ngultrum (Nu) 80 (1.14USD) per month (IQR Nu. 60), and the median amount of pocket money students received was Nu 2000 (28.6 USD) per month (IQR Nu. 2000). Among the participants, 98.6% took a bath during menstruation and 96.9% wrapped used absorbents before disposing them. Among the participants, 24.2% reported missing classes during menstruation (**Table 3**).

The mean number of days missed during menstruation was 1.4 days per month (SD 0.8). Reasons for absenteeism were pain (86.1%), afraid of staining (5.6%), and feeling uncomfortable (4.4%).

Menstrual Hygiene Management Facilities in Colleges and Hostels

Tap water in college toilets was reported to be missing by 21.3% of the participants. Indeed, the data collection team found that over one-third (38.9%) of college toilets did not have water when they visited (**Table 4**).

Soap for hand washing was missing in 77.8% of the college facilities during observation, and 80.1% of the participants also reported the same. The conditions were similar in their hostels.

TABLE 2 | Number and percentage of female college students with appropriate knowledge about MHM (n = 1,010).

Knowledge	Participants with correct knowledge *n* (%)				*P*-value[l]
	Overall	1st Year	2nd Year	3rd Year	
What is menstruation[a,g]	967 (96.4)	326 (94.8)	330 (97.4)	311 (97.2)	0.115
Cause of menstruation[b,h]	863 (87.4)	292 (86.4)	292 (88.0)	279 (87.7)	0.608
Organ from where menstrual blood come[c,i]	743 (76.2)	237 (72.3)	256 (76.9)	250 (79.6)	0.029*
Normal menstruation duration for normal person[d,j]	834 (82.6)	278 (79.7)	278 (81.8)	278 (86.6)	0.017*
Interval between two menstrual cycle in days[e,k]	535 (53.0)	165 (47.3)	188 (55.3)	182 (56.7)	0.015*
Combined					
Comprehensive knowledge[f]	357 (35.4)	108 (31.0)	125 (36.8)	124 (38.6)	0.037*
Having correct knowledge on 1–4 questions	642 (63.5)	233 (66.8)	213 (62.6)	196 (61.1)	0.124
Incorrect answer to all five questions	11 (1.1)	8 (2.3)	2 (0.6)	1 (0.3)	0.028*^

MHM, Menstrual Hygiene Management.
[a]It's "Natural shedding of blood on monthly basis" and it's not ("a disease on monthly basis," "Type of curse received by women," "All of them," "others," and "Don't know").
[b]It's Hormones and it's not ("Curse of God," "Caused by diseases," "Others," and "Don't know").
[c]It's Uterus and it's not (bladder, abdomen, others, and "don't know").
[d]It's 3–7 days.
[e]It's 28–42 days.
[f]Individual having the correct knowledge on all fives questions.
[g]Missing, 7; [h]Missing, 22; [i]Missing, 35; [j]Missing, 16; [k]Missing, 43.
[l]Chi-square test was calculated for the difference between first and final years.
**There is some evidence against the null hypothesis of no difference.*
^Mid-P exact test.

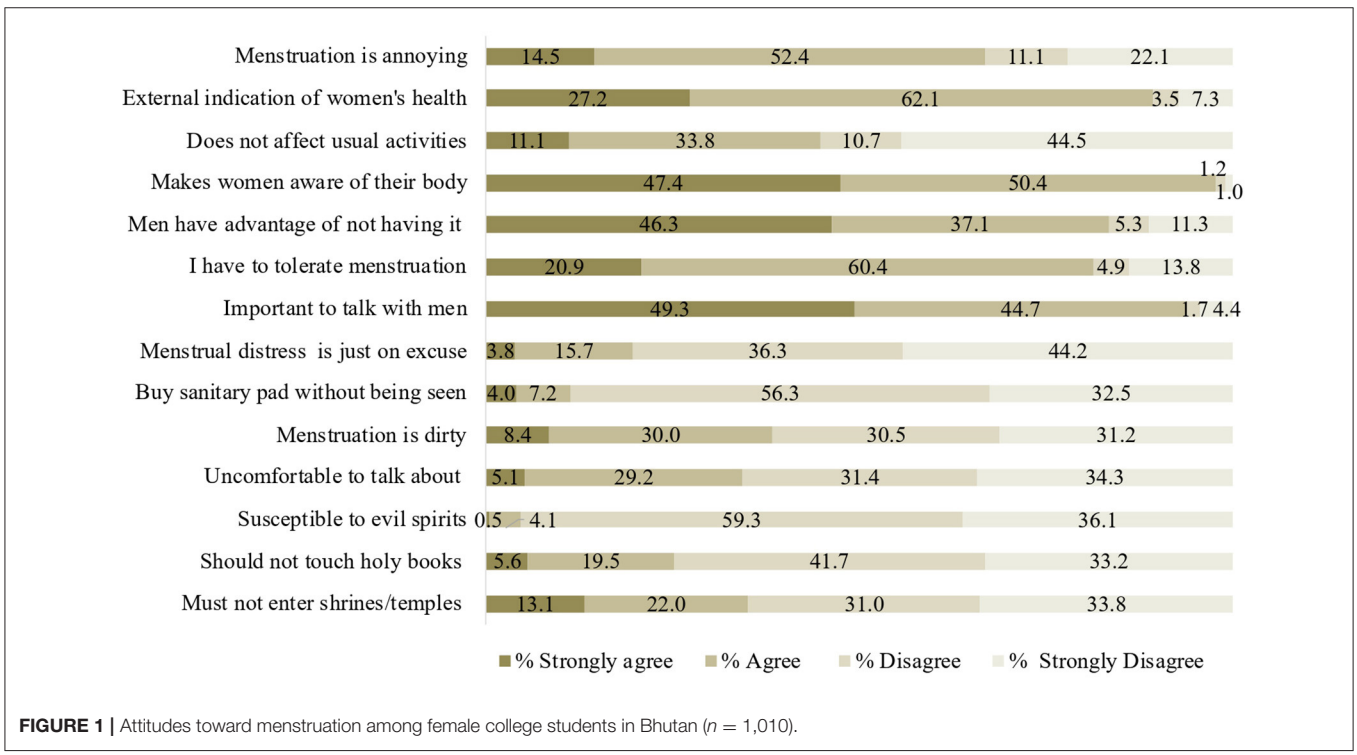

FIGURE 1 | Attitudes toward menstruation among female college students in Bhutan (n = 1,010).

Platform for Discussion and Education on MHM

Among the participants, 916 (91.6%) said that there was a need for platforms to talk about MHM. The preferred platforms were sessions on MHM within the colleges (95.7%) followed by social media groups (26.3%).

DISCUSSION

Our cross-sectional study found inadequate comprehensive knowledge of MHM among female college students in Bhutan. A majority of women (>50%) appeared to be quite knowledgeable although a few students did not know the answer to any of the "knowledge" questions about menstruation. The scores for

TABLE 3 | Practices related to MHM among female college students in all the 10 government colleges of Bhutan, 2018 (n = 1,010).

MHM related practices	Yes *n* (%)				*P*-value[f]
	Overall	1st Year	2nd Year	3rd Year	
Miss college during menstruation[a]	241 (24.2)	61 (17.9)	83 (24.6)	97 (30.7)	< 0.001*
Avoid some food during menstruation[b]	312 (31.1)	110 (31.6)	91 (26.8)	111 (35.1)	0.337
Take bath during menstruation[c]	955 (95.0)	331 (95.1)	324 (95.3)	300 (94.3)	0.654
Clean genitals during menstruation[d]	997 (99.2)	344 (98.9)	339 (99.7)	314 (99.1)	0.817^
Wrap pad before disposing[e]	971 (96.8)	338 (97.7)	325 (95.9)	308 (96.9)	0.509

MHM, Menstrual Hygiene Management.
[a]Missing, 14; [b]Missing, 7; [c]Missing, 4; [d]Missing, 5; [e]Missing, 7.
[f]Chi-square test was calculated for the difference between first and third years.
**There is a strong evidence against the null hypothesis of no difference.*
^Mid-P exact test.

TABLE 4 | Status of menstrual hygiene management facilities in all the 10 government colleges of Bhutan, 2018.

Characteristics of MHM facilities	Self-reported (n = 1,010)		Observed (n = 18)	
	Yes *n* (%)	No *n* (%)	Yes *n* (%)	No *n* (%)
College toilets				
Lockable doors for MHM[a]	597(61.0)	382 (39.0)	14 (77.8)	1 (5.6)
Water for MHM[b]	775 (78.7)	210 (21.3)	9 (50.0)	7 (38.9)
Soap for hand washing[c]	199 (19.9)	803 (80.1)	2 (11.1)	14 (77.8)
Bin for pad disposal[d]	759 (75.9)	241 (24.1)	9 (50.0)	6 (33.3)
Hostel toilets				
Lockable doors for MHM[e]	652 (66.3)	332 (33.7)	13(72.2)	1 (5.6)
Water for MHM[f]	784 (81.0)	184 (19.0)	12 (66.7)	6 (33.3)
Soap for hand washing	Not asked	Not asked	6 (33.3)	12 (66.7)
Bin for pad disposal[g]	805 (81.2)	187 (18.85)	10 (55.6)	8 (44.4)

[a]Missing, 31; [b]Missing, 25; [c]Missing, 8; [d]Missing, 10; [e]Missing, 26; [f]Missing, 42; [g]Missing, 18.

"practices" were found to be better than for "knowledge," almost all students reported that they bathed during menstruation and disposed products properly. Only one-quarter still agreed with beliefs such as not entering shrines or not touching holy books during menstruation and menstruation being dirty. In spite of this, almost all students expressed their interest to talk further on MHM. There was a notable lack of MHM facilities observed at the colleges that correlated with reports from the students.

We expected a higher level of education of the participants to correspond with a higher knowledge of menstruation. This was true, despite an overall low score on comprehensive knowledge. This finding was further confirmed by comparing sub-groups. Final year students had higher comprehensive knowledge compared to first year students, possibly due to the influence of peers. Similar evidence from a study of Saudi nursing students linked an increase in education level to increased knowledge of menstruation, although no reason was given for the association (14). The effect of the practices getting better with years spent in college could be attributed to peer support. Studies in China and Bangladesh found adequate and accurate information on menstruation, which is important to improve practice on MHM (27, 28, 30).

The proportion of the female college students agreeing with socio-cultural beliefs, such as not entering a shrine or menstruation being dirty, is small but similar to a study among school girls in Bhutan (11). This may indicate that beliefs do not change with an increase in educational level, although our study did not find the association between the two. A similar study in Nepal found that cultural beliefs lead girls to practice self-imposed restrictions like not entering temples or joining prayer ceremonies (18). These beliefs and taboos remained as menstruation was not discussed and these perceptions are passed through generations (20, 24). However, our findings show how women that are having at least a little knowledge about menstruation may not endorse such taboos. Gender issues seem to be an important result of this study due to the sense of injustice felt as most participant say that men have an advantage over women of not having menstruation. While menstruation is a healthy and integral part of female identity, the cultural message of menstruation to be gross, troubling, or shameful has created a dominant narrative of menstruation as a negative, troubling, and problematic experience for those who menstruate (22, 31). It indicates that there is a need to provide an adequate information package that will normalize

menstruation, change attitudes, and end negative social norms (24, 31).

Approximately half of our participants claimed that menstruation affected their usual activities. The majority rated pain as the main reason for absenteeism from college. This is comparable to the assessment conducted among school-going girls in Bhutan (11). A similar study in Mumbai found that the most common problems faced during menstruation were menstrual cramps. In the survey, 44.6% of school girls said that menstrual cramps affected their usual activities and 53.6% of them agreed that women feel more tired than usual during menstruation (11). Absenteeism was noted with an increase in education in our study. However, further analyses would be needed to explore the correlation between absenteeism and educational level along with other variables that were not included in our data collection. Absenteeism due to menstrual cramps may affect the academic performance of a student. Studying a correlation between academic performance and menstrual cramps was beyond the scope of our study, and future research studies in this area would be interesting.

Another key finding of this study was inadequate MHM facilities like water, soap, and bins for disposal of absorbents in both hostels and college toilets, compromising the ability of the students to practice proper hygiene. These findings have been corroborated by a systematic review carried out in low- and middle-income countries where women and girls were unable to undertake their preferred menstrual practices due to inadequate MHM infrastructure (6, 32, 33). Lack of safe spaces for MHM may affect the health and dignity of women and girls (32). Issues of access to facilities and attitudes go hand in hand in causing exclusion, stigma, and disadvantage (22). The participants expressed a strong wish for platforms to talk about menstruation in their college, and only a small proportion said they were uncomfortable in discussing it. Studies in China, Bangladesh, and elsewhere have shown that educational sessions have enhanced knowledge, promoted a more positive attitude, and improved practices such as managing menstrual cramps (6, 15, 28). Significant increases in menstrual knowledge and confidence among women were observed following a more open discourse (15).

Strengths and Limitations

This study had some strengths and limitations. First, college women in Bhutan are chosen based on merit and come from different socioeconomic classes. This improves its generalizability. Second, the study sample was taken from the female college population in all Bhutanese government colleges; therefore, it is generalizable at a national level. Third, we used direct observation of WASH facilities to triangulate with the reports from the students.

The main limitation of our study is that it was a self-administered survey, possibly subject to social desirability bias, with no indication of how participants interpreted the questions. We mitigated this limitation considerably by conducting a pre-test to adjust the questions and also by having the research team stay at the site during data collection to clarify any questions for the participants. The questions on "know about menstrual hygiene" and "infection due to poor MHM" might have overestimated the actual knowledge of the participants on menstrual hygiene, since the participants may have avoided replying to questions, which they did not know. Finally, although we observed WASH facilities in each of the colleges, we could not include the associations between the MHM facilities and the practices of the participants in our analyses, as the observations of MHM facilities were not sufficiently representative.

The study has the following implications: First, increased educational sessions in schools and colleges could improve MHM practices. The focus should be on evidence-based hygiene practices and demystifying false beliefs that limit the participation of women and girls in education and other socio-cultural activities, such as eating certain foods (1, 18, 20). Second, adequate physical facilities to practice MHM are crucial in improving hygiene practices. This should be followed by timely monitoring of these facilities (34). Sensitization of men may be a logical outcome. Men on campus and in the community could ensure adequate menstrual supplies are available for female students (35). Also, colleges could ensure that a healthcare provider is available who can help women when they feel unwell, treat the side effects of menstruation, and assess their urogenital health in case of infections. The college and management should take immediate action to ensure the availability of clean running water and soaps, bins with lids for disposal of sanitary bins, and secure, lockable doors in the toilet facilities.

Conclusions

This study of KAP related to MHM found significant knowledge and belief gaps but some encouraging practices among female students in government colleges of Bhutan. It also revealed important inadequate physical and psychosocial facilities to support the practices of these students, leading to absenteeism. There are clear ways forward to tackle these problems, and we encourage college administrations to address them.

DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study are not publicly available due to sensitivity of disaggregated data for each colleges. However, anonymized datasets are available from corresponding author on reasonable request.

ETHICS STATEMENT

The study was reviewed and approved by Research Ethics Board of Health (REBH) (Approval no: Ref. No. REBH/Approval/2017/077) under the Ministry of Health in Bhutan and Médecins Sans Frontières (MSF) Ethics Review Board (MSF approval no ID 1776). The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TT, SS, PM, MG, JT, and RW conceptualized the study. TT collected and cleaned the primary data. TT and JG-C conducted the analysis and interpretation of the data. TT, JG-C, and SS

drafted the manuscript. TT, MG, JG-C, PM, and SS revised the manuscript. All authors approved the final manuscript.

FUNDING

The fund for the data collection was supported by the Health Research and Epidemiology unit, under the MoH in Bhutan. The training program and open access publication costs were funded by the La Fondation Veuve Emile Metz-Tesch (Luxembourg). The funders had no role in study design, data collection and analysis, decision to publish, or the preparation of the manuscript.

ACKNOWLEDGMENTS

This research was conducted through the Structured Operational Research and Training Initiative (SORT IT), a global partnership led by the Special Programme for Research and Training in Tropical Diseases at the World Health Organization (WHO/TDR). The model is based on a course developed jointly by the International Union against Tuberculosis and Lung Disease (The Union) and the Medécins Sans Frontières (MSF/Doctors without Borders). The specific SORT IT program that resulted in this publication was managed by the MSF and the Public Health Agency of Canada. We are grateful to the Ministry of Education, faculties, and students from the RIM, RUB, and KGUMSB for cooperation and support during data collection. We thank data collectors and research officers of each college for support during data collection. We are especially grateful to Tony Reid, Rafael Van Den Bergh, Samuel Sieber, Claire Braun, and Rob D'hondt for their input and guidance during this manuscript preparation. Most of all, we are grateful to the participants who volunteered to be in this study and provide data for the purposes of this study and further learning.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/frph.2021.703978/full#supplementary-material

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frontiers in Reproductive Health

MINI REVIEW
published: 13 October 2021
doi: 10.3389/frph.2021.756704

Menstruation Dysregulation and Endometriosis Development

Kevin K. W. Kuan[1], *Douglas A. Gibson*[2], *Lucy H. R. Whitaker*[3] *and Andrew W. Horne*[3]*

[1] *Medical School, University of Edinburgh, Edinburgh, United Kingdom,* [2] *Centre for Inflammation Research, University of Edinburgh, Edinburgh, United Kingdom,* [3] *Medical Research Council (MRC) Centre for Reproductive Health, University of Edinburgh, Edinburgh, United Kingdom*

Endometriosis is a common gynecological condition characterized by the growth of endometrial-like tissue outside of the uterus which may cause symptoms such as chronic pelvic pain or subfertility. Several surgical and medical therapies are available to manage symptoms, but a cure has yet to be determined which can be attributed to the incomplete understanding of disease pathogenesis. Sampson's theory of retrograde menstruation is a widely accepted theory describing how shed endometrial tissue can enter the peritoneal cavity, but other factors are likely at play to facilitate the establishment of endometriosis lesions. This review summarizes literature that has explored how dysregulation of menstruation can contribute to the pathogenesis of endometriosis such as dysregulation of inflammatory mediators, aberrant endometrial matrix metalloproteinase expression, hypoxic stress, and reduced apoptosis. Overall, many of these factors have overlapping pathways which can prolong the survival of shed endometrial debris, increase tissue migration, and facilitate implantation of endometrial tissue at ectopic sites. Moreover, some of these changes are also implicated in abnormal uterine bleeding and endometrial diseases. More research is needed to better understand the underlying mechanisms driving dysregulation of menstruation in endometriosis specifically and identifying specific pathways could introduce new treatment targets. Analyzing menstrual fluid from women with endometriosis for inflammatory markers and other biomarkers may also be beneficial for earlier diagnosis and disease staging.

Keywords: endometriosis, menstruation, pathogenesis, inflammation, matrix metalloproteinase (MMP), angiogenesis, apoptosis, abnormal uterine bleeding (AUB)

OPEN ACCESS

Edited by:
Fiona L. Cousins,
Hudson Institute of Medical Research, Australia

Reviewed by:
Annelyn Torres-Reveron,
Sur180 Therapeutics, LLC, United States
Moamar Al-Jefout,
United Arab Emirates University, United Arab Emirates

***Correspondence:**
Andrew W. Horne
andrew.horne@ed.ac.uk

Specialty section:
This article was submitted to Gynecology, a section of the journal Frontiers in Reproductive Health

Received: *10 August 2021*
Accepted: *17 September 2021*
Published: *13 October 2021*

Citation:
Kuan KKW, Gibson DA, Whitaker LHR and Horne AW (2021) Menstruation Dysregulation and Endometriosis Development. Front. Reprod. Health 3:756704. doi: 10.3389/frph.2021.756704

INTRODUCTION

Endometriosis is a chronic inflammatory condition characterized by the growth of endometrial-like tissue outside the uterus. Around 10% of reproductive-aged women are affected and symptoms may include chronic pelvic pain, dyspareunia, and subfertility which can impair the patient's quality of life and work productivity (1). Paradoxically, the severity of symptoms does not necessarily correlate with disease presentation and the lack of reliable diagnostic biomarkers contributes to the average diagnostic delay of 7 years from the onset of symptoms (2, 3). Depending on location and depth of tissue invasion, endometriosis can be classified as superficial (peritoneal), ovarian, or deep (infiltrating) endometriosis. If endometriosis is suspected, laparoscopic visualization remains the gold standard for diagnosis and lesions found may be excised or ablated for symptom control. Apart from surgery, medical treatments, such as analgesics, hormonal modulation/suppression with progestins, combined oral contraceptive pills, or Gonadotrophin-releasing hormone (GnRH)

modulators, can be used for pain management. While symptoms may be reduced temporarily, a cure has sadly not been identified.

One of the main challenges for researchers is the uncertainty regarding the exact underlying mechanisms explaining the etiology and natural history of endometriosis. Sampson's theory of retrograde menstruation is the most widely accepted hypothesis describing how disruption to normal menstrual flow may result in endometriosis. Normally, the superficial (functional) endometrial layer is sloughed during menstruation (menses) to prepare the endometrium for the next menstrual cycle resulting in vaginal bleeding for an average of 5 days (4). In retrograde menstruation, shed tissue flows through the fallopian tubes, enters the pelvic cavity and adheres to tissue in the pelvic cavity leading to formation of ectopic endometriosis lesions.

This review focuses on the "theory of retrograde menstruation" as part of the Frontiers "Menstruation: Myths, Mechanisms, Models, and Malfunction" Special Issue. However, we acknowledge that retrograde menstruation is unable to explain all cases of endometriosis and other theories [e.g., stem cells, epithelial-mesenchymal transition (EMT), coelomic metaplasia, etc.] have also been proposed (5–7).

SEARCH STRATEGY

We applied a broad search strategy to the PubMed database using the terms "(endometriosis) AND [menstruation OR (menstrual cycle) OR menses]" for studies published from inception until July 2021 yielding 3,020 manuscripts. Studies not specific to the menstruation dysregulation themes or other narrative review articles were excluded. Reference lists of studies used in this review were searched for additional studies we judged as relevant. Ultimately, 62 studies were included in the review.

REGULATION OF MENSTRUATION

Dysregulation of Inflammatory Mediators

Following ovulation, the corpus luteum produces progesterone which has anti-inflammatory effects to create a suitable environment for embryo implantation. In the absence of pregnancy, the corpus luteum regresses causing a rapid decline in progesterone levels increasing the activation of the NF-kB inflammatory pathway to prepare for menstruation (8, 9). Local endometrial secretion of inflammatory mediators from epithelial and stromal cells are normally upregulated during the secretory and menstrual phase in the presence of tissue necrosis to aid endometrial repair as part of physiologic menstruation (8).

Many studies have investigated the role of inflammatory regulators in the pathogenesis of endometriosis especially the production of interleukin-1, interleukin-8, tumor necrosis factor-a (TNF-a), monocyte chemotactic protein-1 (MCP-1), and macrophage migration inhibitory factor, and it is generally agreed that women with endometriosis display significantly higher cytokine mRNA expression and immunohistochemistry staining in eutopic/ectopic endometria and peritoneal tissue (10–12). Increased cytokine secretion from endometrial tissue and peritoneal fluid may act in an autocrine manner to promote angiogenesis and cellular proliferation in the endometrium prolonging the viability of shed endometrial cells for implantation, but the exact relationship remains unclear (10).

Apart from aberrant cytokine expression, their receptors are also dysregulated in endometriosis. For example, soluble IL-1 receptor II (ILR-II) is normally concomitantly upregulated as a "decoy receptor" inhibiting excess activation of IL-1. In endometriosis, researchers observed a downregulation of ILR-II immunostaining in eutopic endometrial tissue (13) and greater IL-1-induced MCP-1 secretion from endometrial epithelial cells *in vitro* (14). Interestingly, Akoum et al. observed increased ILR-II staining within epithelial cells suggesting that the release of ILR-II to the cell surface may be inhibited (13). TNF-a also has two primary receptors, TNF-RI and TNF-RII, with pro-inflammatory and anti-inflammatory actions, respectively. In endometriosis, decreased expression of anti-inflammatory TNF-RII within endometrial glandular cells favors pro-inflammatory activity and reduced apoptosis, the importance of which will be discussed later (15). The underlying causes for the downregulated receptors are uncertain and improved knowledge of these control pathways could introduce new treatment methods to reduce the exaggerated immune response.

Dysregulation of innate and adaptive immune mediators could also promote the development of endometriotic lesions. Studies found that antigen-presenting cells like dendritic cells (DC) and Foxp3+ regulatory T-cells were downregulated in endometriosis during the secretory and menstrual phases (16, 17). Although their exact role in menstruation is unclear, researchers suggest that they may activate a targeted immune response toward menstrual debris for clearance (16, 17). The function of endometrial macrophages also appears altered in endometriosis which can cause implications toward disease progression (18). Normally, macrophages phagocytose foreign substances but this activity can be suppressed by certain regulators. For example, mRNA expression of the scavenger receptor CD36 is decreased in peritoneal macrophages and may explain decreased phagocytosis in women with endometriosis contributing to the persistence of peritoneal cavity lesions (19). If endometriotic lesions bleed, this can cause peritoneal heme accumulation and increased heme oxygenase-1 (HO-1) expression in ectopic endometrial stromal cells and peritoneal macrophages, both suppressors of phagocytosis (20). Although evidence suggests that heme and HO-1 overload reduces phagocytosis of ectopic stromal cells, whether these factors could affect endometrial macrophage activity in a paracrine manner should be explored (20). Macrophage phenotype can also exhibit pro- and anti-inflammatory properties. A recent study sequenced RNA from eutopic endometrial macrophages from women with endometriosis which exhibited a significantly greater (z-score ≥ 2.00) pro-inflammatory phenotype (activation of NF-kB pathways and increased upstream TNF regulators) not observed in controls (21). Altogether, an inefficient clearance of shed menstrual fragments could prolong the survival of cells increasing the chance for implantation. Furthermore, the presence of uncleared debris and altered macrophage phenotype may further contribute to an inflammatory peritoneal environment promoting the establishment and persistence of endometriosis lesions (19–21).

Limited research has been done analyzing menstrual blood for inflammatory markers in endometriosis. One study found significantly higher myeloperoxidase (MPO) and N-acetyl-B-D-glucosaminidase (NAG) enzymes ($P = 0.0117$ and $P = 0.039$, respectively), both markers of leukocyte accumulation, in the menstrual blood of women with endometriosis compared to peripheral blood which was not observed in controls. However, when menstrual effluent NAG and MPO activity was compared between controls and endometriosis samples, there was no significant difference (22). Nonetheless, the significant difference in inflammatory markers found in the menstrual blood of endometriosis samples should not be undermined because they corroborate with earlier studies that suggest increased inflammatory activity in endometriosis (15). Recent evidence also found a distinct cytokine profile in menstrual blood vs. blood plasma in healthy donors demonstrating the importance of menstrual blood as a non-invasive source for profiling expression of mediators found in endometrial tissue (23). Therefore, future studies should consider utilizing menstrual blood for analyzing other inflammatory markers raised in endometriosis since it would best represent the inflammatory content of menstrual effluents during retrograde menstruation.

MATRIX METALLOPROTEINASES

Matrix metalloproteinases (MMP) are a family of enzymes mainly localized in the functional layer of the endometrium and secreted from stromal fibroblasts and immune cells mediating endometrial breakdown and extracellular matrix remodeling during menstruation. Ovarian steroid hormones regulate MMP activity and endogenous antagonists known as tissue inhibitors of matrix metalloproteinases (TIMP) prevent overexpression (24). Due to their impact on endometrial structure, abnormal expression of some MMPs such as MMP-2 and MMP-9 are implicated in uterine pathologies such as heavy menstrual bleeding (HMB), fibroids and adenomyosis (25–27). In endometriosis, aberrant MMP/TIMP expression may cause excess endometrial tissue migration, endometrial invasion, and recruitment of angiogenic factors in ectopic lesions (28, 29). Furthermore, enhanced proteolytic activity may dislocate the basal layer of the endometrium increasing the amount of basalis cells in menstrual blood with stem cell characteristics that can differentiate into epithelial and stromal endometrial tissue supporting the stem cell theory (5, 30). However, not all subtypes are dysregulated in endometriosis, and several factors could influence MMP expression.

As mentioned earlier, inflammatory mediators have a multifaceted role and regulating MMP activity is no exception. In a study that treated uterine tissue containing both epithelial and stromal cells with cytokines upregulated in endometriosis, tissue derived from patients with endometriosis secreted more MMP-3 following IL-1 treatment ($P < 0.01$) in a dose-dependent manner which was not observed in controls. This showed that endometrial cells from women with endometriosis respond differently to cytokine-induced MMP secretion. However, treatment with TNF-a did not change MMP-3 secretion. Furthermore, MMP-1/2 and TIMP-1/2 levels were not significantly different to controls after cytokine stimulation suggesting that cytokine-specific pathways are present (31). Therefore, future *in vitro* analyses of MMP secretion against an array of cytokine treatments may be useful in identifying specific immune pathways. MMP-27 has also been found to localize near CD163+/CD206+ macrophages especially during the time of menstruation. This inflammatory and degenerative microenvironment can favor endometriosis progression by increasing tissue migration and promoting implantation (32).

Local MMP expression can vary greatly depending on the location of endometriotic lesions and growth patterns. One study analyzed MMP expression in colorectal endometriosis (one of the most aggressive forms of deep infiltrative endometriosis) and reported significantly greater MMP-2,−3, and−11, and lower TIMP-2 expression than endometrial cysts and peritoneal lesions (33). Ovarian endometriomas also had a different MMP profile with increased production of MMP-1,−2,−7, and−9 during the menstrual period (29, 33). Since ovarian steroid hormone secretion lacks the normal cyclic variation in the presence of endometriomas (34), this may explain the different MMP-2 expression compared to other types of endometriosis (29). *In vitro* studies suggest that increased MMP activity is related to disease invasiveness which may explain the different MMP profile in colorectal endometriosis (35, 36). Certain MMP levels in peritoneal fluid are positively correlated to advanced stages of disease according to revised AFS staging, making MMP a promising diagnostic biomarker and is being explored (37). The exact cause for the overall increased MMP-2 expression remains unknown, but it may be due to reduced MMP-2 gene methylation (38). For clarity, **Table 1** summarizes the MMPs upregulated depending on endometriosis location and main study findings.

While many of the studies recognize that MMP is upregulated during menstruation, most of the evidence available is from tissue samples collected during the proliferative or secretory (31, 33, 35, 38) phases. This limits the interpretation that MMP dysregulation during menstruation can cause endometriosis and future research should compare endometrial tissue obtained during the menstrual phase to fully understand the impact on disease pathogenesis. Since the location can also affect MMP expression, endometriotic samples should be compared according to lesion location to minimize potential confounders.

HYPOXIA AND ANGIOGENESIS

During menstruation, the rapid decline in progesterone also causes vasoconstriction of the spiral arterioles which supplies oxygenated blood to the endometrium during the luteal phase. Reduced oxygen supply induces hypoxic stress stabilizing hypoxia inducible factor 1 (HIF-1). Although the exact roles of hypoxia and HIF-1 in the endometrium remains unclear, it is hypothesized to help restore endometrial blood supply and assist in endometrial repair following menstruation (42, 43). However, perturbation of hypoxia in the endometrium may also potentiate gynecological conditions like heavy menstrual bleeding and endometriosis (42).

TABLE 1 | Summary of literature analyzing MMP expression in endometriosis.

References	Important MMP/TIMP subtype(s) identified	Location of endometriosis	Main finding(s)/conclusion(s)
Sillem et al. (39)	TIMP-2	Unspecified	Increased TIMP-2 transcription may increase endometrial cell invasiveness
Mizumoto et al. (29)	MMP-1,−2,−7, -9 TIMP-1	Endometrioma	MMP primarily produced from stromal cells which may cause ECM destruction
Sillem et al. (31)	MMP-1,−2, -3 TIMP-1,−2	Unspecified	IL-1 induces significantly greater MMP-3 secretion from eutopic EM tissue not observed in controls
Chung et al. (35)	MMP-2 TIMP-2	Unspecified	Increased eutopic endometrial MMP-2 expression in EM and significantly lower TIMP-2
Uzan et al. (33)	MMP-2,−3, -11 TIMP-1,−2	Colorectal, endometrioma, peritoneal	MMP profile depends on EM location; Highest MMP-2,−3, and−11 expression in colorectal EM; TIMP-2 expression higher in peritoneal EM
Hudelist et al. (40)	MMP-1	Endometrioma, peritoneal	Significantly increased MMP-immunohistochemistry staining in ectopic lesions; non-significant differences in eutopic MMP-1 compared to controls
Kyama et al. (11)	MMP-3	Unspecified	Significantly higher eutopic MMP-3 mRNA expression in EM
Matsuzaki et al. (41)	MMP-9	Unspecified	Higher eutopic MMP-9 in EM; PFK 115-584 inhibited activity and reduced invasive cells
Cominelli et al. (32)	MMP-27	Endometrioma, peritoneal, rectovaginal	Increased macrophage MMP-27 in endometrioma and peritoneal EM, but not in rectovaginal lesions
Tang et al. (38)	MMP-2, -9 TIMP-1,−2, -3	Unspecified	Decreased MMP-2 DNA methylation in EM cells; Significantly higher MMP-2,−9 and TIMP-1,−2 transcription abundance in EM; Significantly lower TIMP-3 transcription abundance in EM

One of the ways hypoxia may promote endometriosis is by increasing EMT. The EMT theory describes the changes of stationary epithelial cells to migratory mesenchymal cells during tissue repair. Our understanding of EMT in endometrial physiology is mostly derived from murine mice models, but the few studies utilizing human endometrial tissue suggest that factors driving EMT may be increased in endometriosis (44, 45). Rytkonen et al. found that hypoxia upregulated several stromal cell-specific genes that drive EMT (e.g., collagens, fibronectin, and proteases) and increased the expression of transcription factors JunD Proto-Oncogene and CCAAT Enhancer Binding Protein Delta by 18- and 5-fold, respectively, in deep endometriotic lesions (45). These transcription factors could be potential treatment targets, and inhibitors of the Jun pathway like the c-Jun NH_2-terminal kinase inhibitor significantly reduced the amount of active endometriotic lesions in baboon models (46). Other studies also found increased mesenchymal transition markers like N-cadherin and vimentin in endometrial epithelial cells under hypoxic and inflammatory conditions (47, 48). Meanwhile, invasive activity and mesenchymal changes of Ishikawa cells decreased when HIF-1a levels were down-regulated (48). Therefore, hypoxic stress during menstruation may promote EMT and gene expression favoring endometrial cell migration.

Vascular endothelial growth factor alpha (VEGF-a) is an important angiogenic mediator, that is upregulated during menstruation and further activated by hypoxia and inflammation (49). However, excess VEGF-a in menstrual fragments may increase the vascularization potential of shed cells during retrograde menstruation facilitating attachment and growth at extra-uterine sites (9, 50). An interesting point of discussion is the comparison of VEGF expression in red (more active and vascular) lesions vs. black (less active and later-staged) lesions. Khan et al. found significantly higher VEGF expression in red lesions correlating with higher vascular activity (51). Meanwhile, a later study by Takehara et al. found no significant difference in gene expression and it remains unclear whether VEGF differs depending on the type of lesion (52). However, both agreed that women with endometriosis had significantly higher VEGF immunoreactivity in eutopic/ectopic endometrial tissue compared to controls which is well-supported by the existing literature (52–55). The endometrial lesions also exhibited similar proliferative and angiogenic activity as eutopic tissue supporting Sampson's theory of retrograde menstruation (51).

The Notch-induced four jointed box 1 (FJX1) protein has also been considered a possible pro-angiogenic factor in endometriosis. In the primate endometrium, Notch regulates decidualization, cell proliferation, and cell fate (56). Although FJX1 function is poorly understood in humans, its regulatory actions on HIF-1 may influence angiogenic activity. In eutopic tissue from human and baboon models with endometriosis, FJX1 was significantly increased during the secretory phase. During menstruation, FJX1's downstream effects like increased angiogenic activity and HIF-1 expression was observed (57). However, other upstream factors must be dysregulated in endometriosis since FJX1 was not significantly raised in the normal endometrium. Increased activation of the Notch signaling pathway in endometriosis

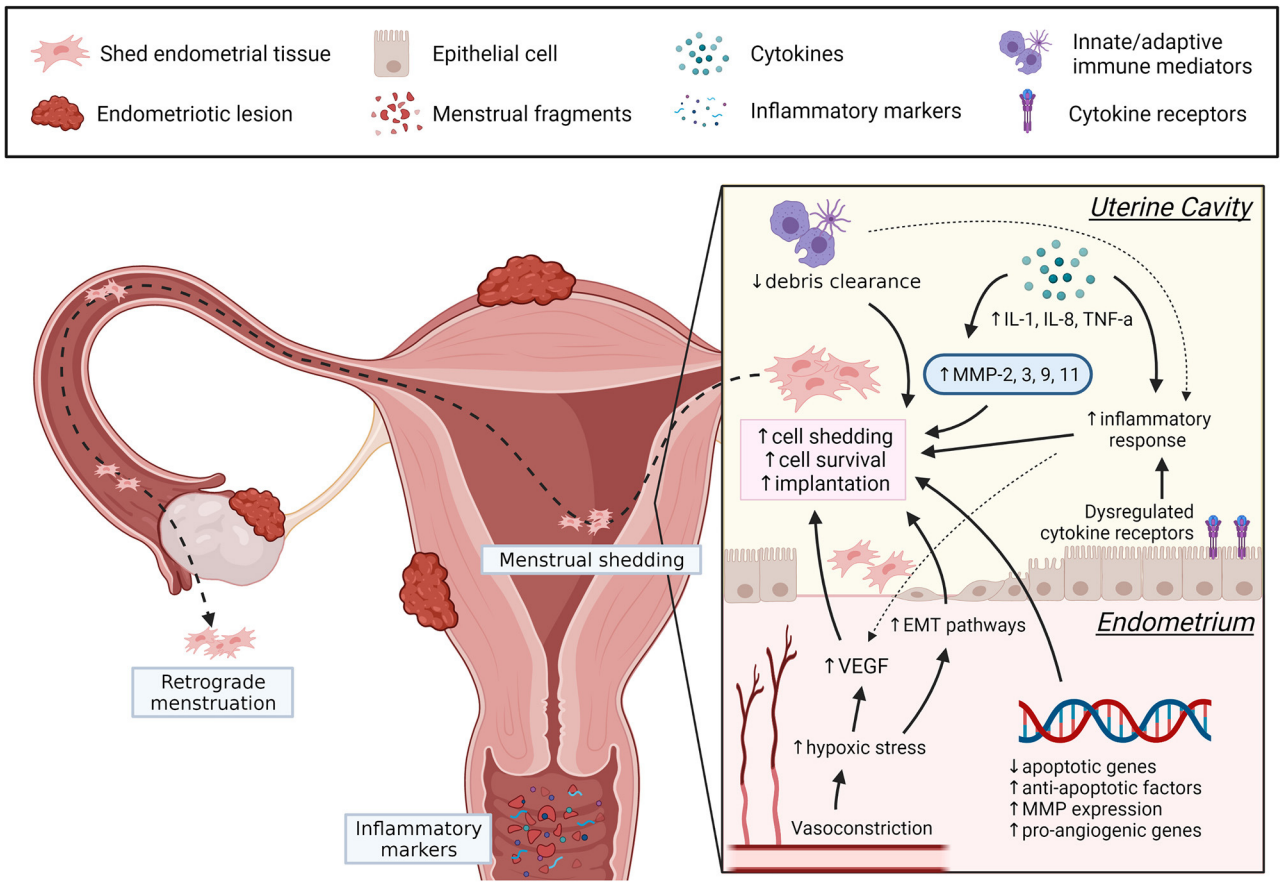

FIGURE 1 | Summary of dysregulated menstrual pathways that can contribute to the pathogenesis of endometriosis. In women with endometriosis, dysregulation of several menstrual processes (angiogenesis, EMT, apoptotic regulators, inflammation, debris clearance, MMP) may promote endometrial cell detachment, prolong endometrial cell survival, and increase the likelihood for implantation following retrograde menstruation. Understanding these pathways may introduce new therapeutic targets and can be achieved by further exploring the underlying mechanisms of menstrual dysregulation in endometriosis. Created with BioRender.com.

could be a reasonable assumption since it induces FJX1 expression (58). In murine models with endometriosis, administration of Notch1 antagonists reduced cell migration and size of lesions and could be a promising therapeutic target (58).

While VEGF activity may be similar between eutopic and ectopic endometrial tissue, other menstrual characteristics may not necessarily be shared. In a recent retrospective study, matched superficial peritoneal endometriotic lesions and eutopic endometrial tissue from 42 patients were compared for histological/morphological analysis throughout the menstrual cycle and only 4% of the endometriotic lesions displayed stromal decidualization during the secretory phase (59). Endometriotic gland profiles (i.e., presence of hemosiderin-laden macrophages outside the menstrual phase) were independent of the menstrual cycle phases also reported by previous studies which may explain intermenstrual pelvic pain symptoms reiterating the complexity of endometriotic tissue (59–61).

CONTROL OF APOPTOSIS REGULATORS AND CELL PROLIFERATION

Apoptosis is a form of programmed cell death and is important during menstruation to eliminate shed cells within the uterine environment. In women with endometriosis, reduced spontaneous eutopic endometrial apoptosis was observed using TdT-mediated dUTP biotin nick end-labeling assay throughout the menstrual cycle which could prolong cell survival for implantation at ectopic sites (62, 63). Interestingly, when Bax, a pro-apoptotic gene, was analyzed using immunohistochemical techniques during the secretory phase, the levels were raised in endometriosis which seems counterintuitive (63, 64). When another study further separated the secretory phase into early and late stages, Bax mRNA expression was significantly higher during the early phase in endometriosis, but decreased by 63% during the late secretory phase accompanied by reduced stromal and epithelial apoptotic activity compared to controls (64). It is unclear why pro-apoptotic factors are upregulated earlier in the

menstrual cycle, but the later downregulation supports the theory of reduced apoptosis during menstruation in endometriosis.

Increased expression of anti-apoptotic factors may also explain the reduced apoptotic activity in endometriosis. For example, the phosphorylated ERK1/2 pathway usually prolongs cell survival and can influence the c-Jun transcription factor described earlier but is abnormally high in endometriosis regardless of the menstrual cycle phase resulting in persistent proliferative changes (65–68). Recently, a study suggested that the ERK pathways may also induce plasminogen activator inhibitor-1 expression, another anti-apoptotic protein that inhibits fibrinolysis, and is hypothesized to assist in the shedding of endometrial cells for attachment elsewhere (69).

The B-cell lymphoma 2 family proteins have also been commonly studied since they comprise of both pro-apoptotic (Bcl-xS) and anti-apoptotic (Bcl-xL and Bcl-2) regulators in the endometrium. In every sample analyzed, Bcl-xL expression significantly exceeded Bcl-xS throughout the menstrual cycle. Furthermore, the anti-apoptotic Bcl-2 form was increased in endometriosis improving endometrial cell survival (70). Overall, the literature suggests that the decreased apoptotic activity during the late-secretory and menstrual phases prolong the viability of shed endometrial cells allowing for implantation. However, the proliferative and early-secretory phases may have increased apoptotic activity and should be further investigated.

CONCLUSIONS

Menstruation is a complex physiological process, and dysregulation of control mechanisms are implicated in abnormal uterine bleeding and endometrial diseases. In endometriosis, increased endometrial invasion, inflammation, angiogenic activity, and MMP have many overlapping pathways facilitating basalis invasion, EMT, stem cells release, prolonged viability of shed endometrial cells and implantation of ectopic lesions (see **Figure 1**), although the exact underlying mechanisms remain unclear. In the future, more studies should assess endometrial biopsies collected during the menstrual phase. The menstrual cycle phase of biopsy collection and the location of endometriosis lesions may introduce confounders when analyzing regulatory factors and should be considered. Evaluating menstrual blood from women with endometriosis could also be a useful non-invasive sample for understanding disease mechanisms and exploring potential biomarkers. It is evident that menstruation is a complex physiological process with many unanswered questions. However, understanding how dysregulation of certain factors contribute to the pathogenesis of endometriosis can help identify new diagnostic markers and therapeutic targets, ultimately improving the patient's quality of life.

AUTHOR CONTRIBUTIONS

KK wrote the manuscript, created the tables/figures, and designed the review with AH. DG, LW, and AH were involved in critically reviewing and editing the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

AH and LW were supported by a grant from the Medical Research Council (MR/N022556/1). DG was supported by a Sir Henry Dale Fellowship jointly funded by the Wellcome Trust and the Royal Society (Grant No. 220656/Z/20/Z to DG). LW was supported by an NES/CSO Clinical Lectureship (PCL/19/01).

ACKNOWLEDGMENTS

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Conflict of Interest: LW receives grant funding from the National Institute for Health Research NIHR and the Chief Scientist's Office. AH receives grant funding from the NIHR, the Medical Research Council MRC, the Chief Scientist's Office, and Roche. He has received honoraria for consultancy for Ferring, Roche, Nordic Pharma, and Abbvie.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Reproductive Health

REVIEW
published: 26 November 2021
doi: 10.3389/frph.2021.784578

The Spiny Mouse—A Menstruating Rodent to Build a Bridge From Bench to Bedside

Nadia Bellofiore[1,2]*, *Jarrod McKenna*[2], *Stacey Ellery*[1,2] *and Peter Temple-Smith*[2]

[1] *The Ritchie Centre, Hudson Institute of Medical Research, Clayton, VIC, Australia,* [2] *Department of Obstetrics and Gynaecology, Monash University, Clayton, VIC, Australia*

Menstruation, the cyclical breakdown of the uterine lining, is arguably one of evolution's most mysterious reproductive strategies. The complexity and rarity of menstruation within the animal kingdom is undoubtedly a leading contributor to our current lack of understanding about menstrual function and disorders. In particular, the molecular and environmental mechanisms that drive menstrual and fertility dysregulation remain ambiguous, owing to the restricted opportunities to study menstruation and model menstrual disorders in species outside the primates. The recent discovery of naturally occurring menstruation in the Egyptian spiny mouse (*Acomys cahirinus*) offers a new laboratory model with significant benefits for prospective research in women's health. This review summarises current knowledge of spiny mouse menstruation, with an emphasis on spiral artery formation, inflammation and endocrinology. We offer a new perspective on cycle variation in menstrual bleeding between individual animals, and propose that this is indicative of fertility success. We discuss how we can harness our knowledge of the unique physiology of the spiny mouse to better understand vascular remodelling and its implications for successful implantation, placentation, and foetal development. Our research suggests that the spiny mouse has the potential as a translational research model to bridge the gap between bench to bedside and provide improved reproductive health outcomes for women.

Keywords: menstruating mouse model, DHEA, abnormal uterine bleeding, preeclampsia, angiogenesis, uterus

OPEN ACCESS

Edited by:
Philippa Saunders, University of Edinburgh, United Kingdom

Reviewed by:
Phoebe Kirkwood, University of Edinburgh, United Kingdom
Niamh Forde, University of Leeds, United Kingdom

***Correspondence:**
Nadia Bellofiore
nadia.bellofiore@hudson.org.au

Specialty section:
This article was submitted to Gynecology, a section of the journal Frontiers in Reproductive Health

Received: *28 September 2021*
Accepted: *08 November 2021*
Published: *26 November 2021*

Citation:
Bellofiore N, McKenna J, Ellery S and Temple-Smith P (2021) The Spiny Mouse—A Menstruating Rodent to Build a Bridge From Bench to Bedside. Front. Reprod. Health 3:784578. doi: 10.3389/frph.2021.784578

INTRODUCTION

The menstrual cycle is a process in which the uterine lining sheds, regenerates, and terminally differentiates in preparation for pregnancy. Unsuccessful pregnancy results in necrosis and shedding of the functional layer of the uterus, the endometrium. This culminates in cyclical uterine bleeding termed menstruation. Menstruation occurs almost exclusively in higher order primates, including humans and Old-World monkeys, with several species of bat and the elephant shrew being the few exceptions (1–5).

As unique as our non-pregnant cycles are, so too are our pregnancies with menstrual species presenting some of the most complex and metabolically demanding gestational processes in mammals (6–9). Consequently, the absence of menstruation as the preferred female reproductive strategy in mammals has restricted the advancement of our knowledge and understanding of the biology of menstruation, and especially the targeted development of therapeutics for menstrual and

associated pregnancy disorders. While the need for a more thorough understanding of women's health is clear, the dearth of appropriate non-human laboratory models has slowed the progression of menstrual research.

The recent discovery that a small rodent, the common spiny mouse (*Acomys cahirinus*), has a naturally occurring menstrual cycle provides a rare opportunity to use a small laboratory animal in studies of female reproductive biology (10). An in-depth characterisation of this singular phenomenon reveals similarities in both physiological and behavioural aspects of menstruation with higher order primates including humans (11, 12). Further, certain likenesses to primate pregnancy (13, 14) places the spiny mouse as a promising candidate for examining various aspects of women's reproductive health. This review will discuss the advantages of using this now underutilised laboratory rodent in menstrual and gestational research, with particular emphasis on correlating the unexplored linkages between menstrual health and pregnancy outcomes. We will examine how the unique endocrinology of this species may also be leveraged for studying reproductive and inflammatory disorders to highlight to the broader scientific community the untapped potential of this rodent for biomedical research.

DISRUPTED CYCLES, DISRUPTED LIVES

In the collaborative Global Burden of Diseases Project (15, 16), almost 500 disabling diseases and injuries were subjected to a comprehensive statistical analysis of prevalence and mortality, including disability-adjusted life years (DALYs). Menstrual disorders, including Abnormal Uterine Bleeding (AUB) were not classified among them. AUB is defined as a change in the frequency, duration and/or amount of blood loss in menstruation. and can be further classified according to the International Federation of Gynaecology and Obstetrics in 2011 PALM-COEIN system (Polyp, Adenomyosis, Leiomyoma, Malignancy and hyperplasia, Coagulopathy, Ovulatory Dysfunction, Endometrial, Iatrogenic, Not yet classified) (17). Under these categories, up to 80% of menstrual bleeding disorders can be accounted for (17). Many ovulatory disorders can be attributed to aetiologies such as polycystic ovarian syndrome, extreme weight fluctuations, stress or other endocrinopathies to result in disrupted cycles. A spectrum of deviations from the "normal" menstrual period manifests in patients from extremely light or infrequent bleeding (amenorrhea) to heavy menstrual bleeding (HMB, replacing previously used menorrhagia). HMB is often the simplest aspect of AUB to assess the burden of disease as it generally has the greatest impact on daily function and quality of life (17).

A systematic review analysed available data from 1980 to 2005 (18) to address a disturbing gap in awareness in the public, medical and political sectors provides conservative estimates of the global burden and Health-related Quality of Life (HRQoL) of AUB. Prevalence of AUB was estimated at 10–30% of patients in regions of Europe and the United States. Unsurprisingly, women who sat below the 25th percentile for HRQoL were negatively impacted in their work productivity. The authors estimated the annual direct financial costs (such as visits to medical practitioners, surgeries and medical interventions) of $1–1.55 billion. Indirect costs (such as workplace absenteeism) could be as high as $12–36 billion, with AUB patients reported to work 3.6 weeks less per year than age-matched women without AUB. Furthermore, hysterectomy or endometrial ablation remain the prominent non-medicinal treatments for AUB, with almost 90% of women hospitalised with AUB undergoing surgery, including hysterectomy, which is the second most performed gynaecological procedure in the US. These values clearly reflect not only the dire need for further research into alternative methods of treatment for AUB, but a shift in global perception of the degree of debilitation caused by menstrual cycle dysregulation. To achieve this, a comprehensive understanding of menstruation across a physiological, cellular, and biomolecular level is critical, though are not easily studied among women. For this research, especially those which inform new treatments, appropriate animal models are required.

THE MENSTRUAL CYCLE IN THE ANIMAL KINGDOM

To understand how we can best examine disorders of menstruation and pregnancy with our current available resources, we first need better to understand the intricacies of the menstrual cycle. The menstrual cycle involves complex hormonal interactions between hypothalamus, pituitary gland, ovaries, and uterus. A comprehensive description can be found in Johnson (19). Briefly, the major uterine morphological and physiological changes governed by ovarian steroids oestradiol-17B (E_2) and progesterone (P_4). The menstrual cycle comprises both uterine and ovarian distinctive phases under direct stimulation from pituitary hormones. The beginning of a new fertile cycle in all menstruating species is marked by shedding of the superficial layer of the endometrium in the uterine cycle; i.e., menses or menstruation. Menses corresponds with the follicular phase of the ovarian cycle, where follicle-stimulating hormone (FSH) secreted from the anterior pituitary causes a gradual increase in follicular recruitment and growth. As follicles mature, they in turn secrete increasing levels of E_2, resulting in a rapid thickening of the endometrial stroma during the corresponding proliferative phase of the uterine cycle. High E_2 initiates a surge of luteinising hormone (LH) from the anterior pituitary gland, triggering the release of the oocyte mid-cycle during the ovulatory phase. The remnant of the ovulatory follicle forms a functional corpus luteum, which secrets both sex steroids, with P_4 the dominant hormone of the luteal phase in the ovarian cycle (19). It is this process that delineates menstrual species from most other mammals (20).

Viable placental and foetal development rely on the functional transformation of the endometrium under the influence of P_4 from the corpus luteum. The current leading theory suggests that menses is the by-product of spontaneous decidualisation of the endometrium. During the secretory/luteal phase of the

uterine/ovarian cycle (respectively), endometrial stromal cells undergo spontaneous decidualisation, a terminal metamorphosis to facilitate embryo implantation (21–24), which is a rare and pre-emptive process occurring only in menstruating species. This terminal differentiation of the endometrial stroma allows cells to form a hospitable niche for the implanting embryo, while also selecting out those of poor quality and reducing the risk of non-viable pregnancies (22, 25). Simultaneously, endocrine signalling promotes substantial angiogenesis; the creation of new blood vessels from the uterine artery and results in the formation of spiral arteries (26). The unison of these cyclical processes is crucial in preparing the endometrium for a successful pregnancy.

Concurrently, uterine angiogenesis during the menstrual cycle culminates in the development of large, visibly coiled spiral arteries. Further substantial vascular changes during early gestation, known as spiral artery remodelling, are essential to support adequate gas and nutrient exchange with the growing foetus. Without a human chorionic gonadotrophin (hCG) rescue signal secreted from an implanted blastocyst, the corpus luteum degenerates, and P_4 is rapidly withdrawn. This withdrawal causes necrosis of endometrial decidual cells, and the degradation of the stromal matrix in an inflammatory event similar to the tissue repair mechanism seen in wound healing. As the supporting uterine architecture is broken down, so too are the newly formed blood vessels, resulting in the flow of blood that we observe during menses. A new fertile cycle is now initiated.

Of nearly six thousand identified mammals in the world, <2% have adopted the reproductive strategy of cyclical shedding and rebuilding of the endometrium (10). Almost all identified menstruating species belong to the primate order. The handful of exceptions, which includes a few species of bats and, perhaps, the elephant shrew (1, 3–5, 27), do little to help resolve the evolutionary enigma of menstruation. While most have common traits suggesting the existence of a phylogenetic link, a "one-size-fits-all" explanation for why some species adopted this strategy has yet to be elucidated. An in-depth comparison of biological commonalities is reviewed in Bellofiore et al. (20) from which we can infer that while spontaneous decidualisation, mode of placentation and offspring maturity at birth are often comparable in menstruating mammals, there are a number of grey areas. For example, all menstruating species identified to date appear to have haemochorial placentation, but not all species with haemochorial placentation menstruate, for example mice and guinea pigs. This intent to find common ground among menstruating species is further obscured by the spiny mouse.

MENSTRUATING MICE

Rodents do not naturally menstruate, the only exception to date being the spiny mouse (10). Currently, there are few established captive colonies of spiny mice across the world, with various species used to study different aspects of biology. The species described in this review, unless otherwise specified, refers to that of an in-house derived research colony of common spiny mice at Monash Medical Centre, Melbourne, Australia. This colony was established in 2001 from 5 breeding pairs, and since then have not had new genetic material added. The implications of this potential genetic bottle-necking have been previously discussed elsewhere (28), as has a comparison of husbandry with that of other known colonies (20, 28).

The common or Egyptian spiny mouse (*Acomys cahirinus*) is a ground-dwelling rodent native to regions of Africa and the Middle East. It is one of over 20 species in the genus *Acomys*, with names often reflecting geographical dispersion or coat colours (29). The species' name is derived from their dorsal exterior coat; thick, rigid, and spine-like hairs displacing the soft neonatal fur at ∼30–60 days. The spiny mouse is still a relative novelty in scientific research and possesses many remarkable traits for a rodent. Spiny mice were initially used to study diabetes mellitus due to their tendency for obesity and pancreatic hyperplasia when fed a high sugar diet (30, 31). More recently, spiny mice have been discovered to have skin autotomy, demonstrating scar-free wound healing for the first time in a mammalian species (32).

Their distinctiveness extends further to their reproduction. Dams have a relatively long gestational period (38–39 days), approximately twice that of a standard laboratory mouse (*Mus musculus*), and deliver on average 2–3 developmentally mature (precocial) pups per litter (33). In stark contrast to conventional rodents, spiny mouse pups are born in a similar advanced state of development to human babies, having completed organ development *in utero*, being completely mobile, and with eyes and ears open (**Figure 1**). The lower numbers of offspring in spiny mouse pregnancy likely reflects a greater placental investment, including increased ratio of labyrinth to spongy zone regions (13), and their surprising capacity to synthesise adrenal hormones cortisol and dehydroepiandrostenedione (DHEA) (34, 35), which result in *in utero* maturation of most organs, including lungs, kidneys and ovaries prior to birth (36–38). The precocious nature of the spiny mouse is preferred to the laboratory mouse to study foetal development. We have used this rodent to establish models of perinatal injury, including intrauterine growth restriction and birth asphyxia (39, 40).

The recent observation of cyclical spontaneous decidualisation and ensuing menstrual bleeding in spiny mice (10) confirmed *A. cahirinus* as the only rodent with a naturally-occurring menstrual cycle. In other rodents, decidualisation does not occur until embryo implantation. However, artificial decidualisation and menstruation can be experimentally induced in *M. musculus,* as fist described by Finn and Pope (41). Briefly, a decidual reaction in ovariectomised mice was initiated using E_2 and P_4 injections, followed by physical stimulation of the endometrial stroma via arachis oil injection into the uterine lumen. When hormonal support was withdrawn, the decidualised cells were no longer supported, resulting in menstrual-like shedding. This model has been further refined using E_2 priming prior to insertion of slow-release progesterone implants, allowing for a more rapid hormonal withdrawal and greater physiological relevance to true menstruation (42). Further alterations to have been made to the mouse model of

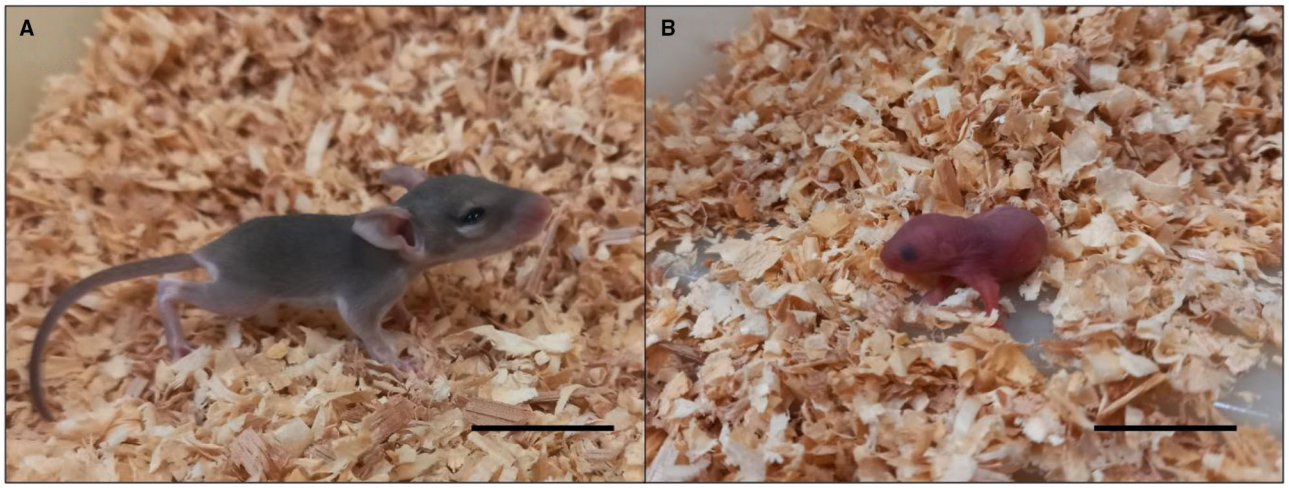

FIGURE 1 | Spiny mouse **(A)** and mouse **(B)** pups on the day of birth. Spiny mice are covered in fur, eyes and ears opened, mobile with limbs fully developed and functional, weighing ∼5–6 g in litter sizes of 1–5. The mouse pup has not developed fur, or mobility, with eyes and ears closed. Mice weigh ∼1–2 g in litter sizes of 4–12. Scale bars = 1 cm.

menstruation which have contributed to a greater understanding of the role of hormones. One study used in-tact females and induction of pseudopregnancy to elevate endogenous P_4 and demonstrate menstrual shedding following a natural declination (43), while others have generated data supporting that P_4 support is time-sensitive, and shedding becomes irreversible after a time-critical window (44). Variations of the mouse model of menstruation have since been used to establish new models of endometriosis through intraperitoneal injection of menstrual debris from experimentally induced donor mice (45). Importantly, these models have made substantial contributions in our understanding of androgens in endometrial repair (46), neutrophil and macrophage promotion of angiogenesis during endometriotic lesion development through cytokine secretion (47) and altered expression profiles for genes regulating the immune system, cell adhesions, proliferation and angiogenesis in the endometriosis phenotype (48). Undoubtedly, these induced models have led to vital insights into the menstrual regulation and pathologies of menstrual disorders. However, artificial models have their limitations, especially regarding menstruation as a lifetime recurrence, the relevance of induced decidualisation and use of ovariectomised models. These limitations have been extensively discussed elsewhere (11, 20, 28), as have the advantages of a using naturally menstruating rodent, the spiny mouse.

Relative to other menstruating species, the spiny mouse menstrual cycle is brief, lasting on average 9 days and ranging from 6 to 12 days in healthy, sexually mature females. The morphology and function of the decidual cells in the spiny mouse closely mimics decidualisation in primates; it is stimulated by a significant rise in progesterone, and recognised by secretion of biomarkers prolactin and interleukin-11 (10, 11). Comprehensive histological and morphological analysis of the spiny mouse uterine architecture across the menstrual cycle confirmed similarities to primate menstruation in the breakdown, repair, and rebuilding of the endometrium. The spiny mouse demonstrates focal "piecemeal" shedding in progressive waves along the uterus, with peak inflammatory influx of neutrophils occurring mid-menstruation and simultaneous repair occurring in the epithelium (11). Similarities were also evident in the uterine secretion of inflammatory and repair markers interleukin-8 and macrophage inhibitory factors, as well as the localised focal shedding and adjacent repair of the endometrium (11). These studies also demonstrated behavioural and physiological similarities between spiny mice and other naturally menstruating species, in that changes in food consumption, weight fluctuations, anxiety, and exploration are driven by menstrual cycle stage and suggestive of a human-like premenstrual syndrome (PMS) (12). Having a non-primate model of PMS in a species with a short menstrual cycle allows an unexpected opportunity for a comprehensive examination of the influences of molecular and endocrinological changes during the menstrual cycle on the behavioural repertoire of individuals during the transition from sexual maturation into adulthood. Such potential warrants further investigation and is discussed more thoroughly by Bellofiore et al. (12, 20).

AN EMERGING MODEL OF ABNORMAL UTERINE BLEEDING

Studies using the spiny mouse have identified the natural formation of spiral arteries prior to menstrual onset for the first time in a rodent (11, 20). A combination of histological techniques, including immunofluorescent double-labelling for alpha-Smooth muscle actin and vascular endothelial growth factor, has provided evidence of cyclical vascular remodelling concurrent with spontaneous decidualisation occurring naturally

in the spiny mouse. This strongly encourages the use of the spiny mouse as a model for uterine vascular studies.

Individual spiny mice have notable variation in menstrual blood loss (11). Within the well-characterised Monash colony, menses is best identified under light microscopic analysis of haematoxylin and eosin-stained vaginal lavages, with frank menstrual blood observed at the vulva a rarer occurrence. In fact, the level of individual bleeding between spiny mice is remarkably varied, with up to 10% of females exhibiting what is most appropriately describe as heavy menstrual bleeding. In these females, menstrual blood can be viewed either at the vagina or in the lavage sample itself, for prolonged periods of time. The reverse is also true, in that a similar proportion of females demonstrate light menstrual bleeding whereby relatively few erythrocytes can be identified in lavage samples or a menstrual bleed that lasts less than a day. Differences in menstrual blood loss between individuals is also observed in women (49). A small animal model of natural AUB, not an experimentally induced AUB, may provide interesting new insight into the genetic or environmental origins of such deviations from the norm, or even help us to better understand why these variations exist at all.

The evolution of a wide spectrum of menstrual blood loss, which exists among women, has yet to be definitively explained, as is the variation in the timing, onset, and heaviness of an individual's menstrual period when an underlying pathological cause is not identified (17). It has been argued that menstrual bleeding has evolved not necessarily as a selective advantage in itself, but rather as a by-product of another biological process (spontaneous decidualisation); it has merely evolved because it does not provide a disadvantage to survival (23). However, logic would then suggest that a small rodent species, likely to be the target of numerous ground and aerial predators, would find it a significant disadvantage to leave a trail of blood each menstrual cycle, providing both visual and olfactory signals. We must then assume that the risk of predation is outweighed by the benefit of spontaneous decidualisation and spiral artery formation prior to conception. The question remains: what are these benefits? Having observed such a large natural variation of bleeding in a species which has not been subjected to selective pressures of environment and predation, we hypothesise that females with heavy menstrual bleeding have a selective advantage for breeding. Their vessels undergo more extensive remodelling, resulting in optimal placental nutrient and gas exchange and, hence, larger and/or more offspring can be supported.: The subsequent excessive blood loss during menstruation stems from increased spiral artery formation and a thicker endometrial lining, both of which are stimulated by the androgen DHEA.

DHEA: MAKING MENSTRUATORS AND AUGMENTING ANGIOGENESIS

DHEA and Reproduction

Current knowledge emphasises the roles of sex steroids oestrogen and progesterone in female fertility, but largely ignores the importance of DHEA. DHEA and its metabolite for storage in the bloodstream, DHEA-Sulphate (DHEA-S), are among the most abundant circulating steroids in humans (50). A ubiquitous androgen primarily derived from the adrenal glands, and perhaps by the gonads, DHEA production is significantly increased during the early stages of sexual maturation and is an important precursor in the synthesis of sex steroid hormone testosterone and oestrogen (51).

DHEA is one of the main androgens elevated during adrenarche. This is a process of sexual maturation related to puberty during which the production of androgens is increased, and is typically specific to higher order primates (52). The association between adrenal maturation, time to menarche (the first period), and female fertility, while undoubtedly intrinsically linked, has not been well-examined in menstruating species. While Old World monkeys (humans included) have a distinctive prepubertal adrenarche, New World monkeys and other non-menstruating species lack enzymes in the steroidogenic pathway which reduce the ability of the adrenal glands to produce DHEA. Previous studies have confirmed that the spiny mouse has P450c17 activity and produces DHEA *in utero* (35). In contrast, non-menstruating marmosets have significantly reduced circulating DHEA due to a deficiency in the key enzyme P450c17 (53).

A further commonality between menstruating species is the production of 1–2 offspring per pregnancy. This is a repeated theme in the menstruating spiny mouse, littering on average 2–3 pups (though this can range from 1 to 5). Conventional laboratory rodents which lack the capacity to synthesise DHEA (34, 36) do not spontaneously decidualise or menstruate, and have litters ranging from 8 (mouse) to 18 (Rats). This demarcation between species suggests DHEA has a central role in the menstrual cycle [reviewed in detail in (28)] and has an important advantage for conception and maintenance of precocial foetal development.

Decidualisation of the uterine stroma during the menstrual cycle in preparation to support pregnancy relies in part on the actions of androgens. In a recent *in vitro* study, androgens were shown to play a pivotal role in enhancing decidualisation through intracrine action (54), as well as their role in regulating repair mechanisms during menstruation (46). Capable of acting as an oestrogen receptor agonist, DHEA is thought to be a key substrate for oestrogen biosynthesis in postmenopausal women (55, 56). Interestingly, through oestrogenic conversion, it also displays immunomodulatory properties, dampening excessive inflammatory responses in mouse models of impaired wound healing (57). The importance of DHEA has also been previously highlighted in regards to the central nervous system and neurodevelopment of the foetus, as well as for placental biosynthesis of oestrogens (58).

DHEA: An Angiogenic Androgen

Links between adrenal regulation of the uterine vascular network and the corresponding degree of menstrual bleeding have not been identified. However, previous studies have shown that DHEA promotes angiogenesis in many species and tissue types (59, 60). The stimulatory actions of DHEA on

uterine vessel growth have yet to be extensively examined, as methods for visualising the feto-placental unit vasculature during pregnancy in naturally menstruating species have not been possible. Furthermore, not without some merit, DHEA has been labelled a "human hormone"; distinct in its synthesis and circulating levels from other mammals. This makes it extremely challenging to study this natural hormonal interaction in less immediate target tissues, such as the uterus, to follow long-term biological processes, such as puberty and pregnancy. Thus, our understanding of how altered androgenic input may impact vascularity and what the implications are for foetal development has been significantly slowed.

DHEA promotes angiogenesis. It was recently identified to increase endothelial proliferation by 30% in bovine aortic vascular endothelial cells *in vitro*, as well as promoting the growth of new primary capillaries (60). The investigators also demonstrated that DHEA enhances angiogenesis by measure of increased vessel density in an *in vivo* chick chorioallantoic membrane assay. DHEA has also been implicated in stimulation of vascular endothelial growth factor (VEGF), a potent angiogenic protein in the human menstrual cycle (26) and identified in the endometrium and spiral arteries in the spiny mouse (11). In a study of natural killer cells obtained from healthy and Alzheimer's patients, incubation with DHEA-S increased VEGF production in a dose-dependent manner (59). This is of particular importance, as VEGF deficient mice are incapable of developing the appropriate vascular network needed to support pregnancy and subsequently abort (26). VEGF production and vascular permeability in the endometrial stroma is also increased in response to oestradiol (26). DHEA has been shown to act through both the oestrogen receptor and as a substrate for oestrogen synthesis (56). This is suggested in androgen receptor knockout ($AR^{-/-}$) mice, whereby $AR^{-/-}$ females were still able to conceive, but produced less pups and had significantly reduced uterine area (including the endometrium). This may be due to DHEA only exhibiting actions as the oestrogen receptor (61). Furthermore, endometrial hypertrophy due to oestrogenic overstimulation has not been associated with DHEA treatments (62). Thus, the discovery of the role of DHEA as a potential regulator of the uterine microenvironment presents an exciting research opportunity. This is particularly pertinent in the menstruating spiny mouse, given they retain enzyme P450c17 for DHEA synthesis.

Developing Preeclampsia

Preeclampsia (PE) is a severe disorder affecting 1 in 20 pregnancies (63), and characterised by dangerously high maternal blood pressure, organ failure, and foetal compromise. Complications from PE is a crisis spanning even developed countries; it accounts for 20% of maternal deaths in the United States and 60,000 maternal deaths annually (64–66). As the primary intervention is delivery of the placenta, PE is also the largest cause of preterm birth, inflicting adverse neurological outcomes and respiratory disorders upon already vulnerable babies (67). Therefore, PE poses an imminent threat to the immediate and long-term health of many mothers and babies.

The foundations for PE are laid prior to pregnancy, with poor functional transformation of the endometrium in preceding menstrual cycles (68). In turn, the endocrine signalling needed to promote sufficient decidualisation, angiogenesis and spiral artery remodelling, are lost. The absence of these interactions ultimately leads to shallow invasion of the endometrium by the embryo within the first week of pregnancy, and subsequently PE (26, 68, 69).

In healthy pregnancies, extravillous trophoblasts migrate through the decidua to colonise the myometrial spiral arteries, forming intraluminal plugs before eventually replacing the maternal endothelium. During this proteolytic invasion, the elastic and muscular tissues of the spiral arteries are destroyed to incorporate the cytotrophoblasts into the vessel walls before finally reconstituting these vessels without maternal vasomotor control (70, 71). These changes facilitate a low-resistance, high volume blood flow system to meet the vascular demands of a growing foetus.

PE is a disease of heterogeneity, most commonly diagnosed between 20 weeks gestation and up to 48 h postpartum (64, 71). Superficial penetration of the cytotrophoblast results in widespread endothelial dysfunction and placental malperfusion and ischemia; phenotypes shared by both PE and intrauterine growth restriction (IUGR) pregnancies (often simultaneously) (71). Pinpointing the failure of the uteroplacental arteries to dilate has been the subject of much controversy. Zhou et al. suggested that the extravillous trophoblast of preeclamptic patients have reduced expression of adhesion molecules, including E-cadherin, platelet- and vascular-endothelial adhesion molecule-1 (PECAM-1 and VECAM-1, respectively) (72). However, Lyall et al. could not recapitulate this, finding no differences in expression in the trophoblast cells between normal and preeclampsia or IUGR patients (73). The role of nitric oxide synthesis is also in question, as uteroplacental arteries in guinea pig models dilate when stimulated by the invading trophoblast, as well as inducing preeclampsia and IUGR phenotypes in guinea pigs and rats when nitric oxide synthase was inhibited (74–76).

Furthermore, maternal factors must be considered in preeclampsia development, as chronic hypertension, diabetes, renal disease, very young or advanced age are all maternal risk factors contributing to likelihood of disease onset (64, 71). Immune maladaptation is also a theory, with Kaufman and Huppertz hypothesising that reduced trophoblast invasion of uteroplacental arteries leads to an accumulation of apoptotic interstitial trophoblast and excessive recruitment of macrophages. Macrophage activation then leads to further macrophage attraction in a positive feedback cycle, preventing further endovascular invasion.

Brosens urged consideration of the role of progesterone-dependent decidual cells in preparation for the inflammatory event of early pregnancy (77). Decidual cells, which are resistant to oxidative stress, form a cuff around the spiral arteries during the secretory phase of the menstrual cycle. This cuff then provides potential histotrophic support of the early conceptus and for local chemokine secretion to trigger an influx of specialised uterine Natural Killer (uNK) cells; immune cells that aid in vascular remodelling through secretion of growth and angiogenic factors.

Brosens also advocates that PE is undoubtedly a disease of early pregnancy, and caused by the imbalance of low angiogenic factors and high antiangiogenic factors (64). Brosens suggested that inadequate blood vessel remodelling may be attributed to impaired decidualisation and uNK function. Additionally, immune imbalance is also implicated in PE pathogenesis as a result of placental ischemia (77). Uncontrolled, chronic inflammation results from a T-helper (Th) cell reversal, whereby the ratio of Th-1 to Th2 cells is high. Th-1 cells increase secretion of proinflammatory cytokines, including interleukins (IL)-6 and−17 and Tumour Necrosis Factor-alpha (TNF-α). A subsequent decreased regulatory immune cells influenced by Th-2 cell action, when combined with this, perpetuates the unchallenged production of reactive oxygen species culminating in the hallmark symptoms of PE, hypertension and endothelial dysfunction (78).

Importantly, there are hints of a relationship between endometrial and pregnancy pathologies, including an increased risk of PE based on the "lightness" of a woman's menstrual period, as these women may have inadequate remodelling of uterine blood vessels (77, 79, 80). A recent theory suggests that cyclical menstruation evolved as a means of preparing the uterus for the impending hyperinflammation and tissue ischemia associated with invasive placentation (77). This protective process, known as uterine preconditioning, would explain why the prevalence and severity of preeclampsia (PE) is highest in first pregnancies and adolescents who have had insufficient decidualisation and corresponding vascular preparation (81). In first pregnancies, multiples (i.e., carrying more than one baby), and in women conceiving through *in-vitro* fertilisation, the risk of developing PE is increased 2–3-fold. In multiple pregnancies, increased placental mass causes increased soluble fms-like tyrosine kinase-1 (sFlt-1), impairing angiogenesis (82–85).

Combining the knowledge that DHEA is a stimulant of angiogenesis and decidualisation with Brosens' preconditioning theory, we propose a novel theory: women with low DHEA have impaired preconditioning, endometrial and vascular remodelling, resulting in increased risk of preeclampsia. Our hypothesis is presented in **Figure 2**.

Kaufman and Huppertz astutely summarise the most prominent drawbacks in using human tissue alone to study preeclampsia. Placental samples, which are readily available after delivery, do not cover the primary zone of interest, and placental bed biopsies obtained during caesarean section are not robust representatives of the entire organ. Even for non-preeclampsia uterine studies, whole uterine samples are difficult to obtain. If hysterectomised organs are available, often control cases are limited, as patients rarely elect to have a hysterectomy in uncomplicated circumstances (71). These areas could be greatly benefitted from using an appropriate animal model in this area of research. O. Indeed, in current animal models, often only a single clinical symptom of PE (i.e., high blood pressure or renal dysfunction only) can be induced through genetic manipulations or administration of compounds. However, these models do not sufficiently mimic disease progression (86). The success of developing novel therapeutics as either prophylactic measures or treatments for PE in humans relies largely on extensive preclinical trials in appropriate whole-animal or artificial organ systems (e.g., 3D cell-culture or organoids). Here lies obvious advantages of the spiny mouse, including in the context of preeclampsia; the possibility of studying control or manipulated organs in their entirety, which may also allow the possibility of whole feto-placental unit dissection and analysis with modern imaging technology.

As menstruation and PE are almost exclusive to higher order primates (23) there have been limited advances in understanding this disease pathophysiology, particularly in being able to use the menstrual cycle to screen for PE and developing new predictive tests and treatments. There are no current medical tests to identify women at increased risk of developing PE before pregnancy. Therefore, non-invasive monitoring of the menstrual cycle and subsequent degree of bleeding presents a potentially underutilised, yet pivotal, predictor in the development of PE. PE has is not yet been confirmed in spiny mice, though idiopathic maternal death during or immediately following the birth of pups in our colony has been observed. Nonetheless, the spiny mouse may provide fundamental preclinical data on long-term menstrual physiology and associated birth outcomes, enabling identification of new biomarkers and aid in developing surveillance protocols for at-risk females.

A HYBRID MODEL FOR PREGNANCY AND IMPLANTATION

The recent discovery of a naturally menstruating laboratory rodent, the spiny mouse, provides a new investigative tool to address pregnancy and implantation-related disorders. Not only has the cyclical assembly of spiral arterioles in the secretory phase of the menstrual cycle been confirmed in this species through histological assessment, but further work characterising early pregnancy reveals further primate similarities. Immunohistochemistry using alpha smooth muscle actin (aSMA) and cytokeratin enabled visualisation of vascular and epithelial structures, respectively, during the menstrual cycle (11) and pregnancy (87). The early pregnant spiny mouse (day 10 of a 38-day gestation) demonstrates an absence of aSMA staining during early placental development, whereby trophoblast invade the maternal vasculature. As in humans, the breakdown of the vascular smooth muscle surrounding spiral arteries allows for high-pressure, low-resistance placental perfusion (87). Observing this in spiny mice is indicative of potentially conserved vascular mechanisms for the development of a viable feto-placental unit, and may be used to study the resulting phenotypes from arteriole remodelling impairment and dysfunction.

Spiral arteriole remodelling is not mutually exclusive from spontaneous decidualization; the latter in fact is thought to support the unique angiogenic process. The correlation between species which exhibit a spontaneous decidual response, as opposed to those which do not, demonstrates a potential commonality between seemingly unrelated menstruating mammals. This suggests that spontaneous decidualisation

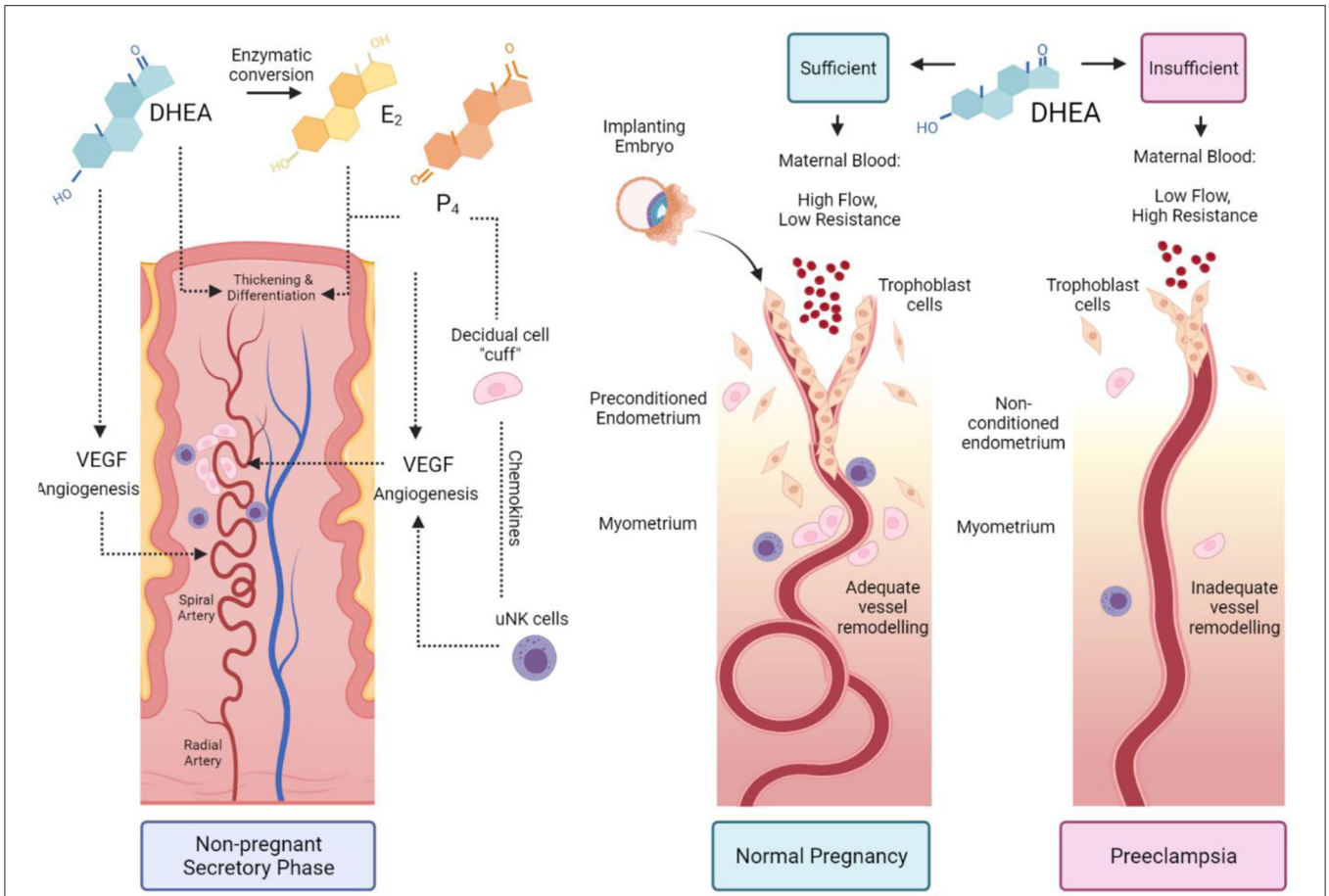

FIGURE 2 | The proposed role of DHEA in preconditioning the uterus for pregnancy. During the secretory phase of a normal menstrual cycle, DHEA acts via an androgen receptor or through conversion to oestrogen and through the oestrogen receptor to stimulate angiogenesis and spiral artery remodelling. Oestradiol (E_2) and progesterone (P_4) promote endometrial thickening and differentiation during spontaneous decidualisation. The decidual cells form a cuff around spiral arteries and recruit specialised uterine natural killer (uNK) cells to further promote vessel remodelling (*Left-hand panel*). Sufficient DHEA primes the uterus in the preceding menstrual cycles for embryo implantation. Trophoblasts invade through the remodelled vessels up to the myometrial layer and enable high maternal blood flow with low vascular resistance (*Middle panel*). Insufficient DHEA causes poor uterine preconditioning; low DHEA and reduced conversion to E_2 impairs decidualisation, uNK recruitment, angiogenesis and vessel remodelling. Trophoblast invasion is shallow, with high blood pressure due to vascular resistance, resulting in preeclampsia (*Right panel*).

evolved as a pre-emptive defence mechanism against the aggressive nature of embryonic invasion during pregnancy and the placental type of the species (23). However, recent reports show that, despite the presence of spiral arterioles and remodelling during early pregnancy, the spiny mouse has a shallow, eccentric embryo invasion of the endometrium similar to their murid relatives (87). The spiny mouse therefore neither recapitulates the primate nor the rodent reproductive strategies perfectly, but demonstrates aspects of both in terms of vascular remodelling (primate) and implantation (rodent).

The depth of trophoblast invasion can differ dramatically between species. Menstruating primates and guinea pigs present with aggressive, interstitial embryo implantation (20, 88), whereas most rodents show eccentric implantation with moderate trophoblast invasion (**Figure 3**). Moreover, adhesion to, and invasion of, the uterine wall occurs within 6 h in rodents, which limits the use of this order for understanding the underlying physical mechanisms involved in early stages of human implantation (89). Cows and ewes, on the other hand, show centric embryo implantation with no invasion of the uterine epithelium. In these species the foetus grows within the uterine lumen. Non-menstruating primates such as common marmosets have been used as *in vitro* models of primate embryo adhesion and protein secretion (90). Marmosets also present with centric implantation (91) and do not reflect the implantation events of higher-order primates. Moreover, while guinea pig embryos invade deeply within the endometrial stroma, there is no post-ovulatory rise in progesterone levels and decidualisation of endometrial stromal cells is induced, rather than spontaneous; characteristics unique to true menstruating species (20). Together, these studies

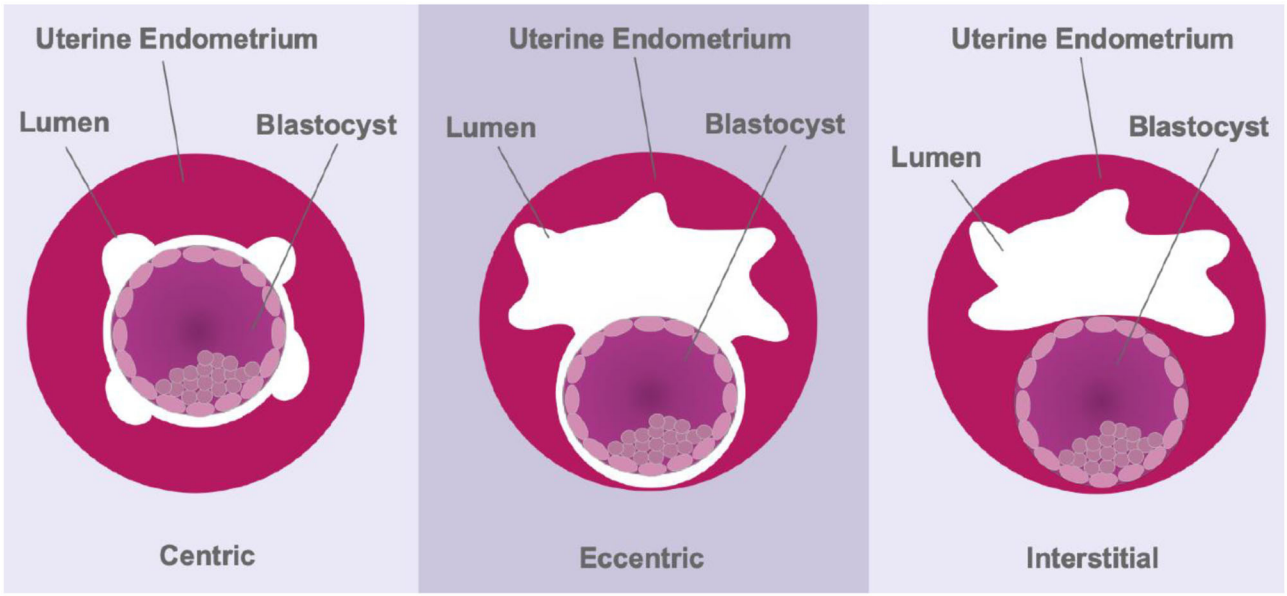

FIGURE 3 | Schematic representing the three modes of implantation. During centric implantation, the embryo is superficially attached to the uterine epithelium and trophoblasts do not invade the endometrium (cows and ewes). Eccentric implantation involves the formation of a tear-drop shaped cavity within the endometrium with a moderate degree of trophoblast invasion (most rodents). During interstitial implantation, the blastocyst completely embeds itself within the endometrial stroma with trophoblasts invading to the basal endometrium (primates).

highlight the significant species variations in implantation in the mammals.

While old-world monkeys clearly present with aggressive trophoblast invasion, great-ape embryos invade even deeper within the endometrial stroma. Recently, trophoblast invasion and subsequent spiral artery remodelling in chimpanzees (92) and gorillas (93) were simultaneously described, both strongly resembling the processes in later stages of human implantation. Considering our phylogenetic relatedness, it is perhaps no surprise that deep trophoblast invasion extending to the inner myometrium are features shared between all great apes, and implantation is most suitably modelled in these species.

Clearly, *A. cahirinus* presents with a unique combination of rodent- and human-like reproductive characteristics. It is the only menstruating rodent that also exhibits postpartum ovulation, an absence of lactational amenorrhea and shallow implantation, and yet it clearly shows remodelling of spiral arteries during pregnancy (87); these are perhaps the most puzzling combinations of reproductive traits observed to date. This not only questions the dogma of the origins of menstruation in mammalian species, but also challenges the assumption that menstruation evolved alongside aggressive trophoblast invasion. It remains to be determined the depth of invasion and role of early formation of the placenta in this species. These observations will be vital in establishing this species as a relevant and accessible animal model of female reproductive health.

ASSISTED REPRODUCTIVE TECHNOLOGIES: A NEED FOR FEMALE FOCUS

Infertility is an incredibly heterogenous, multifaceted condition. Today, it is estimated that 8–12% of couples of reproductive age experience infertility and require various interventions and technologies to conceive (94). The use of Assisted Reproductive Technologies (ARTs) have been used extensively to treat failures in human reproduction since their rapid development, refinement, and accessibility over the past half century. ARTs involve the manipulation of male and female gametes to achieve a viable pregnancy and, ultimately, a live birth. The most commonly used of these reproductive techniques include *in vitro fertilisation* (IVF) and intracytoplasmic sperm injection (ICSI), caused by a growing population of subfertile individuals. Despite growing popularity and treatments becoming more affordable, the success rate, as determined by live birth rate, of ARTs plateaued at around 30% for over the previous decade, and is now declining (95). This is likely to stem from a fundamental knowledge gap in the multiple steps of human fertilisation and implantation, and is further complicated by an inability to conduct invasive procedures and long-term research, particularly in women.

Gamete collection is at the apex of many ART procedures. While semen is relatively easy to collect and study, collection of mature oocytes suitable for *in-vitro* research is far more difficult, and much of our understanding of female-factor infertility

has relied on comparative research in farm and laboratory species. Collecting healthy, mature oocytes is a critical step for *in-vitro* research, animal colony management, and treatment of infertility. However, in most cases, a low natural ovulation rate is inefficient and has led to the development of controlled ovarian stimulation (COS) or superovulation. This is turn has led to a significant increased incidence in COS-related complications such as ovarian hyperstimulation syndrome (OHSS) (96). Further research is therefore required to mitigate or prevent the potentially lethal outcomes of COS. While clinical studies are possible, the ability to screen for these complications and develop novel treatment regimens in an appropriate animal model would not only increase likelihood of success but reduce physical and emotional discomfort for infertility patients.

Another hurdle in understanding human infertility is the fertilisation of mature oocytes *in vitro* (IVF) and *in vivo*. While human IVF is a broadly successful technique, fertilisation rates in humans rarely exceed 70% (97, 98), and some patients, although rare, still present with total fertilisation failure (TFF) (99). Deciphering the aetiology of TFF remains technically challenging; particularly as progress in human IVF is hindered by ethical and legal restrictions limiting access to human tissues. IVF in farm and laboratory species has provided substantial evidence for male- and female-factors affecting fertilisation success including DNA fragmentation, inadequate sperm capacitation, aneuploidy or poor oocyte activation (100). In some patients, however, ICSI is sought as the course of action, even if evidence of male-factor fertility is lacking, in a bid to overcome cases of idiopathic infertility (101). While ICSI is an effective treatment for severe male-factor infertility, ICSI does not confer any benefit in cycles with female factor infertility (97, 98, 101), which ignores half of the contributing parameters for fertility failure.

Of non-primate models, bovine, murine and cricetinae oocytes are typically obtained for *in vitro* studies, with rodents often preferred for their ready availability in most research settings. However, compared to the primate, cow or even hamster oocyte, mouse oocytes have a poor "wound healing" capacity following mechanical manipulation linked to ICSI, and survival rarely exceeds 50% (102, 103). Similarly, ICSI in cows is very rarely performed due to the technical complexity, and additional requirement of oocyte activation following manipulation (104). Although ICSI is successful in non-human primates (105–107), the combined cost of maintaining captive primate colonies and the complexity of ICSI limits their use in biomedical research. Clearly, current animal models of ICSI and fertilisation failure present several technical and biological issues that limit their application as models for clinical use. The spiny mouse has demonstrated the ability to wound heal regions of damaged skin and hair follicles completely scar-free (32). The potential relationship between this and the similar scar-free healing of the endometrium during the menstrual cycle has been contemplated elsewhere (28), but certainly warrants further exploration. Whether or not these wound healing capabilities apply to the oocytes of the spiny mouse remains to be seen.

Most of the current library of ART knowledge has been derived from non-menstruating animal models. The history of assisted reproduction is now more than 120 years old (108). Much of the early, ground-breaking work on *in-vitro* fertilisation (IVF) and embryo culture were performed in rodents and cattle (100, 109). The obvious relevance issue for these models is that they exhibit an oestrous, not a menstrual, cycle, and that they do not experience a lifetime of cyclical endometrial shedding and regeneration that may have unforeseen implications for the ability of a couple to conceive and carry a baby to term. An influx of inflammatory cytokines, proteases, repair molecules triggered by cascading hormones (110) are all important in the preparation of the uterus for implantation, but paired with foreign invading spermatozoa and lifestyle factors, the ability to conceive varies greatly within our own species. Even great apes, our closest evolutionary relatives, present copious challenges, largely due to the complexities of their husbandry and welfare needs, high running costs for their maintenance and logistical challenges in combating societal and ethical concerns to justify their usage (100).

The spiny mouse provides many novel features that are important and useful in providing an alternate model for ARTs research. Their small size and relatively low cost combined with similarities to human uterine physiology in terms of cyclical endometrial differentiation provides a relevant model to comprehensively investigate currently ambiguous clinical practises. One such practise is the use of endometrial scratch in ART, where the uterine lining is subjected to physical injury prior to or during an ART cycle. The initial observation of improved pregnancy and live birth rates led to a rapid adoption of the procedure (111, 112). Lensen et al. determining 83% of clinics in the UK, Australia and New Zealand recommend this to ART patients (113), despite conflicting evidence of its efficacy in multiple randomised controlled trials (RCTs). In fact, a recent systematic review comparing women undergoing endometrial scratching with no prior IVF/ICSI treatment, one or two failed cycles found no significant differences between scratching and controls (114). The authors advocate at best, endometrial scratching provides false hope for patients and with such poor evidence, could in fact be harmful. They also conclude that the heterogeneity of patients in the analysed RCTs is a major limitation. Furthermore, the behaviour of the endometrium in terms of vascular and stromal inflammation and repair in response to the scratch would likely be associated with that seen during menstruation; thus, subjective to the immune and hormonal profile of each individual [see (11)]. Here is where the spiny mouse as a preclinical model presents obvious advantages for ART procedure development and refinement.

CONCLUSION

The discovery of menstruation in the spiny mouse offers an exciting opportunity to advance studies in many areas of female reproductive biology. We now have access to a more physiologically relevant, small animal model for menstrual research. In a broader sense, it also provides us with a unique opportunity to develop new disease models for AUB,

infertility and preeclampsia and to provide a new and, in some areas of research, better model to test and develop new ideas to improve our understanding of other difficult issues in women's health.

AUTHOR CONTRIBUTIONS

NB developed theories and drafted manuscript. JM contributed to manuscript subsections, figures, and editing. SE and PT-S refined ideas and edited manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

Hudson Institute of Medical Research was supported by Victorian Government Operational Infrastructure funding.

ACKNOWLEDGMENTS

Figure 2 created using BioRender.com.

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frontiers in Reproductive Health

ORIGINAL RESEARCH
published: 08 December 2021
doi: 10.3389/frph.2021.787277

Menstrual Equity Initiatives at USA Universities: A Multiple Case Study of Common Obstacles and Enabling Factors

Caitlin Gruer[1]*, *Taylor Goss*[2], *Margaret L. Schmitt*[1] and *Marni Sommer*[1]

[1] Department of Sociomedical Sciences, Mailman School of Public Health, Columbia University, New York, NY, United States, [2] Department of Health Policy and Management, Mailman School of Public Health, Columbia University, New York, NY, United States

Background: In recent years there has been growing momentum in the USA around addressing issues of "menstrual equity" and "period poverty," including a proliferation of university-level initiatives seeking to provide access to free menstrual products. This multiple case study examined four such efforts at a diversity of tertiary institutions to identify the factors that facilitated or impeded success.

Methods: We conducted a qualitative multiple case study, including a desk review and key informant interviews with student and administrative actors from universities with free menstrual product initiatives. We sought to identify key learning regarding common challenges and obstacles, enabling factors which supported success and sustainability, and practical learning for future initiatives. From the desk review, four schools ($n = 4$) were purposively selected to represent a range of geographic regions, student population size, and university type. Purposive sampling was used to identify students and administrators engaged in the menstrual equity initiatives on each campus ($n = 20$; 4–6 per school). Data from the desk review and interviews were analyzed using thematic analysis.

Results: Key themes included (1) the critical role of champions, (2) the importance of social and financial support, (3) challenges diffusing menstrual equity from pilot to scale, and (4) recommendations for future initiatives. University initiatives varied greatly in terms of their scope, funding, and implementation strategy.

Conclusion: This multiple case study provides valuable insights regarding the facilitating factors and obstacles faced by initiatives providing free menstrual products at universities. To date, these initiatives have proven successful across the four case studies; however, in most cases, the scope of the initiatives was constrained by limited resources and sustainability concerns. Future campus menstrual equity strategies would benefit from cross-institutional learning and dialogue highlighting design and implementation successes and challenges.

Keywords: menstruation, menstrual equity, period poverty, menstrual products, university, tertiary education

OPEN ACCESS

Edited by:
Philippa Saunders, University of Edinburgh, United Kingdom

Reviewed by:
Dewi Rokhmah, University of Jember, Indonesia
Tamaryn Crankshaw, University of KwaZulu Natal, South Africa

***Correspondence:**
Caitlin Gruer
caitlin.gruer@columbia.edu

Specialty section:
This article was submitted to Gynecology, a section of the journal Frontiers in Reproductive Health

Received: *30 September 2021*
Accepted: *15 November 2021*
Published: *08 December 2021*

Citation:
Gruer C, Goss T, Schmitt ML and Sommer M (2021) Menstrual Equity Initiatives at USA Universities: A Multiple Case Study of Common Obstacles and Enabling Factors. Front. Reprod. Health 3:787277. doi: 10.3389/frph.2021.787277

INTRODUCTION

In recent years there have been growing reports in the United States of America (USA) of student-led initiatives to address period poverty and menstrual equity on university campuses, most commonly through the provision of free menstrual products (1, 2). Such initiatives emerged from the larger movement to address menstrual inequities across the USA and globally. These have included efforts to remove taxes on period products in the USA and other high-income countries in particular, and to tackle the menstruation-related challenges that girls face in school in low- and middle-income countries (3–5). The latter includes a lack of access to menstrual products, private and supportive bathroom facilities, menstrual health and hygiene (MHH) education, and inadequate support for menstrual pain and anxiety, all of which in turn negatively impact the health, well-being and educational experiences of menstruating students (3–8).

Although limited research exists on MHH in the USA, studies have identified vulnerable populations including girls in high-school (9), young women in college (10), low-income women (11), and people experiencing homelessness (12–14), all of whom regularly struggle to access menstrual products. In addition, there is a small but growing evidence base about the other challenges faced by menstruating students in the US that are very similar to those seen in low- and middle-income countries, such as inadequate MHH education, problematic school bathroom usage policies, and menstrual pain (10, 15–17). A parallel growing legislative movement in numerous cities and states seeks to provide access to menstrual products for vulnerable populations. For example, numerous states such as California, Illinois, Maryland, Oregon, Washington, and New York have passed legislation to provide girls in school with access to free menstrual products (18). Similarly, legislation mandating that homeless shelters provide menstrual products to their clients and that prisons provide free menstrual products to female inmates exists in a small but growing number of states (19). To date, however, there is minimal evaluation of the implementation and impact of these policy initiatives that would serve to inform future resource investment.

Less is known about the menstrual needs and challenges faced by students who menstruate at the university or college level. In the USA, "college" and "university" are often used interchangeably although colleges are typically smaller institutions that focus on undergraduate education, while universities tend to be larger institutions with both undergraduate and graduate education programs (20). This paper will use both terms depending on the language used by the specific institution to describe itself. One recent study conducted with 471 undergraduate university women across the USA found that 14.2% had been unable to afford the menstrual products they needed at some point in the past-year, and an additional 10% were unable to afford menstrual products every month (10). Inability to afford menstrual products differed significantly by race, immigration status, and familial college history. Latina and Black women reported period poverty within the last year more often than White women and women of another race (10). Similarly, those respondents born outside of the USA were more likely to report period poverty in the last year and the last month than those born in the USA, and first generation college students were more likely to have experienced period poverty than non-first generation students (10). Across the study sample, the inability to consistently afford menstrual products was associated with negative mental health outcomes, including depression (10). Although more evidence is needed, this study serves to highlight how access to menstrual products may be an important issue on numerous university campuses, a conclusion further shored up by the attention it has gained from student activists.

There is a long history of university-level student activism and advocacy in the USA, both on and off campus. This has illustratively included wartime opposition in the 1930s and 1960s, racial justice and desegregation in the 1940s and 1950s, the rights of women's and the lesbian, gay, bisexual, transgender, and queer (LGBTQ) communities in the 1960s, and for individuals experiencing homeless and hunger in the 1970s (21). Modern examples of student advocacy are often continuations of earlier fights for equality, including the growing number of menstrual equity advocacy initiatives occurring on university campuses today (21). These student-led menstrual equity movements combine prior advocacy issues related to gender, socioeconomic status, homelessness, and LGBTQ equity with a component of health and bodily autonomy that has historically not been directly addressed in campus activism (21, 22). Menstrual equity is an issue of gender equity and of social justice, assuring those with lesser means can access products and manage their periods with dignity and comfort.

This paper describes a multiple case study that was conducted using qualitative methods to examine four diverse student-led menstrual equity initiatives from across the USA. Given the large range of higher educational institutions in the USA (e.g., public, private, large or small student populations), it was important to explore these initiatives in varying contexts. The aim was to understand how these initiatives organized, mobilized financial and social support, and developed plans for sustainability.

MATERIALS AND METHODS

We conducted a qualitative multiple case study, including a desk review to explore existing university-based menstrual equity movements in the USA and key informant interviews ($n = 20$) with actors engaged in initiatives focused on the provision of free menstrual products on four diverse university campuses. The application of a multiple case study approach enabled us to compare the differences and similarities across initiatives. The desk review was used to identify the four case study schools, and through the subsequent interviews, we sought to identify key learning regarding common challenges and obstacles faced by the architects of these initiatives, enabling factors which supported success and sustainability, and practical learning for future initiatives.

All study procedures were approved by the Columbia University Medical Center Institutional Review Board.

Desk Review

A desk review of the existing peer review and gray literature was conducted to gain an overall understanding of the menstrual health movement landscape at USA universities and enable the compilation of an initial list of universities with past or ongoing menstrual equity movements. This included a review of gray literature (news media, social media, internal reports) that described menstrual equity initiatives on university and college campuses. Google internet searches were conducted using a variety of search terms including "menstruation," "menstrual," "period," "college," and "university." News media results related to the provision of menstrual products on university or college campuses were cataloged, yielding a list of 36 universities and colleges reporting a successful or proposed menstrual product initiative. For the purposes of this study, initiatives were considered successful if they had launched and were actively providing free menstrual products.

Selection of Four Case Study Schools

The 36 schools identified through the desk review were categorized by (1) type of institution (Public or Private), (2) USA geographical location, including Northeast, Southeast, Midwest, Southwest, and West Coast, and (3) undergraduate student population size. These categories were chosen to ensure that a diversity of types of schools and student body populations were represented. Of the 36 schools identified, there were 19 private schools and 17 public schools. The schools ranged in geographic location, with 16 located in the Northeast, 9 in the Southeast, 6 in the Midwest, 1 in the Southwest, and 2 on the West Coast. The schools varied in size from an undergraduate population of 2,000 to 55,000 students.

As shown in **Table 1**, four schools were then purposively selected to represent a range of geographical regions, undergraduate population size (7,000–32,000), and two private and two public institutions.

Key Informant Interviews

Across the four campuses, key informant interviews were subsequently conducted with two groups of actors involved in the development and implementation of the menstrual equity initiatives. This included *Group 1,* comprised of students engaged in an existing campaign ($n = 10$); and *Group 2* which included campus administration, faculty, and staff ($n = 10$). **Table 2** shows the breakdown of key informants by group and university.

Two semi-structured interview guides were developed to capture the range of participant's expertise, knowledge, and engagement in the menstrual equity initiatives. The Group 1 guide explored the experience of campaigning for campus menstrual equity, including the student's motivation for engaging in the topic and the process, successes, and challenges of spearheading such an initiative. The Group 2 guide focused on the administrative perspectives of menstrual equity campaigns, why they supported the campaigns, the challenges they faced, and what hesitations they had for implementing a program for increased menstrual product access.

Sampling and Recruitment

Key informants were selected across the four schools utilizing purposive sampling ($n = 20$). Participants were identified based on their past or present involvement in initiating, implementing, or supporting an existing menstrual equity initiative. Initial participants were identified through the desk review. Recommendations were solicited from the initial participants to generate a list of additional actors for inclusion. The researchers recruited potential participants via email or telephone. Individuals who responded were provided with background on the study and invited to participate in an interview.

Data Collection

Three researchers conducted semi-structured qualitative interviews with the 20 key informants in-person, over the phone and via videoconferencing (Zoom) to accommodate schedules, geographic distances, the social distancing constraints of the COVID-19 pandemic, and participant preference. Data collection took place between November 2019 and April 2020. Interviews ranged from 20 to 60 min in length and were recorded after receiving informed consent from the participant. The recordings were transcribed for analysis.

Data Analysis

Two researchers reviewed the documents from the desk review and the written transcripts from the qualitative interviews. Malterud's "systematic text condensation" method for qualitative analysis was used to identify key themes of interest (23). This included the following steps: (1) identification of preliminary themes; (2) creative development of qualitative codes; (3) condensation of coded text; (4) synthesis and reconceptualization (23). A simple codebook of the key themes was developed, and Dedoose qualitative analysis software was used to code the interviews. The key themes identified in the data were shared with the larger research team for discussion, refinement

TABLE 1 | Basic characteristics of selected case study schools.

School Type	A Private	B Private	C Public	D Public
Undergrad Population	7,000	18,000	17,000	32,000
Geography	Midwest	Northeast	Southeast	West

TABLE 2 | Study participants by university and group.

University Code	Group 1	Group 2
A	3	2
B	2	4
C	2	2
D	3	2

TABLE 3 | Characteristics of the menstrual equity initiatives across the four case study universities.

Characteristic	University code			
	A	**B**	**C**	**D**
University characteristics				
School Type	Private, religious	Private	Public	Public
Undergrad Population	7,000 (small/S)	18,000 (medium/M)	17,000 (medium/M)	32,000 (large/L)
Geography	Midwest (MW)	Northeast (NE)	Southeast (SE)	West (W)
Menstrual equity initiative characteristics				
Process	Student proposal for an annual autonomous wellness grant approved by the student government	Student developed a proposal for the administration with support from a school-run entrepreneurial think tank	Students gained administrative support and administrators began a small-scale implementation	Student government developed a referendum to increase the student wellness fee to cover the cost of menstrual products and other initiatives
Budget Source	Annual wellness grant	Administration	Administration	Student wellness fees
Labor Source	Student-run volunteer group	Custodial/facilities staff	Custodial/facilities staff	Joint (students + custodial)
Recruitment Methods	Social media; informational meetings; word of mouth	High student involvement from the beginning including multiple student groups and student governments	Partnered with campus sorority leadership	Social media-based campaigning for referendum votes
Primary Champions	2 student leaders with administrative connections; 1 staff member	Multiple student government leaders; 1 administrator; campus think tank	2 student leaders; 1 facilities administrator	Multiple student government leaders; on-campus student health center admin
Locations	10 classroom buildings plus recreation center, ∼20 bathrooms	5 major classroom buildings, 160 bathrooms (undergraduate campus only)	3 buildings (2 major common areas and 1 large classroom building), 18 bathrooms	Health Center, LGBTQ Center, housing offices of some dorm buildings, and some libraries
Type of Location	Female-assigned and single-use bathrooms	Female-assigned, male-assigned, and single-use bathrooms	Female-assigned bathrooms	Outside of bathrooms and in common areas
Initiative Status	Student implementation and student upkeep; some struggles due to dependency on student volunteer availability	Staged rollout by university; permanent funding source unclear	University facilities-run pilot; searching for permanent funding	Joint rollout in select locations
Unique Features	Entirely student-run; utilized commercial menstrual product company for advice and supplies	Proposal supported by entrepreneurial think tank; city passed a menstrual equity bill during student movement	Strong administrative support and leadership	Initiative is now institutionalized; disjointed facilities permissions per building

and validation (23). The key analytical themes identified during analysis are presented below, along with illustrative interview excerpts.

RESULTS

Each of the universities in the study had a menstrual equity initiative providing free menstrual products operating on their campus. **Table 3** provides an overview of the university demographics and initiative characteristics, including the process for establishing the initiative, the budget source, the labor and maintenance source, and the locations targeted for distributing the free products. When defining the locations of menstrual product distribution, participants used the terms "bathroom" and "restroom" interchangeably; however, for the purposes of this paper, the term "bathroom" is used throughout to refer to sanitation spaces housing both toilets and handwashing facilities.

The specific mechanisms and implementation processes of the menstrual equity initiatives differed across the four schools, however patterns emerged around four thematic areas: (1) the critical role of champions, (2) the importance of social and financial support, (3) challenges diffusing menstrual equity from pilot to scale, and (4) recommendations for future initiatives.

Critical Role of Champions

Across all four schools, champions played a critical role in the success of these initiatives from initiation through implementation. In all cases, the menstrual equity initiative was initiated and championed by undergraduate students, typically with one to two students identifiable by fellow students and administrators as the initiative leaders. These student champions were primarily motivated by what they perceived to be an important issue of inequality. While some champions were motivated by a personal interest in reproductive health and women's health issues, most were extrinsically motivated by stories from their fellow classmates about challenges accessing menstrual products on campus or news articles about period poverty. As a student champion from School A (S, Private, MW) described:

. . . we had someone reach out to us via email, basically explaining her story about how as a commuter student 1 day she had gotten her period and was basically unable—she didn't have any menstrual products on her and there were none available at the school. And so, she essentially had to go home for the day and it interrupted her education. –KII 16

The presence of one to two clear student leaders associated with the initiative seemed to facilitate success, providing unity to the student's messaging and a singular point of contact for conversations between the administration and student body. Conversely, a lack of unity posed a significant threat to success. Before the existing sustained menstrual equity initiative at School B (M, Private, NW), there were a number of unsuccessful initiatives that emerged from various student groups to promote free menstrual products. While there was reported to have been significant interest among the student groups, there was a lack of unity across these differing but simultaneous efforts, leading to fragmentation and stagnation. When the multiple student initiatives were instead united under one clear student leader or champion, the initiative was found to be more successful in communicating with the administration and furthering the overall goal of advocating for free menstrual products on campus.

At all four schools, the student champions were members of the student government. These positions enabled them to build student support, and to engage with the university administration to move the initiative forward. The student participants reported that their role in student government facilitated their success in championing the menstrual equity initiative as it provided unique access to members of the administration and a strong understanding of the university bureaucratic systems. As one of the student champions from School C (M, Public, SE) described:

I was pretty well versed in university budget issues, you know, school budgets, division budgets, and that kind of stuff, you know what the bureaucracies were, when deadlines had to be made, something that most students and particularly student leaders were never quite interested or capable of delving into. –KII 13

While the student champions played an integral role in initiating and developing support for the idea of a menstrual equity initiative, their role in obtaining and managing funding and implementation differed across schools. For example, at School A (S, Private, MW) the two student champions not only conceived the idea for the initiative and ran the pilot, but they also acquired funding via a student government wellness grant to implement the initiative, and, with a small team of other students, were responsible for stocking the menstrual products in the dispensers across campus. Similarly, at School D (L, Public, W), the student champions were able to pass a referendum through student elections to levy a small increase to the student wellness fee to cover the cost of the free menstrual products, with little need for input from the administration. However, at School C (M, Public, SE) and School B (M, Private, NE), the student champions were heavily involved in the conception and initiation of the initiative, but the administration was primarily responsible for funding and implementation.

The support of a champion within the staff or administration was also a crucial facilitating factor in generating buy-in and support beyond the student body in three of the four schools. The exception to this was School D (L, Public, W) where the need for an administration champion was less crucial due to the ability of the student government to cover the cost of the initiative by levying stable and recurring funds via student fees. The role of these administration champions varied, and included securing funding, helping students to navigate the university bureaucracy, providing guidance on proposal development, and gathering support and approval from other members of the university bureaucracy. As a staff member from School B (M, Private, NE) described:

I think they needed a champion on campus, so I think [high level administrator] helped play that. He heard the students, he heard their concerns, he recognized their perspective that this was a human rights issue, and he became a conduit to connect them with facilities. –KII 11

Across the cases, this administration champion was seemingly more effective when they were more senior, had budgetary control, and were better-connected to senior leadership whose approval and buy-in was required for implementation (e.g., operations or facilities staff).

Importance of Social and Financial Support

Critical to the success of all four menstrual equity initiatives was obtaining social and financial support, with student champions describing the importance of generating support from both the student body and the administration. As one student respondent explained:

I think the biggest roadblock was getting the stakeholders on board because without their support, I could fight for like 3 years and they were never going to—like they could have just never, never taken it on and then it wouldn't have happened. –KII 04

To generate initial support, the student champions utilized numerous methods, including surveys of the student body to ascertain level of need, petitions to document the extent of student body support, and campus-level social media campaigns to generate awareness and mobilization for the initiative. The student champions noted a difference in how certain audiences responded to various strategies of framing the issue of menstrual equity. While undergraduate students seemed more responsive to language that framed the issue as one of equity, social justice, or morality, university administrations were generally more responsive to proposals framing the initiative as addressing period-related emergencies (e.g., an unexpected menstrual period or running out of supplies). As one student described:

I think when I was trying to market the program to students and things like that, we're definitely kind of making it more like "This is a necessity. We recognize this is a necessity. Please take it because this is something that is just systemically wrong and needs to be addressed." But definitely in terms of like the administration, like we kind of talked about this moral argument and we kind of said

that like, "yes, this is a necessity and people need it." . . . but kind of framing it almost as in an emergency situation for them to feel like, "oh, people aren't going to just let's take it." And like the "if you need them" kind of thing was kind of our approach. So, I definitely took different framing depending on the population you were talking to. –KII 06

In addition, at each school, the students conducted a student-run pilot, typically consisting of providing free menstrual products from a basket in a small number of centralized locations on campus. These pilot programs were typically implemented by the students themselves, including the restocking of the menstrual products, and were funded via discretionary funds from student government or other student body organization budgets. These small pilots were used in conversations with administration and the broader student body to demonstrate both the need and usefulness of the products by the campus population, as well as proof of concept. In some cases, these pilots also proved to mitigate fears of misuse, as in most cases there was no evidence of tampering with the supplies, or of students taking an excess number of products.

Despite these measures, there was some resistance to the menstrual equity initiatives across all four campuses. Most frequently the opposition came from members of the administration. As one administrator explained:

There were like a couple people that I encountered that were kind of like "this isn't important, or this isn't something that the whole student body can benefit from, so you shouldn't be spending your time on it." —KII 10

Similarly, some administrators expressed concern about the viability of a free product distribution system, including the potential for vandalism of the dispensers or misuse of the free products. As a student from School D (L, Public, W) described:

. . . there was a lot of concern that students would steal these products. . . when they were made available for free. They were like, 'oh, what if, you know, somebody takes the whole box or someone just like decides to walk off with all the products' and we were like "well doesn't that mean that people need them?" —KII 03

Although there were very limited reports of vandalism or product misuse during the implementation of the initiatives, these detractors had to be either won over or overruled before the initiatives could progress to the point of implementation. Addressing this opposition from the administration required understanding and addressing their concerns. This often required stepping away from the idealism or moral framing of the issue of menstrual equity. As one administrator described:

[The students] said, "Well, this is just right. Everybody should be into it and want to do it." And I had to say, "Well, not necessarily. Not everybody sees it your way. And look, I'm behind you but part of being behind you means we've got to educate the community." –KII 19

In addition, one of the most common concerns was the potential cost of the initiative. Across the universities, students and administrators alike struggled to estimate the real implementation costs. While administrators tended to overestimate how much the initiatives would cost, student's initial budgets often failed to account for less visible expenses such as the cost of labor to maintain the stock of the menstrual products and maintenance of the dispensers in cases of damage or malfunction. This underscores the necessity of a detailed, evidence-based budget proposal that accounts for all associated resource requirements, and the importance of good collaboration between the students and the administration.

Challenges Diffusing Menstrual Equity From Pilot to Scale

Although the pilot projects were all successful, each school faced significant challenges in relation to diffusing the menstrual equity initiatives from the small number of locations included in the pilot to a more widespread distribution due to the increased funding, labor, and maintenance requirements when operating at scale.

During the effort to move beyond the initial pilots to operate in more locations across campus, each initiative had to determine the source of labor for distributing and maintaining a consistent stock of menstrual products across the target locations, with the two primary options being the custodial staff or the student groups. Each option presented benefits and drawbacks, as relying on the custodial staff required the buy-in and support of multiple levels of the administration, while student-managed systems placed a large burden on the students responsible. Three of the schools (B, C and D) opted to work with the administration to utilize staff-managed distribution systems. As a student from School D (L, Public, W) described, this was not the fastest or easiest option, but was more sustainable over time:

I think one of the biggest issues that we faced after the funding was secured was just getting the administrative buy-in. . . But I think that was one of the things that really made it successful, because if we don't really have the buy-in then, it becomes a little bit tougher to get this kind of rolled out because. . . there are a decent amount of us [students] but there's also not enough to continuously replenish all the sources like, yes, we can buy them and then we can go and distribute it at the center, but we can't really be there every single day to replenish it. So that kind of takes a lot of administrative buy-in. –KII 06

In line with this, School A (S, Private, MW), which utilized a student-run system, encountered implementation challenges due to competing student priorities and turnover of the student leaders with no clear successor. While the students were typically able to maintain the menstrual products stock, it constrained the number of locations in which the initiative could be feasibly implemented and placed significant stress on the student implementers.

Due to resource limitations, both financial and human, each of the initiatives were forced to make difficult decisions and compromises about where the free products would be located.

While students across all four universities began with the idea that all bathrooms or all locations across the school should have free menstrual products, this was challenging in practice due to budget and labor constraints. Instead, all four initiatives focused on centrally located or high-trafficked buildings with the intention of making the menstrual products as accessible as possible.

Also central to this decision was a discussion about what types of bathrooms (i.e., female-assigned, male-assigned, or gender-neutral) should be targeted for the free product distributions. This included deciding whether it should be those frequented by the highest concentration of people who menstruate (i.e., female-assigned bathrooms), or an equal number of female- and male-assigned or single-use bathrooms to meet the needs of all people who menstruate. Student champions from all four universities initially proposed universal implementation, targeting gender-neutral, single-use bathrooms, and/or male-assigned bathrooms in addition to female-assigned bathrooms. As one of the student champions described:

> *I personally came into the room saying "This should be in every single restroom, unisex, men's restrooms, and women's restroom. They should be in every single restroom."—KII 13*

However, in some cases, these proposals faced resistance from members of administration who questioned whether the level of need in male-assigned locations justified the required maintenance and upkeep. As an administrator from School B (M, Private, NE) described:

> *…we provide products in all of the single-occupant because they're gender-neutral…The other half of them that are multi-occupant restrooms, I have about eight hundred of them and if I assume that half of them are currently labeled men and the other half are currently labeled women, those four hundred [that are labeled men] are the ones that I'm curious about whether or not we need to supply all of them. –KII 10*

Similarly, some administrators expressed concern that misuse of products would be more common in male-assigned bathrooms due to curiosity and lack of understanding, although there are conflicting reports of whether this concern was valid based on actual implementation experiences.

Ultimately, the initiatives were forced to make compromises in terms of what locations would be included in the distribution, due to budgetary constraints and in some cases administrative opposition. As one student described the trade-offs involved in this discussion:

> *…[inclusivity] is like a two-edged sword and it's awful, but not being able to put them in the male-assigned restrooms because we were able to spread them out to more buildings. So, we were able to include them in the school of engineering, which is something we wouldn't have been able to do. We were able to put four on the med campus instead of just two. So that definitely helped us out. But it wasn't kind of in the way that we wanted, but it is definitely the overall distribution we needed. But that was a hard thing. We also chose to put them only in academic buildings and the student union. –KII 01*

At the time of data collection, three out of the four universities had implemented some form of inclusive distribution: University D (L, public, W) implemented public dispensers outside of bathrooms that could be accessed regardless of the gender-assignment of the bathroom used; University A (S, private, MW) installed formal dispensers inside single-use bathrooms; and University B (M, private, NE) was perhaps the most inclusive by providing menstrual products in all bathrooms (female-assigned, male-assigned, and gender neutral) in the selected buildings.

Recommendations for Future Initiatives

The student and administration respondents shared a number of ideas for other schools or student champions considering initiating similar projects to provide access to free menstrual products. The most common recommendations made by student participants were inspirational sentiments surrounding the amount of hard work a project like this might entail but encouraging future advocates to remain dedicated and resilient.

> *So, yeah, the advice is you should really just do it. I think it will really, really be something that, you know, is necessary…So I think there is a lot of momentum specifically right now for these projects to kind of take flight. And I do think students should take advantage of it and really use all these initiatives and these bills of laws to really kind of swing that and just, you know, start the project. —KII 07*

Notably, almost every student respondent mentioned the immense time commitment required to champion one of these initiatives, a commitment that should not be overlooked by student champions seeking to undertake similar initiatives. To increase the feasibility and chances of success, one student champion recommended utilizing the student government structures, even if the champion is not a member of student government.

> *…really talk to student leaders and hold them accountable. I think that's something that's important. Being a student leader, if that one student hadn't reached out to [us as members of student government], we would have never started this, you know? So, I think, if you're on the other end, be the person that's like, "Hey, why aren't you doing something about this or let's get together and do something?" –KII 16*

This commitment did appear to be less significant in Schools B and C in which the administration was more involved in the pilot and implementation processes, indicating that the required student commitment could be mitigated by earlier administration involvement.

In addition, many of the student respondents recommended considering how inclusivity could be incorporated into the initiatives, including ensuring access to menstrual products for transgender and non-binary student populations and avoiding the use of gendered language, such as "feminine hygiene products."

A second set of recommendations, primarily offered by the administration respondents, focused on practical considerations for gaining support from key stakeholders with political, budgetary, and implementation power. These recommendations centered on understanding the university context and constraints, so that any initiative could be designed to address or avoid them. While these recommendations were mostly directed toward prospective student champions, they could also be relevant to more junior administrators interested in implementing free menstrual products on campus–although the desk review did not uncover any examples of an administrator initiating such advocacy without pressure from students. As one administrator recommended:

> *Approach this as a question of why do we currently—what were the constraints that led to us not providing these products? And have they changed? And if so, what are others doing that they've figured out? —KII 10*

This includes the use of data and evidence from both the student body and other initiatives, and a well-conceived and researched proposal. As an administrator from School B (M, Private, NE) suggested, this is not only critical for generating consensus but also for overcoming any opposition:

> *…the best advice I could give is that the only way to fight misperception is with data. And the more fact-based, one can be in the discussion—about costs, about availability, about theft, about all these sorts of petty excuses that people use not to do something—the better off you are, the more strength of the argument is. —KII 09*

Additional recommendations from administrative participants included finding an administrative champion, being realistic about funding expectations and increasing student body awareness about the initiative to gain support and aid students in understanding the rationale for menstrual product dispensers in male-assigned bathrooms.

Finally, both the administration and student respondents emphasized that it was critical to think about the sustainability of the initiative from the onset to ensure that the initiative was designed in a manner that allows for and promotes success in the long-term. As one student respondent described:

> *…there needs to be longevity to it, like you need to make it a sustainable program. And so, you need to think about more than just right now and how will it work. Even if that's like the starting point, there has to be some way for it to continue beyond you and whatever group you're working with. –KII 03*

Sustainability was primarily described in terms of obtaining a permanent source of funding to maintain access to free menstrual products, administrative oversight of the stocking and maintenance of the dispensers, and potentially expanding free menstrual product access to more buildings or bathrooms.

DISCUSSION

Menstrual equity initiatives on university campuses are appearing with increasing regularity (24), and likely will continue to do so as attention to menstrual equity and period poverty continues to gain momentum in the USA and around the world. These initiatives may be particularly pressing as more reports emerge documenting the challenges that many college students, particularly students of color, face in accessing and affording basic necessities, including menstrual products (10, 25, 26). The case studies shared here provide important insights into common challenges and enabling factors encountered by students and administrators seeking to enact this type of programming, providing critical learning for other localities and stakeholders seeking to create and implement similar initiatives. Importantly, these initiatives were all launched by students, however as recognition of menstrual equity gains attention, universities may themselves initiate policies equipping all on-campus bathrooms with menstrual products. This study was not able to extract any patterns based on university type (private/public), geography, or size. The budget source, size and scope of the four initiatives studied varied across types with no within-group comparisons for type, geography or size.

Overall, the four case studies illustrate the critical importance of having champions both within the student body and the administration. These key players were necessary for generating support, navigating the university bureaucracy, and providing a singular point of contact for the administration or other students to direct questions and concerns. In some cases, the centrality of the student champion to the initiative implementation proved problematic when they graduated, studied abroad, or became preoccupied with other priorities. The effectiveness of a champion is evident within the broader menstrual equity movement as well. In Scotland, the grassroots efforts to address period poverty nationwide were spearheaded in part by Victoria Heany who started the #FreePeriodScotland campaign and garnered sufficient interest for the issue to capture the attention of the Scottish government, leading to the eventual passage of legislation mandating universal access to menstrual products across the country, the first legislation of its kind globally (27–29). Also critical was a staunch champion within the Scottish parliament, Monica Lennon, who took up the issue, introduced the bill to parliament, and advocated for the legislation internally until its passage (27).

The findings also highlight the importance of generating both social and financial support and the multiple methods that can be used to do this, such as small-scale pilot projects to show proof of concept, surveys to demonstrate need, and advocacy and social media campaigns to generate interest. These approaches are important to consider not only within university settings, but also at the state or national level as they have also been found to be effective in the push for broader menstrual equity legislation. For example, during the menstrual equity campaign in Scotland, activists developed a multi-pronged campaign utilizing surveys, testimonies from Scottish women and girls, social media, and a pilot initiative to not only provide evidence of need but also to generate and showcase broad public support for the effort (30,

31). Also critical was the ability to tailor the message framing as needed, with the student champions modifying their messaging as needed to be most compelling for their target audience; equity was the main concern for student peers while "emergency-use" was a more convincing argument for many administrators.

Similarly, multiple localities have utilized small-scale pilots to test the feasibility and impact of initiatives to provide free menstrual products (32, 33). For example, before mandating that schools across the city provide access to free menstrual products to schoolgirls, New York City conducted a small-scale pilot first at one school, and then in 25 middle- and high- schools in primarily low-income neighborhoods in two areas of the city (34, 35). In addition to providing evidence of the positive impact, these small-scale pilots can also be useful to mitigate fears of misuse or potential hoarding of the menstrual products (36). Fears of vandalism or misuse are common across contexts; however, there have been few examples of these challenges to date, with those that have occurred primarily limited to the free products being thrown out in male-assigned restrooms (37, 38). Future advocates should gather supporting evidence for their cause, including but not limited to figures from similar universities with successful initiatives and internal figures from within the university about student body need and interest. Although only one of the case study universities utilized evidence from another university, external experience, including practical lessons learned, may serve as a critical source of information and guidance.

These case studies also draw attention to some of the common challenges to the successful initiation of a menstrual equity initiative. Two frequently identified hurdles included addressing opposition from members of the administration and securing a sustainable and sufficient source of funding. In some cases, well-developed proposals can be used to overcome these challenges, particularly when they include details regarding the size and scope, timeline, labor and product sources; however, initiative leaders must also be flexible and open to the suggestions and compromises offered by other stakeholders. This is evident within the broader menstrual equity movement as well. For example, while the proposed Scottish legislation was very detailed in terms of the scope, timeline and proposed budget, the policy was still amended to simplify, clarify and address the concerns of some Scottish Ministers (39). Similarly, in Brazil, youth activists developed a period poverty policy proposal that they then shared with local officials. In the state of Rio de Janeiro, this proposal attracted the attention of a state representative who became a staunch advocate, leading to the passage of a bill to reduce the tax on menstrual products and make them more accessible (40).

The resource challenges within the four school initiatives raised important conversations around diversity and inclusion, such as whether male-assigned bathrooms would be included in the distribution scheme to meet the needs of transgender or gender non-binary (TGNB) members of the student body. A recent study found that menstruating TGNB individuals who were assigned female at birth (AFAB) described female toilets as confrontational spaces where they receive incriminating stares and body policing (41). As such, to meet the needs of these individuals, it may be particularly important to provide access to menstrual products in locations beyond female-assigned bathrooms. These conversations are part of a larger effort by universities, brought on by growing social pressure, to create more inclusive environments and meet the needs of TGNB students. This has included for example, the hundreds of colleges and universities that have added gender identity and/or gender expression to their policies on non-discrimination (42), and the hundreds who have created or are in the process of creating gender-inclusive restrooms on their campus (43–45). These conversations were also prevalent within this study's sample, with the administration participants from multiple schools highlighting the ongoing initiatives on their campus to increase the availability of single-use bathrooms or to create "all-gendered" or "gender neutral" bathrooms spaces. It is important to note that despite these ongoing conversations and efforts toward creating inclusive environments for TGNB students, overt and covert discrimination is still present on university campuses and further work is needed in this area of MHH research (42).

While the number of these university and college menstrual equity initiatives seems to be on the rise, there is also a simultaneous mobilization for legislation mandating that public universities provide free menstrual products to their students. Already this legislation was passed in Scotland (29) and the United Kingdom in 2020 (46) and in several states in the USA (e.g., Washington State, Illinois, California) in 2021 (47–49). Although it remains unclear how these mandates will be operationalized or monitored, they represent an important step toward the standardized integration of menstrual product provision into normal campus operations. This legislation may render the campus-level initiatives described in this study unnecessary as it seeks to institutionalize this practice at the state or national level. However, these initiatives may provide useful guidance to policymakers and administrators in the states with these mandates on the cost, implementation models, distribution schemes, and challenges of providing free menstrual products on campus.

Limitations

There are three limitations to note. First, although the study included four diverse universities, there are numerous other university student-led menstrual equity initiatives which might have varying factors impacting their implementation. Second, this study only explored successful initiatives, and so may miss important challenges or obstacles that caused other such initiatives to fail. Third, given the relatively recent and short period of menstrual equity initiatives on the four campuses, it was not possible to assess their longer-term sustainability.

CONCLUSION

The examined university menstrual equity initiatives varied greatly in their scope, funding, maintenance, and labor sources, yet all four noted the presence of student champions, the importance of gaining administrative support, and the necessity of addressing inclusivity. Three recommendations emerge: (1) sustainable menstrual equity initiatives are more likely to occur when university administration commits resources and

capacity to assuring menstrual products are widely available, (2) more research is needed to understand the most effective implementation approaches for menstrual equity initiatives on diverse campuses, and (3) additional learning is needed to understand how such initiatives may positively or negatively impact TGNB students, students of color, and those experiencing homelessness while in university. Finally, these menstrual equity initiatives play an important role in meeting the needs of students on college and university campuses; however, to fully address the issue of menstrual equity, structural solutions are required such as the institutionalized provision of menstrual products within all university bathrooms.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

This study involving human participants was reviewed and approved by the Columbia University Medical Center Institutional Review Board. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

CG participated in study conception, conducted the data collection and analysis, and drafted the manuscript. TG participated in study conception, conducted the data collection and analysis, and contributed to drafting the manuscript. MS and MLS conceived the study and contributed to the drafting the manuscript. All authors read and approved the final manuscript.

FUNDING

This project was funded with generous support by the Sid and Helaine Lerner MHM Faculty Support Fund.

ACKNOWLEDGMENTS

The authors are grateful to the key informants who so generously provided their time and insights.

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frontiers in Reproductive Health

REVIEW
published: 20 December 2021
doi: 10.3389/frph.2021.699133

Computational Models for Diagnosing and Treating Endometriosis

Wangui Mbuguiro[1,2]*, *Adriana Noemi Gonzalez*[1,2] *and Feilim Mac Gabhann*[2,3]

[1] Department of Biomedical Engineering, Johns Hopkins University School of Medicine, Baltimore, MD, United States, [2] Institute for Computational Medicine, Institute for NanoBioTechnology, Johns Hopkins University, Baltimore, MD, United States, [3] Department of Biomedical Engineering, Johns Hopkins University, Baltimore, MD, United States

Endometriosis is a common but poorly understood disease. Symptoms can begin early in adolescence, with menarche, and can be debilitating. Despite this, people often suffer several years before being correctly diagnosed and adequately treated. Endometriosis involves the inappropriate growth of endometrial-like tissue (including epithelial cells, stromal fibroblasts, vascular cells, and immune cells) outside of the uterus. Computational models can aid in understanding the mechanisms by which immune, hormone, and vascular disruptions manifest in endometriosis and complicate treatment. In this review, we illustrate how three computational modeling approaches (regression, pharmacokinetics/pharmacodynamics, and quantitative systems pharmacology) have been used to improve the diagnosis and treatment of endometriosis. As we explore these approaches and their differing detail of biological mechanisms, we consider how each approach can answer different questions about endometriosis. We summarize the mathematics involved, and we use published examples of each approach to compare how researchers: (1) shape the scope of each model, (2) incorporate experimental and clinical data, and (3) generate clinically useful predictions and insight. Lastly, we discuss the benefits and limitations of each modeling approach and how we can combine these approaches to further understand, diagnose, and treat endometriosis.

Keywords: endometriosis, hormone therapy, computational, machine learning, systems biology, mechanism, biomarker, pharmacokinetics

OPEN ACCESS

Edited by:
Philippa Saunders, University of Edinburgh, United Kingdom

Reviewed by:
Tea Lanisnik Rizner, University of Ljubljana, Slovenia
Chloe Hogg, GlaxoSmithKline, United Kingdom

***Correspondence:**
Wangui Mbuguiro
wangui@jhmi.edu

Specialty section:
This article was submitted to Gynecology, a section of the journal Frontiers in Reproductive Health

Received: *22 April 2021*
Accepted: *23 November 2021*
Published: *20 December 2021*

Citation:
Mbuguiro W, Gonzalez AN and Mac Gabhann F (2021) Computational Models for Diagnosing and Treating Endometriosis. Front. Reprod. Health 3:699133. doi: 10.3389/frph.2021.699133

INTRODUCTION

Endometriosis: A Complex Disease

Although observations of endometrial-like cells growing outside of the uterus were made as early as the nineteenth century (1), endometriosis remains a significant and understudied public health challenge. Endometriosis is estimated to afflict 10% of menstruators and 20–25% of women undergoing surgery due to infertility or pelvic pain (2, 3). One challenge to estimating this prevalence is the variability in endometriosis presentation—with some only discovering endometriosis incidentally during surgery and others living with a wide range of debilitating symptoms (4). People with symptomatic endometriosis suffer an average of 7 years before diagnosis, a delay exacerbated by the lack of a non-surgical diagnostic for the disease (5). There is no cure for endometriosis. Rather, those with suspected or diagnosed endometriosis must decide how to combine interventions that primarily address symptoms (e.g., hormonal contraceptives and hysterectomy) and those that target endometriosis lesions specifically (e.g., ablation or

excision surgeries). Unfortunately, all of these interventions affect a patient's ability to conceive and have 5 year symptom recurrence rates ranging from 10 to 62% (6).

Endometriosis patients are typically staged by the visual appearance of lesions and adhesions according to the American Society for Reproductive Medicine's revised system. However, this staging does not correlate with patient symptoms or treatment outcomes (7). Looking beyond visual characteristics, clinical and experimental studies suggest that the growth and survival of lesions is enabled by a combination of immune dysfunction, hormone dysregulation, and aberrant blood vessel development (8–10). Specifically, endometriosis patients have been observed to have differences in progesterone receptor expression and functioning (11, 12), in peritoneal cytokine profiles, and in immune cell functioning (13, 14)—which all have the potential to interfere with the efficacy of pharmacological and surgical interventions.

To understand how such complex systems can contribute to patient symptoms and treatment outcomes, we need to integrate quantitative and computational approaches with clinical and experimental techniques. Researchers have created mathematical models to predict patient diagnoses and treatment outcomes based on symptoms, measurements, and medical history. However, as the success (and failure) of therapies is increasingly recognized as dependent on system-wide biological differences, computational models will need to expand in order to understand the mechanisms connecting these differences to clinical presentations and treatment outcomes.

In this review, we will first summarize how mathematical models have been used and modified over the years to study, diagnose, and treat endometriosis (in section "Systems Biology and Computational Models of Endometriosis"). We will then explore three mathematical modeling approaches to endometriosis that each take advantage of increasing detail in biological mechanisms. For each modeling approach, we will investigate their design, use of experimental and clinical data, and the insight they provide. Lastly, we will discuss current limitations in mathematical modeling of endometriosis and possible future directions in the conclusion section.

Systems Biology and Computational Models of Endometriosis

Systems biology is an integrative approach to investigating how genetic, cellular, and tissue level differences can influence an organism's physiology. This could include using quantitative measurements, ranging from *in vitro* cell culture experiments to various clinical observations, to extensively characterize a biological system. These experimental and clinical data can then be analyzed using mathematical and computational modeling approaches to make predictions about how the biological system behaves under various conditions (**Supplementary Table 1**). But how do we represent this system complexity meaningfully in a model? There are several ways, with different levels of mechanistic detail.

Regression and Machine Learning

Early computational models of endometriosis to have impact on the clinic were **regression models** that helped develop non-surgical screening tools for endometriosis in symptomatic women [reviewed in (15)]. Regression is a form of **machine learning** and is primarily **data-driven**, basing predictions (e.g., the probability of a patient having endometriosis) on measurable characteristics (e.g., differences in age, weight, pain qualities, subfertility, etc.) without including any causal relationships. More advanced forms of regression modeling, such as mixed-effects modeling, have been used to identify symptom-based subtypes of endometriosis patients using electronic heath records (16) and patient self-reporting (16, 17). The findings of these models have aided in diagnosing endometriosis (discussed in section "Diagnosing Endometriosis—Regression Modeling") and evaluating endometriosis treatment strategies (section "Gaps in Modeling Endometriosis and Opportunities for Future Models").

With the advent of techniques to collect and analyze patient samples, researchers have identified possible biomarkers for endometriosis using measurements from the peritoneal fluid, blood, urine, eutopic endometrium, and more [reviewed in (18)]. Regression modeling has been used here to identify associations between endometriosis and gene expression regulators (19), cytokines, angiogenic factors, and growth factors (20). Additionally, other machine learning techniques have been used to identify and explore the significance of molecular abnormalities found in endometriosis (14, 21–23).

Mechanism-Based Modeling

In contrast to data-driven models, which base predictions on how biological *components* (e.g., patient features, protein levels, etc.) may be associated with a *phenomenon* (e.g., diagnosis or therapy response), **mechanism-based models** incorporate and attempt to understand the "how" in these associations. In other words, mechanism-based models use equations that reflect how *components* interact in *space* and *time* within a specific *context* (e.g., drug or antigen exposure) to affect said *phenomenon* (24) (**Table 1**). In applying a mechanism-based approach, systems biologists can synthesize experimental data from independent studies as they simulate experiments done in cell culture, animal experiments, and clinical trials. This has enabled the prediction of drug interactions, establishing the fields of quantitative systems pharmacology (28) and systems toxicology (29).

Early mechanism-based modeling relevant to endometriosis predicted ovarian follicle maturation in response to hormone cycling (30). Since then, several papers have expanded these models to predict the effects of exogenous hormones in people with normal menstrual cycles and in people with polycystic ovary syndrome [reviewed in (31)]. More recently, models with increasing levels of mechanistic detail have been developed to optimize hormonal therapies for treating endometriosis and other estrogen-associated conditions while minimizing adverse events [discussed in sections "Treating Endometriosis—Pharmacokinetic and Pharmacodynamic (PK-PD) Modeling" and "Modulating the Menstrual Cycle—Quantitative Systems Pharmacology (QSP) Models"].

TABLE 1 | Overview of mechanism-based modeling and discussion in this review.

Defining feature of "mechanism"	Presence in regression modeling to diagnose endometriosis (25)	Presence in PK-PD modeling in treating endometriosis (26)	Presence in QSP modeling of menstrual cycle modulators (27)
Phenomenon	Endometriosis diagnosis	Therapy delivery and effect on ovarian cyst formation	Therapy delivery and effect on ovulation
Context	Patients seeing clinicians for pain and/or infertility, without previous diagnosis	Patients receiving therapy (Intravaginal ring containing anastrozole and levonorgestrel)	Patients receiving therapy (Gonadotropin-releasing hormone analogs)
Components	Patient attributes (e.g., symptoms, characteristics, medical history) that may contribute to diagnosis	Patient attributes, drug, and endogenous molecules that affect response to therapy	Drug, cells, and endogenous molecules (e.g., hormones and receptors), that affect response to therapy
Spatial arrangement & Temporal relationships	(Not modeled)	Drug transport from intravaginal ring to non-specific body compartments	Synthesis, transport, and interactions between components throughout the hypothalamus, pituitary, and ovaries

A biological mechanism includes all five features in the first column of this table [defined in (24)].

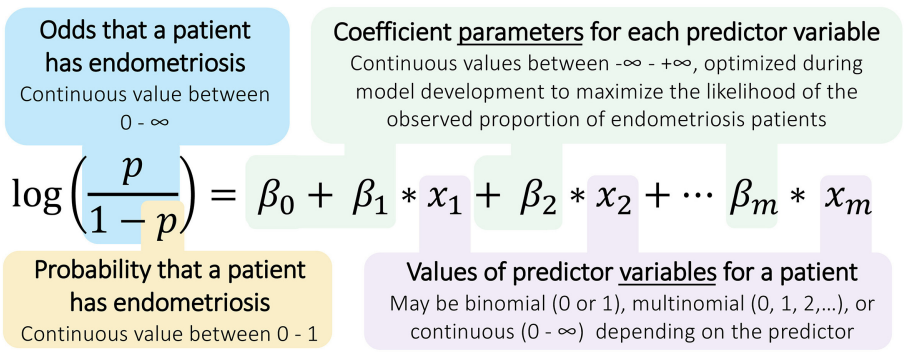


FIGURE 1 | Structure of logistic regression models for diagnosing endometriosis. Logistic regressions calculate the odds and probability of a binary outcome (e.g., positive endometriosis diagnosis) using measurements taken across several predictor variables (e.g., patient observations). The model parameters, the β coefficients, are identified by applying the logistic regression model to a many-patient data set for whom the outcomes are known, and these coefficients can then be used with new patient data to predict the likelihood of endometriosis in that patient.

Comparing Modeling Approaches (Scope, Data, Impact)

For all models, careful selection of scope is key—in other words, modelers choose which variables and parameters are included and which are not. What's included in the model will in turn affect how clinical and experimental data are used to create and validate the model. As a result, these modeling approaches will differ in the insight they can provide to clinical decisions and the impact this may have on patients. In this review, we compare how three computational studies design their model scope, use data, and impact clinical decisions.

DIAGNOSING ENDOMETRIOSIS—REGRESSION MODELING

Motivation for Logistic Regression Modeling

The current "gold standard" for diagnosing endometriosis is laparoscopic surgery followed by histology to identify endometrial-like growths in the abdomen (5), but this surgery has several limitations that have led to it being commonly postponed or avoided. These include: its high cost, potential complications, and the need for a highly skilled endometriosis surgeon (32). Instead, blood tests, pelvic examinations, and ultrasound imaging are done to rule out other disorders. Of these methods, only ultrasound imaging can detect endometriosis; however, it is limited to only detecting one form of disease (ovarian endometriotic cysts) (20). As a result, researchers have turned to logistic regression to answer the question: Can a combination of clinical observations reliably predict endometriosis?

Use of Logistic Regression Modeling to Guide Diagnosis

A logistic regression model estimates the probability of a binary outcome, such as having or not having endometriosis, using a set of independent observations about a patient as predictors (33). Regression models include components (**Figure 1**), in the form of these predictor variables, but they are not modeled as having any *spatial* or *temporal* relationships with one another; hence, these models are not considered mechanism-based (**Table 1**).

In medicine, logistic regression modeling is commonly applied to establish clinical scales, to identify risk factors for a disease, and to develop recommendations for treatment. For endometriosis, findings from logistic regression and related

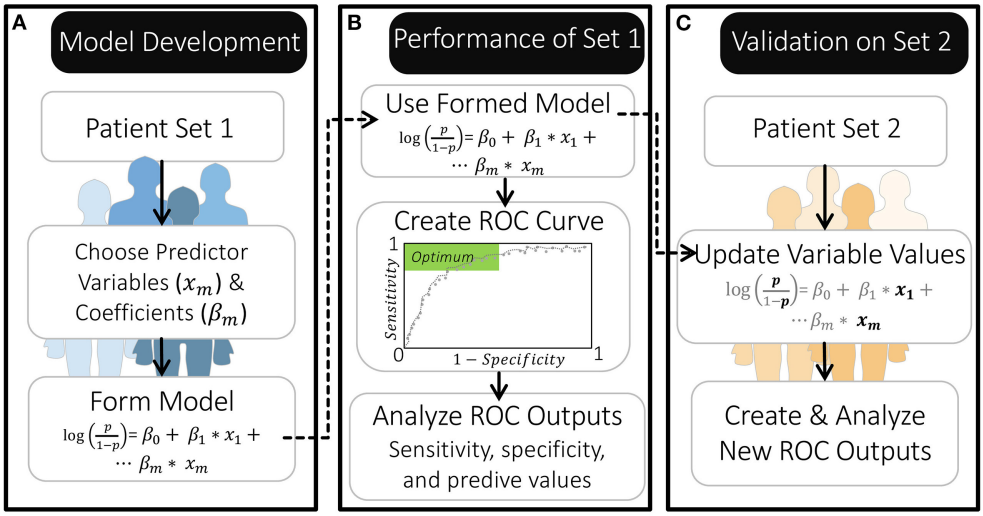


FIGURE 2 | Overview of model development and validation by Nnoaham et al. (25). **(A)** The authors created their models using a set of 771 patients. **(B)** They then evaluated the performance of this model using a ROC curve to identify probability thresholds for classification that produce a specificity and sensitivity within the optimal range. **(C)** They further validated the model by first updating it with new predictor variable values for a separate set of 625 patients (leaving the β coefficients as they were) and then creating a new ROC curve.

modeling have been cited as evidence for diagnosis guidelines [e.g., guidelines in (5) reference modeling in (34, 35)]. Specifically, researchers have used regression modeling to predict endometriosis from symptoms and medical history alone, blood tests alone, imaging alone, and a combination of these data sources (15).

Logistic regression does not require extensive prior knowledge of the mechanistic underpinnings of the disease, which can be difficult to ascertain. Instead, these models are entirely data-driven, using patient data that includes their known outcomes (e.g., diagnosis result) to predict the likely outcomes for other patients. Given sufficient data, logistic regression can identify the key elements that are predictive of endometriosis.

In this section, we will outline key points in creating a logistic regression model, using modeling by Nnoaham et al. (25) to illustrate these points. We discuss this study because it is one of the largest efforts so far to develop a non-surgical diagnosis for endometriosis, including more than 1,000 patients from 19 hospitals in 13 countries. The considerations detailed here will serve as a comparison point in later sections, where models are increasingly mechanism-based.

Example: Logistic Regression Modeling to Identify Predictors of Endometriosis

As part of the Women's Health Symptom Survey study in 2012, Nnoaham et al. (25) developed and validated symptom-based predictive models to predict the probability of a patient having endometriosis prior to any diagnostic surgery (**Figure 2**). The patients in their study all suffered from pelvic pain and/or infertility and answered over 200 questions, detailing their demographics, medical history, and symptoms. The effect of including any preoperative ultrasound data was also explored (25).

The authors used logistic regression to calculate the probability that a patient would visually be diagnosed with endometriosis at laparoscopy based on a combination of the patient observations. The authors also calculated the probability of finding "moderate" to "severe" endometriosis, according to the revised American Society for Reproductive Medicine classification system (r-ASRM stages III-IV) (25).

Model Scope

For logistic regression models, researchers identify and include only the strongest predictor variables. Although models with many predictor variables may appear more accurate in fitting the training data, they can struggle to predict outcomes for new patients. To avoid this overfitting, researchers narrow the number of predictor variables included in their model, ideally having at least 10 patients for each predictor variable included (33).

Nnoaham et al. (25) identified which of the 200+ patient characteristics to include as predictor variables in their model by first grouping clinically-related predictors and then iteratively removing the predictor(s) in each group with the least significant association with endometriosis. Each of these reduced predictor groups were then combined, and the process was continued until each of their models included 18–25 predictor variables (i.e., one predictor variable for every 30–43 patients in their first patient set) (**Figure 2A**). These predictor variables had differing influence on the model's odds prediction, both in terms of sign and magnitude, which was reflected by their estimated regression coefficients (β). This approach to selecting model variables allowed the authors to minimize redundancy in predictor variables while maximizing how well the reduced model fit the data. Importantly, this process of forming model equations was primarily data-driven; meaning, mechanistic knowledge of how variables interact or contribute to disease was not used in selecting model variables or parameters (regression coefficients).

Data Usage

Logistic regression models are typically created using data from one study. If multiple studies are modeled, these studies must measure the model predictor variables and outcomes in a similar manner.

In constructing these models, Nnoaham et al. (25) used data from the Women's Health Symptom Survey study. As part of model development, all 1,396 patients in this study completed the same survey prior to their diagnostic surgery. This survey could capture a wide range of the patients' experiences, including predictor variables that were linear (e.g., age, average cycle length, menstrual flow) and categorical (e.g., ethnicity). Importantly, it was necessary for patients across the 19 hospitals to undergo the same assessment for the modelers to form a single estimate of the model parameters (the regression coefficients) that could predict the outcome (the diagnosis result) for all patients.

Clinical Impact

Through creating logistic regression models, researchers can identify a combination of characteristics that are highly predictive of a disease or treatment outcome. Clinicians can then use these findings to motivate further actions for patients with these characteristics. Hence, regression modeling aims to aid in the development of a less invasive diagnostic that correctly predicts endometriosis in those that have it (i.e. has a "high sensitivity"). Correctly identifying non-endometriosis patients ("specificity") is also important—although less so if using this diagnostic to prioritize patients with subfertility for laparoscopic surgery, since laparoscopy can also identify other factors affecting fertility (36).

By constructing their model on one patient population, and evaluating it on a second, Nnoaham et al. (25) could assess how well their models would perform if applied to new patients. To evaluate their models, Nnoaham et al. (25) generated ROC curves (**Figures 2B,C**, **Box 1**) and found that their best model for diagnosing r-ASRM stage III-IV endometriosis achieved a sensitivity of 82.3% and specificity of 75.8% for their second set of patients. This sensitivity and specificity are sufficient if this predictive model is applied to develop recommendations for performing surgery to diagnose and treat endometriosis earlier—which is the usage that Nnoaham et al. (25) advocates for. This sensitivity would be insufficient if these model predictions were to be considered as exclusion criteria for diagnostic surgery or treatment, as ∼18% of endometriosis patients would be missed.

Summary

As shown here, regression models serve as valuable tools for identifying patient characteristics that can predict disease or treatment outcomes. Importantly, this modeling does not explain the "how" in this association, as in: "how do these patient characteristics contribute to endometriosis and treatment outcomes?" To answer this question, researchers must model the mechanisms by which components within the system affect each other.

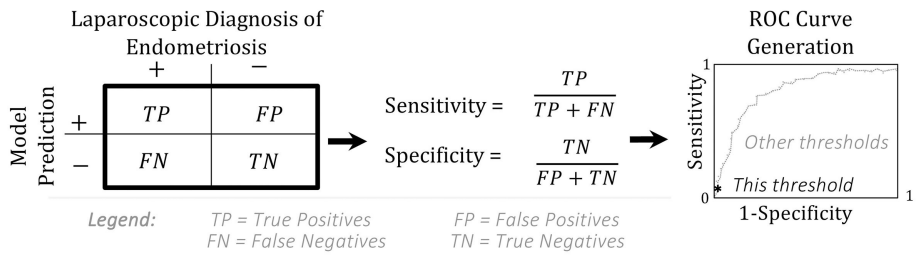
TREATING ENDOMETRIOSIS—PHARMACOKINETIC AND PHARMACODYNAMIC (PK-PD) MODELING

Motivation for PK-PD Modeling

Medicinal approaches for treating endometriosis primarily aim to manage symptoms but have limited efficacy, with symptoms often recurring once a patient stops treatment (38). As a first-line therapy, many patients presenting with a combination of chronic pelvic pain or pain during menstruation, sex, or urination will take medications such as NSAIDS and hormonal contraceptives (38). For those with persistent pain and confirmed endometriosis, therapeutic options can include gonadotropin-releasing hormone (GnRH) analogs and aromatase inhibitors (5). These second- and third-line therapies are effective in treating chronic pelvic pain through suppressing estrogen, thereby inhibiting the growth and survival of endometriosis lesions (8, 39). However, GnRH analogs and aromatase inhibitors can be associated with severe hypoestrogenic effects, such as decreases in bone mineral density (38). Emerging clinical trials aim to identify novel therapeutic strategies for treating endometriosis with increased safety through applying an array of pharmacokinetic (PK) and pharmacodynamic (PD) modeling approaches. Here,

BOX 1 | Sensitivity, Specificity, and ROC.

A model is assessed by creating a receiver operating characteristics (ROC) curve. The ROC curve shows the values for the true positive rate ("sensitivity") vs. the false positive rate (1-"specificity") at every possible probability threshold for classification (37).



PK modeling is applied to answer the question: How much drug will a patient be exposed to over time? PD modeling then considers: As drug exposure varies, how much of a physiological response can be expected?

Use of PK-PD Modeling to Treat Endometriosis

Although treatments for endometriosis are monitored in circulating blood ("centrally") many drugs are delivered to or act throughout peripheral sites. To predict drug exposure or efficacy, we need a way to connect these sites. Pharmacokinetic modeling connects these central and peripheral sites through equations that predict the concentration of a drug as it is absorbed, distributed throughout the body, and eliminated via metabolism or excretion. Pharmacodynamic modeling uses these estimated and monitored drug levels to predict the onset, duration, and intensity of response to that drug (40). Population PK-PD models incorporate variability in select model parameters based on differences between patients (e.g., body mass, age, and genetic background), allowing for simulations of larger virtual populations to better inform recommendations (**Figure 3**).

The commonly applied two-compartment (central and peripheral) pharmacokinetic model incorporates all five elements of mechanism (**Table 1**)—modeling how a *component* (drug) moves through *space* and *time* in the *context* of drug dosing in order to predict *phenomena*, such as drug efficacy or toxic effects. Unlike regression modeling (**Figure 1**), pharmacokinetic models are composed of differential equations, where the variables are the concentration (or amount) of each component and the parameters are the rate constants representing how fast reactions and transitions between components and compartments occur (**Figure 4**).

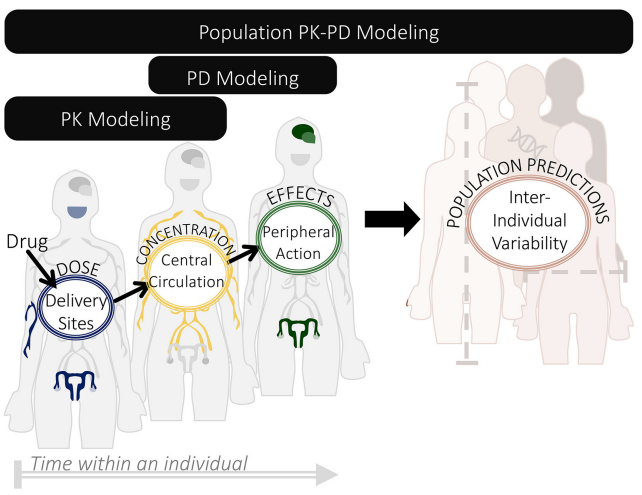


FIGURE 3 | Relationship between pharmacokinetic (PK), pharmacodynamic (PD), and population PK-PD modeling. These three modeling modalities can be used to make predictions about treatment from drug dosing to resulting effects, on an individual and population scale.

Because of this structure and level of mechanistic detail, pharmacological models are ideal for simulating and comparing different dose amounts, regimens, and delivery sites for endometriosis therapies under development. By developing PK-PD models with additional mechanistic detail, researchers have been able to identify endometriosis patients with a genetic favorability for a GnRH antagonist (41), predict changes in bone mineral density following long-term GnRH antagonist treatment of endometriosis (42), and interrogate the role of chosen delivery method in the efficacy of combination progestin therapies (43).

As we discuss the unique considerations in population PK-PD models, we will use (26) as an example. This study by Reinecke et al. applied PK-PD modeling to select doses to be used in phase 2 of a clinical trial for an endometriosis therapy. In addition to modeling the distribution of the therapy throughout the body, this model predicted the influence of endogenous proteins on drug efficacy and adverse events in patients. This study is also of interest because of its application of multiscale data—ranging from *in vitro* experiments to animal experiments and previous phase 1 studies—to select the equations and parameters for this model.

Example: PK-PD Modeling to Design Clinical Trials

In 2017, Reinecke et al. (26) used population PK-PD modeling to guide the development of an intravaginal ring (IVR), delivering the aromatase inhibitor anastrozole (ATZ) and the progestin levonorgestrel (LNG) for long-term, localized treatment of endometriosis and associated pain (26). This new approach to treating endometriosis targets estrogen production in endometriotic lesions through local inhibition of aromatase, thereby minimizing systemic hypoestrogenic effects. This therapy also includes a progestin to provide contraception because ATZ is a teratogen (44).

Population PK-PD modeling was used to identify doses that would achieve therapeutic levels of ATZ and LNG while minimizing the risk of ovarian cysts in a phase 2 clinical trial (EudraCT 2013-005090-53; NCT02203331) (26). These PK-PD models use ordinary differential equations (ODEs) to predict the levels of drugs in the body over time and the associated risk of developing ovarian cysts.

Each drug was modeled using a two-compartment model as a basis (**Figure 4**). Using data from *in vitro* and animal studies alongside their mechanistic understanding of the system, Reinecke et al. (26) amended the ATZ and LNG base models to more closely match the outcomes of a phase 1 clinical study in humans (EudraCT 2011-005620-18). As a result, these models included delivery via an intravaginal ring. In addition, since LNG predominantly binds to and influences the production of sex hormone binding globulin (SHBG) in serum, the LNG model also included the influence of LNG on SHBG (and vice versa) and the additional influence of circulating estradiol (E2) on SHBG (**Figure 5**).

Model Scope

In contrast to regression models, where deciding the scope was fully data-driven, the equations in pharmacological models

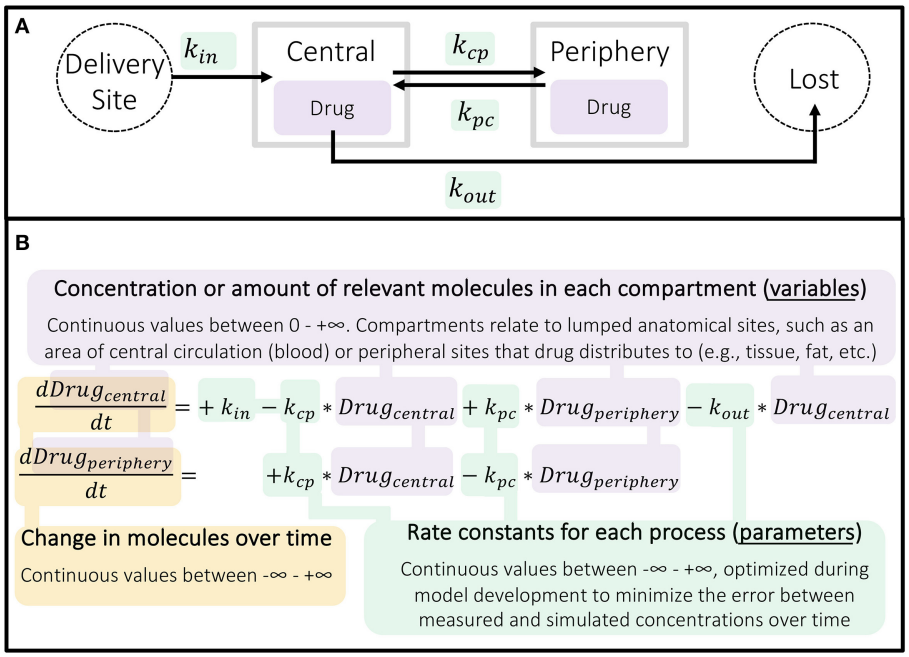


FIGURE 4 | Structure of basic two-compartment pharmacokinetic model. **(A)** Schematic of continuous processes represented in a two-compartment model. **(B)** The two ordinary differential equations (ODEs) used here describe the rate of change in concentration of the drug in the central and peripheral compartments over time as a result of these processes occurring. PK models can be more or less complex, with different compartments and processes included as needed to fully describe the drug being investigated in the simplest reasonable form.

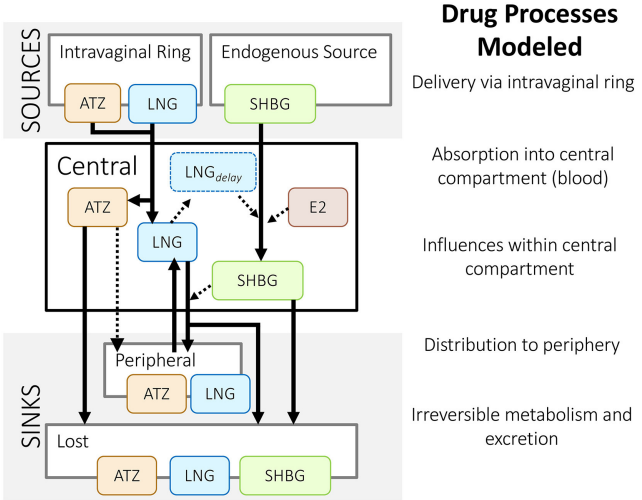


FIGURE 5 | Structure of Reinecke et al.'s (26) population pharmacokinetic models for an intravaginal ring that delivers anastrozole (ATZ) and levonorgestrel (LNG). These models include the influence of estradiol (E_2) and LNG on sex hormone binding globulin (SHBG), and vice versa. Solid lines represent a mass flow; dashed lines represent an indirect influence – as described in Reinecke et al. (26).

can be developed in both a mechanism-based and data-driven manner. In constructing a pharmacokinetic model, researchers can consider the biology of a drug and its interactions within the body to better understand and improve upon the therapy.

For example, Reinecke et al. (26) chose to include sex hormone binding globulin (SHBG), a circulating protein that binds the delivered LNG and endogenous estradiol (E2). LNG and E2 were both modeled as indirect influences on the rate that SHBG is produced (**Box 2**). Inclusion of these molecules allowed the researchers to explore the role of SHBG in contraceptive efficacy and ovarian cyst formation. As a result, the simulations were able to capture fluctuations that appeared in clinical measurements. By including E2 and SHBG in their model, Reinecke et al. (26) could also explore the influence of observed inter-individual variability, such as variability in SHBG and E2 baseline levels, as they made population-level predictions.

Data Usage

Pharmacological models are created using data that characterize the mechanisms contributing to drug delivery and response. Unlike regression modeling, this data can come from multiple independent studies that assess different aspects of the biological system. Hence, processes affecting a drug can be evaluated in isolation prior to being incorporated into a pharmacokinetic model.

Reinecke et al. (26) used data from *in vitro* experiments measuring daily release from an intravaginal ring to create and parametrize equations describing delivery via the intravaginal ring, specifically. In using this data, they assumed that a ring under their bench-top conditions releases drug in a similar manner to a ring within a vagina, which they support using

BOX 2 | Differential equation for SHBG from Reinecke et al. (26) model.

The model for SHBG in the blood over time [$SHBG\,(t)$] includes terms that affect its production and loss, which have rate constants k_{in} and k_{out}, respectively. The production term is influenced by delayed inhibition by LNG and induction by E2, which scale rate constant k_{in} by a factor of $-r_i$ and $+r_s$, respectively. The loss term is linearly proportional to the level of SHBG in the blood.

$$\frac{d}{dt}SHBG\,(t) = +k_{in} * \left(1 - r_i * LNG_{delay}\,(t) + r_s * E2\right) - k_{out} * SHBG\,(t)$$

evidence from a preclinical study conducted in cynomolgus monkeys. This *in vitro* data was used in combination with phase 1 clinical data, which included plasma drug concentrations and drug remaining in the ring at the end of treatment, to create and parametrize their model. As a result, this model can predict multiple patient outcomes over time, including: the level of drug in the intravaginal ring, serum concentrations of the delivered drugs (LNG and ATZ), as well as the levels of influencing molecules (E2 and SHBG).

Clinical Impact

Clinical researchers use pharmacokinetic modeling to explore drug dosing in populations or treatment groups. In addition, by integrating these PK models with pharmacodynamic (PD) models, clinical researchers can predict drug effects and identify predictors for adverse events.

Once Reinecke et al. (26) confirmed their simulations matched the phase 1 (EudraCT 2011-005620-18) results for three intravaginal ring formulations, they used their models to simulate additional doses of ATZ and LNG. These researchers were then able to identify three additional ATZ doses for a phase 2 trial. They used modeling to identify doses that could achieve the minimum effective concentration for all patients, while having minimal overlap between treatment groups, thereby maximizing the potential insight gained. Remarkably, the predictions from Reinecke et al. (26) closely matched results from a subsequent phase 2 study in endometriosis patients (45) (**Figure 6**).

Furthermore, Reinecke et al. (26) created a PK-PD model in order to predict the effect of LNG and ATZ exposure on ovarian cyst formation. They compared the predicted probability of developing ovarian follicles $\geq$ 30 mm between several PD models, which varied in the relative influence of ATZ and LNG exposure. They selected the best model by comparing the predicted probabilities to the observed fraction of patients with enlarged follicles found during ultrasound. In the end, they found that increasing unbound LNG levels are more predictive of large follicle formation than increasing ATZ levels. This model could be used to predict the risk of developing ovarian cysts for the doses they were selecting for phase 2 of their clinical trial.

Summary

As shown through the Reinecke et al. (26) example, population PK-PD modeling can be useful in deciding study treatments, simulating population heterogeneity, and predicting treatment response using information from *in vitro*, animal, and human studies. These insights inform the design of clinical trials and, ultimately, how a drug is used to treat disease. Differing from the logistic regression model, PK models predict changes in component concentrations over time, painting a dynamic picture of the system. Although the base two-compartment model is quick to create and often resembles typical drug exposure, this approach limits the questions researchers can address through modeling. As such, modelers often choose to include more mechanistic detail in their PK model and incorporate population variability in parameter values, as Reinecke et al. (26) has done.

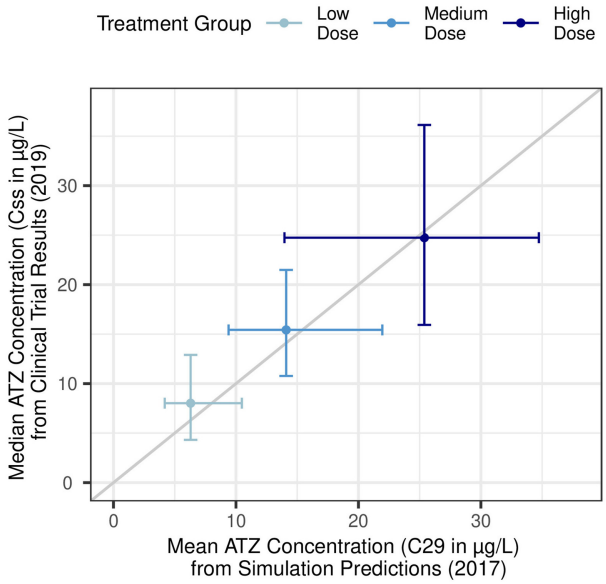


FIGURE 6 | Agreement between pharmacokinetic simulation predictions and subsequent clinical trial results. X-axis: Simulation predictions for mean plasma ATZ concentrations 28 days following ring placement (C_{29}) in low-dose (290 µg/day), medium-dose (630 µg/day), and high-dose (1,080 µg/day) treatment groups. Horizontal error bars represent the 5th and 95th percentiles. Adapted from Reinecke et al. (26). Y-axis: Observed median plasma ATZ concentration as average of measurements taken 28, 56, and 84 days following first ring placement (C_{ss}) in low-dose (300 µg/day), medium-dose (600 µg/day), and high-dose (1,050 µg/day) treatment groups from a phase 2b clinical trial. Vertical error bars represent the 10th to 90th percentile. Adapted from Nave et al. (45).

MODULATING THE MENSTRUAL CYCLE—QUANTITATIVE SYSTEMS PHARMACOLOGY (QSP) MODELS

Motivation for QSP Modeling

Endometriosis treatment is complicated by the systemic effects of estrogens, gonadotropins, and related hormones throughout the menstrual cycle. Therefore, there is significant interest in understanding and predicting the effects of therapies that perturb the cycle, such as gonadotropin-releasing hormone (GnRH) analogs, aromatase inhibitors, and progestins, on endometriosis

and subfertility. To do so, mechanism-based systems biology models have been created using differential equations to describe systemic hormone fluctuations that occur during the menstrual cycle (31). Quantitative systems pharmacology (QSP) connects pharmacokinetic (PK) models of hormone-modulating therapies with models of those hormones and of endogenous protein signaling in order to further study the effect of these drugs on the body. In contrast to pharmacodynamic (PD) modeling, which predicts the change in magnitude of a physiologic response, QSP modeling allows us to consider: What are the underlying mechanisms contributing to a physiological response and how can they be best therapeutically targeted?

Use of QSP Modeling to Develop Treatments for Endometriosis

Quantitative systems pharmacology (QSP) integrates systems biology approaches with both data-driven and mechanism-based computational techniques to understand and optimize therapies (28). Upon first glance, the structure of QSP models resembles that of PK-PD models, using differential equations to represent changes in proteins in the system over time (**Figure 4**). But while PK-PD models tend to be drug-centric (predicting the distribution and effects of exogenous compounds), QSP models also focus on processes endogenous to the body. QSP models thus allow us to answer questions that are more mechanism-focused than typical PK-PD models, because they model the influence of molecules from the sub-cellular to multi-organ levels, thereby including more *components* interacting over more *spatial* and *temporal* scales (**Table 1**).

QSP models have been created to explore the effects of therapies on protein signaling that impacts endometriosis. For example, Riggs et al. (46) expanded upon a mechanism-based model of bone remodeling (47) to study the effects of therapeutic estrogen-suppression to treat endometriosis (46). Importantly, Riggs et al. (46) combined their QSP model with a logistic regression model to assess how well patients' estrogen levels could predict their endometriosis-related pain severity—illustrating how the models discussed in this review can be used in harmony (46). In addition, QSP models have been used to predict *in vivo* treatment outcomes from *in vitro* systems, such as novel microphysiological systems that include the endometrium (48).

Röblitz et al. (27) created a QSP model of hormone cycling to aid in the development of GnRH analog therapies. GnRH analogs are critical in treating several conditions, including: cancers, uterine fibroids, and infertility (27). Although this study was not focused on endometriosis, we are discussing it because they model the GnRH antagonist, cetrorelix, which is used to treat endometriosis (38). Here, we will explore how these researchers integrated a highly mechanistic model of the menstrual cycle with pharmacokinetic models of GnRH analogs in order to compare treatments.

Example: QSP Modeling to Guide Menstrual Cycle Modulation

Röblitz et al. (27) modeled key hormones that travel and signal between the brain, ovaries, and the blood (**Figure 7A**). In the body, and specifically included in the model, GnRH is formed in the hypothalamus and transported to the pituitary gland where it stimulates the release of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), into the bloodstream. LH and FSH exert their effects on processes in the ovaries, which include follicular development, ovulation, and the development of the corpus luteum. Through these processes, the production and release of progesterone (P4), estradiol (E2), and inhibins A and B (IhA, IhB) are regulated. These circulating hormones signal back to the hypothalamus and pituitary, affecting the formation and release of GnRH, LH, and FSH.

Röblitz et al. (27) also created pharmacokinetic models of GnRH analog delivery to connect to these highly mechanistic models of the menstrual cycle. The delivery of GnRH agonist and antagonist are modeled using a one- and two- compartment PK model, respectively—similar to those previously described (**Figure 4**). Röblitz et al. (27) incorporated the pharmacokinetic model of the GnRH agonist, nafarelin, by modeling the drug in the central compartment (circulating blood) as being able to bind to and activate GnRH receptors, as natural GnRH does. In contrast, the GnRH antagonist, cetrorelix, is modeled as being able to bind to but not activate GnRH receptors (**Figure 7B**). In this way, the administered drugs either act alongside or compete with GnRH, thereby affecting the level of GnRH receptors available to activate downstream signaling.

Model Scope

How much physiological detail to include in a mechanistic model is often a balance of the questions being explored and the computational resources and data available.

This delicate balance is illustrated in comparing Röblitz et al. (27) to Reinecke et al. (26). As discussed in the previous section, Reinecke et al. (26) was primarily interested in predicting drug exposure over time and how that affected the odds of ovarian cyst development. As such, drug-protein interactions were primarily modeled as indirect influences, either increasing or decreasing the level of free drug in central circulation (**Figure 5**). In contrast, since Röblitz et al. (27) sought to predict both drug exposure and the effects on signaling throughout the menstrual cycle, these researchers more directly modeled the physiologic processes that together impact the delivery and effect of GnRH analogs. This included hormone-receptor interactions in the brain and ovaries, as well as ovarian follicle maturation (**Figure 7**). In contrast to how Reinecke et al. (26) models the effects of E2 and LNG on the level of SHBG (**Box 2**), Röblitz et al. (27) uses mass action kinetics to represent each interaction and process that alters the level of GnRH receptor on the cell surface (**Box 3**). By creating similar equations for the processes affecting GnRH, other hormones, and their receptors, Röblitz et al. (27) could accurately predict the timing of ovulation under various treatment scenarios.

Although Röblitz et al. (27) models major hormonal and physiological components of the menstrual cycle in significant detail, they do limit their model scope to minimize computational load. For example, as Röblitz et al. (27) created equations to model GnRH signaling, they avoided operating on the small time scales (minutes) that had been previously used to model GnRH

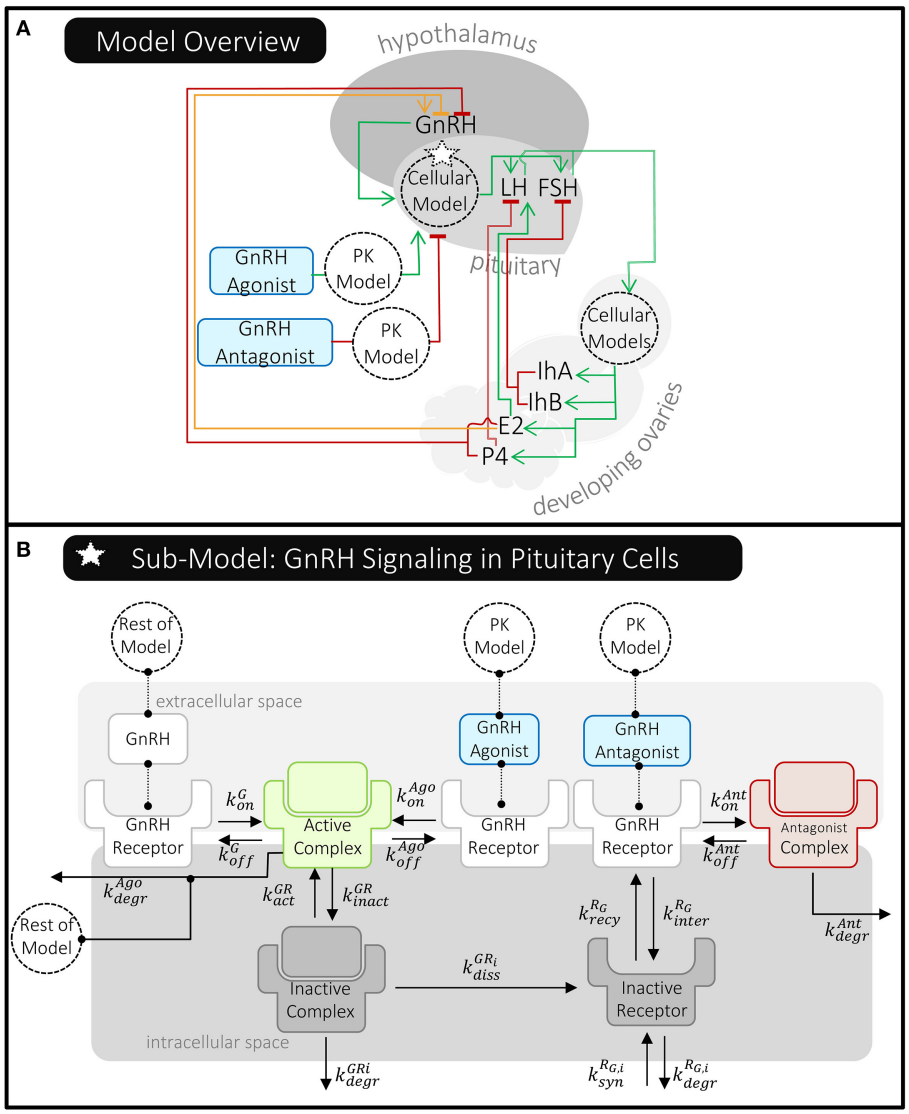


FIGURE 7 | Overview of Röblitz et al.'s (27) quantitative systems pharmacology model of the menstrual cycle and gonadotropin-releasing hormone (GnRH) therapies. **(A)** This schematic shows where molecules are produced and whether they stimulate (green line/arrow head), inhibit (red line/flat head), or have a mixed effect (orange line/both) on the production of other molecules in this model. The dotted circles labeled "Cellular Model(s)" represent processes affecting pituitary GnRH receptors and ovarian LH/FSH receptors that have been modeled in detail. The delivery of GnRH agonist and antagonist are modeled using PK models that feed into the pituitary cellular model. **(B)** The pituitary cellular model is summarized here. Each reaction has a unique reaction rate constant (k) that can depend on the receptor state (e.g., whether it's internalized or the specific molecule it's bound to). For simplicity, reactions involving an active complex have just been shown once; however, the rates of these processes do depend on the receptors' states, as described in Röblitz et al. (27).

pulsations. Röblitz et al. (27) acknowledges that not including a more detailed GnRH pulsing model may be limiting their model's accuracy. However, this reduction in model parameters may also be allowing for a more robust model—sacrificing a model's ability to perfectly predict one component (or scenario) often produces a model that is better able to predict many components (and scenarios).

Data Usage

Similar to PK-PD models, QSP models are created using data from independent studies to characterize drug delivery and effects. However, these researchers must use additional data to model biological mechanisms on multiple scales, even more-so than typical PK-PD models.

Like pharmacokinetic approaches, Röblitz et al. (27) created and parametrized their model of hormone cycling (without GnRH treatment) using daily hormone measurements taken from 12 people with normal menstrual cycles. However, Röblitz et al. (27) connected these models to cellular models of LH and FSH in the ovaries, as well as GnRH in gonadotropic cells of the pituitary (**Figure 7B**). At the cellular level, the rate of GnRH receptor binding and trafficking were estimated using data from

BOX 3 | Structure of differential equation for GnRH receptor from Röblitz et al. (27).

This sub-model uses mass action kinetics to predict cumulative effect of each process on the level of free (unbound) GnRH receptor on the surface of pituitary cells over time [$R_{G,a}$ (t)]. These processes include (listed in order they appear in this equation): binding and unbinding to endogenous GnRH, receptor internalization and recycling—from and to the cell surface, and receptor binding and unbinding to GnRH agonist ("Ago") and antagonist ("Ant"). The rate constant for each reversible reaction is represented by each "k" term below, which are also shown in **Figure 7B**. Refer to Röblitz et al. (27) for full details and equations.

$$\begin{aligned}\frac{d}{dt}R_{G,a}(t) &= -k_{on}^{G} * G(t) * R_{G,a}(t) + k_{off}^{G} * GR(t) \\ &- k_{inter}^{R_G} * R_{G,a}(t) + k_{recy}^{R_G} * R_{G,i}(t) \\ &- k_{on}^{Ago} * SF_{Ago} * Ago_c(t) * R_{G,a}(t) + k_{off}^{Ago} * AgoR(t) \\ &- k_{on}^{Ant} * SF_{Ant} * Ant_c(t) * R_{G,a}(t) + k_{off}^{Ant} * AntR(t)\end{aligned}$$

an earlier model by Blum et al. (49)—this model estimated these reaction rates using experimental measurements of gonadotropes in culture. Through applying both clinical and *in vitro* data, Röblitz et al. (27) was able to not only track the levels of cycling hormones (e.g., LH, FSH, E2, P4, etc.) over time, but they could also predict the concentrations of proteins that aren't currently measured in patients (e.g., LH-receptor and GnRH-receptor complexes). This allows for a multi-scale understanding of how treatments are affecting patients and can be further analyzed to identify alternative therapeutic approaches.

Clinical Impact

QSP models include more biological components, such as endogenous protein or hormone networks, than a typical PK-PD model. Because of this, clinical researchers often use QSP models to compare multiple therapeutic strategies and diseases.

The Röblitz et al. (27) model was successful in simulating not only the levels of each drug over time, but also the resulting fluctuations in patients' hormone levels. This produced a versatile model that could be used by clinical researchers to compare the effects of dosing GnRH agonists and antagonists on hormone cycling and the resulting effects on ovulation. As one example of this utility: Through modeling, Röblitz et al. (27) found that the GnRH antagonist, cetrorelix, delays ovulation in a manner that is highly dependent on each patient's drug clearance rate. This suggests that if a patient's plasma drug concentrations are monitored in the first day of dosing, then a clinician may be able to more accurately predict when ovulation will occur and when subsequent doses may be necessary.

Furthermore, because the Röblitz et al. (27) model includes both the direct targets of GnRH analogs (e.g., bound receptors) and the indirect targets (e.g., developing follicles, circulating hormones), this model can make predictions about system behavior when anything in the model is perturbed. For example, endometriosis is characterized as a hyper-estrogenic state. Because estradiol is included in the model, this model could be used to examine how elevated estradiol affects ovulation and signaling within the menstrual cycle. In addition, exogenous molecules that affect the hormones and receptors already present could be explored with minimal adjustments or additions to the model.

Summary

The Röblitz et al. (27) model combines approaches from traditional PK-PD models with a highly mechanistic, QSP model to compare the effects of GnRH agonists and antagonists on people with normal menstrual cycles. This allows their model to efficiently predict clinical measures while supplying more insight into the biological processes affected by perturbations caused by disease or treatment than a PK-PD model alone could. Importantly, QSP models can be adapted to study different disease or treatment cases. This may involve applying the model to a new set of patients and/or adapting the model to include additional disease-related biological processes, such as in Riggs et al. (46). Ultimately, these highly mechanistic, systems biology models aim to expand (in both number and complexity) the biological questions researchers can explore.

CONCLUSION

Benefits and Limitations of Each Computational Modeling Approach

In this review, we've explored three mathematical modeling techniques that have been applied to improve endometriosis diagnosis and treatment: regression, pharmacokinetics/dynamics (PK-PD), and quantitative systems pharmacology (QSP). Below, we'll summarize the benefits and challenges of each modeling approach and outline opportunities for future modeling of endometriosis.

Regression models represent a data-driven approach; meaning, they can be created without needing to start with a detailed mechanistic understanding of the system. As a result, regression models excel in identifying associations in data (e.g., which measured variables or combinations of variables are strong predictors of endometriosis or of clinical outcomes) without requiring advance knowledge of how these associations contribute to disease. However, these models are limited in their ability to explore the "how" in these associations.

PK±PD and QSP models both represent mechanism-based approaches that can be used to predict how biological factors will influence patient treatment. PK models are especially useful for deciding drug dosing in clinical studies. Although base compartmental PK models only predict the distribution of a drug throughout the body, researchers can add details about drug interactions within the body to the model (if that data is available). This leads to the creation of a more mechanistic PK-PD model. However, to better understand the role that endogenous pathways play on any disease and treatment, a QSP model is used.

QSP models closely resemble PK-PD models; however, QSP models add more focus on the biological mechanisms endogenous to the system. This leads to the inclusion of a wider range of experimental data to parametrize a QSP model (e.g.,

from molecular and cellular to tissue and multi-organ levels). As a result, QSP models can simulate changes within a biological system without any drug introduced—this is something PK models do not do. QSP models thereby become increasingly useful in interrogating the mechanisms underlying a drug response and contributing to disease. QSP models are also well-suited for comparing multiple disease and treatment scenarios. However, these models can be more time- and knowledge-intensive to create.

Gaps in Modeling Endometriosis and Opportunities for Future Models

There are still many opportunities for the development and improvement of computational models to diagnose and treat endometriosis. Regression models need a large (many-patient) dataset across multiple clinical centers [such as in (25)] in order to have findings that can be generalized to other endometriosis patients. As discussed in previous reviews (15, 18), many studies on diagnostic indicators of endometriosis either have too few patients to be generalized or have yet to be validated with an independent patient population. Additionally, regression models predicting treatment outcomes are less common, so have not been discussed here. However, recent studies have used regression modeling to predict the efficacy of assisted reproductive technology and surgery on the fertility outcomes for endometriosis patients (50, 51).

The limitations of PK-PD studies often relate to the availability of sufficient data. How much data, and which data, is needed to model a therapy's delivery and effects will depend on the properties of that specific therapy. Models can be augmented with pre-clinical data and data from previous trials, as in Reinecke et al. (26). Additionally, there has been increased use of more mechanistic PK models, such as physiologically-based PK models, for investigating drug-drug interactions of therapies for endometriosis (42, 52). This could be due to the expanded tools for establishing, analyzing, and submitting these models for regulatory review (53, 54).

QSP models in general are a more recent approach. Several QSP models have been created to investigate the effects of hormone-modulating therapies on cell signaling in people with normal menstrual cycles and in people with polycystic ovary syndrome. So far, few of these models have directly modeled the effects of these therapies in endometriosis—with Riggs et al. (46) being one of the few. These researchers created a mechanism-based model to predict the effects of therapies on endometriosis symptoms and bone mineral density (46). As endometriosis is known to involve dysregulation in hormone, vascular, and immune signaling networks, there are several opportunities to use highly mechanistic computational modeling, such as QSP, to further our ability to understand, diagnose, and treat endometriosis.

For instance, the mechanism-based models of hormone signaling outlined in this review could be adapted to study the effects of hormones on endometrial tissue. One recent study has connected a hormone signaling model to a newly developed mechanistic model of endometrial changes during the menstrual cycle, including terms to represent growth, shedding, and blood vessel development (55). This and future studies can be used to explore the effects of endometriosis-associated hormone dysregulation on the endometrium.

Focusing on vascular and immune influences, researchers can adapt mechanism-based models of protein-signaling in blood vessel development (56) to study the impact of endometriosis lesions producing pro-angiogenic cytokines [e.g., VEGF, IL-1β, IL-6, IL-8, etc. (9)]. Additionally, mechanism-based, systems biology models can help us interrogate the interactions between endometrial and immune cells in endometriosis. Since endometriosis lesions and cancerous tumors share some immune and vascular abnormalities, cancer models may serve as a basis for this. For instance, macrophages are known to affect endometriosis lesions as they differentiate, secrete cytokines, and promote angiogenesis (13)—Mahlbacher et al. (57) have modeled these macrophage behaviors within cancerous tumors. Lastly, agent-based models (another mechanism-based approach) have been created to study signaling and development of epithelial tissues (58), such models can be adapted in order to investigate the functioning of healthy and endometriotic epithelia within organoid cultures.

Since each modeling approach yields distinct insight, data-driven and mechanism-based modeling can and have been used in concert to identify associations in biological data and interrogate the underlying mechanisms of disease, respectively. The harmony of these approaches was demonstrated as we discussed previous QSP models (27, 47). By using a multitude of computational modeling approaches, researchers can synthesize multiscale experimental and clinical data to identify predictors of endometriosis and design therapies. Furthermore, there are exciting opportunities for developing mechanism-based models to discern how disruptions in cell signaling affect immune, vascular, and hormone systems, and ultimately, contribute to endometriosis.

AUTHOR CONTRIBUTIONS

WM performed literature search for and wrote the final draft of this manuscript. AG wrote a section for the first draft of this manuscript. FM, WM, and AG contributed to the conceptualization of this manuscript, manuscript revision, read, and approved the submitted version. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the National Science Foundation Graduate Research Fellowship Program under Grant No. DGE1746891 (to WM) and the National Institutes of Health Medical Scientist Training Program under Grant No. T32GM136577 (to AG).

ACKNOWLEDGMENTS

The authors would like to thank Andrianna Ayiotis, Mark Iskarous, Inez Lam, and Julie Shade for their constructive feedback on this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/frph.2021.699133/full#supplementary-material

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frontiers in Reproductive Health

REVIEW
published: 21 December 2021
doi: 10.3389/frph.2021.779979

Menstrual Fluid Factors Mediate Endometrial Repair

*Lois A. Salamonsen**

Centre for Reproductive Health, Hudson Institute of Medical Research and Department of Molecular and Translational Science, Monash University, Clayton, VIC, Australia

Menstruation is a process whereby the outer functionalis layer of the endometrium is shed each month in response to falling progesterone and estrogen levels in a non-conception cycle. Simultaneously with the tissue breakdown, the surface is re-epithelialized, protecting the wound from infection. Once menstruation is complete and estrogen levels start to rise, regeneration progresses throughout the proliferative phase of the cycle, to fully restore endometrial thickness. Endometrial repair is unique compared to tissue repair elsewhere in the adult, in that it is rapid, scar-free and occurs around 400 times during each modern woman's reproductive life. The shedding tissue and that undergoing repair is bathed in menstrual fluid, which contains live cells, cellular debris, fragments of extracellular matrix, activated leukocytes and their products, soluble cellular components and extracellular vesicles. Proteomic and other analyses have revealed some detail of these components. Menstrual fluid, along with a number of individual proteins enhances epithelial cell migration to cover the wound. This is shown in endometrial epithelial and keratinocyte cell culture models, in an *ex vivo* decellularized skin model and in pig wounds *in vivo*. Thus, the microenvironment provided by menstrual fluid, is likely responsible for the unique rapid and scar-free repair of this remarkable tissue. Insight gained from analysis of this fluid is likely to be of value not only for treating endometrial bleeding problems but also in providing potential new therapies for poorly repairing wounds such as those seen in the aged and in diabetics.

Keywords: menstrual fluid, endometrial repair, Re-epithelialization, scar-free repair, endometrium

OPEN ACCESS

Edited by:
Philippa Saunders, University of Edinburgh, United Kingdom

Reviewed by:
Jan Brosens, University of Warwick, United Kingdom
Jacqueline Maybin, University of Edinburgh, United Kingdom

***Correspondence:**
Lois A. Salamonsen
lois.salamonsen@hudson.org.au
orcid.org/0000-0003-0558-7325

Specialty section:
This article was submitted to Gynecology, a section of the journal Frontiers in Reproductive Health

Received: *20 September 2021*
Accepted: *15 November 2021*
Published: *21 December 2021*

Citation:
Salamonsen LA (2021) Menstrual Fluid Factors Mediate Endometrial Repair. Front. Reprod. Health 3:779979. doi: 10.3389/frph.2021.779979

INTRODUCTION

The endometrium, the inner lining of the uterus, provides the maternal support for embryo development and undergoes remarkable remodeling on a cyclical basis. In humans and a few other species including old world primates, the endometrium develops more extensively during each menstrual cycle than in other placental mammals. In particular the process of decidualization, which provides the basis for the decidua of pregnancy, is initiated whether or not the cycle is one in which conception occurs. As this process is not reversible, the endometrium is shed in the process known as menstruation, then fully repaired and reconstructed during the subsequent cycle. Importantly, in contrast to most adult wounds, the endometrium repairs very rapidly (days) without scarring (otherwise seen only in fetal tissues) and occurs over 400 times during a woman's reproductive lifespan. Most aspects of menstruation are discussed elsewhere in this volume. The focus of this article is the role of menstrual fluid (MF) in the scar-free repair of the endometrium.

Menstruation: Endometrial Shedding

Understanding the cellular and molecular events of menstruation provides insights critical to understand the likely composition of menstrual fluid.

During menstruation, the outer functional layer of the endometrium is shed in a piecemeal manner, with breakdown and rapid repair occurring simultaneously at adjacent sites (1, 2). Shedding is finely controlled so that while most or all of the functional layer is shed, the basalis remains *in situ*. re-epithelialization occurs rapidly but it is from the basalis that the full thickness endometrium subsequently regenerates (3). What is not yet known is the mechanism preventing degradation of the basalis although it has been proposed that the horizontal network of glands forming a "rhizome-like" layer may be limiting (4).

Menstruation is considered to be a highly regulated inflammatory response to progesterone withdrawal. Initiation of menstrual events occurs in the decidualized endometrial stromal cells, which express progesterone receptors and hence sense hormone withdrawal. These cells initiate a sequence of inter-dependent inflammatory events including nuclear translocation of NF-κB, a transcription factor that regulates both innate and adaptive immune responses. NF-κB signaling induces the progressive production of many inflammatory cytokine and chemokine mediators, increased prostaglandin synthetic enzymes and production of pro-inflammatory prostaglandins (5). The released chemokines recruit and activate leukocytes (predominantly granulocytes) into the endometrium. Variable numbers and types of immune cells are present in the functional layer throughout the menstrual cycle, but are in low abundance until the pre-menstrual stimulus that initiates a massive and highly selective influx of leukocytes. Perimenstrually, these comprise up to 50% of the total cells population. 6–15% of all nucleated cells in the stromal compartment of the functional endometrial layer are neutrophils, the same abundance as for macrophages ($CD16^+$) and uterine natural killer (NK) cells ($CD56^+/CD16^-$) but eosinophils, mast cells (both 3–5%) and *T* lymphocytes (1–2%) are less common [review; (6)]. Importantly, these cells are phenotypically different from their counterparts in peripheral blood indicating the effects of the local microenvironment. For example, production of active elastase is much reduced and alpha1-anti-trypsin highly elevated in endometrial neutrophils compared with peripheral blood neutrophils (6). Together these leukocytes, many detectable in activated forms, establish an inflammatory cascade which results in tissue breakdown. These complex phenotypic changes in a highly dynamic physiological setting in women, severely limit investigation of their individual functions. It is also possible that cellular senescence, particularly of the decidualized stromal cells, plays a role; this has been recently described in human endometrial assembloids (7) but any contribution to menstruation remains to be established.

Importantly for the tissue breakdown at menstruation, each non-migratory cell in the functionalis epithelial, stromal, decidualized stromal cells (5, 8) also directly or indirectly responds to progesterone withdrawal by releasing an array of proteolytic enzymes including matrix metalloproteinases, plasminogen activator family members and other molecules, with considerable interactions occurring that initiate self-activating cascades. These can be between products of both resident cell and leukocytes. For example, *in vivo*, endometrial-derived immune cells produce a wide range of enzymes important for other cell activation (e.g., degranulation of eosinophils induced by neutrophil elastase), or molecular processing such as conversion of latent to active matrix metalloproteinases by elastase or cathepsin G. These combined actions result in degradation of the extracellular matrix (ECM) and tissue breakdown (9, 10). Tissue shedding during menstruation is piecemeal; fragments of endometrial tissue can be found in menstrual fluid (MF) along with single endometrial cells, blood, and ECM debris.

Endometrial Repair

Endometrial repair is uniquely scar-free, is initiated almost immediately as shedding starts and is complete by the time bleeding ceases (up to 8 days) (11). Degrading tissue and re-epithelializing sites are seen adjacent to one another in the menstruating tissue by histology and scanning electron microscopy (1, 2) and once bleeding ceases (when re-epithelialization is complete), regeneration of the entire tissue thickness is initiated. The rapid re-epithelialization serves to protect the tissue from bacterial invasion as it regenerates. Initially, re-epithelialization is observed as migration of epithelial cells from the exposed stumps of glands and these can be seen by scanning electron microscopy to expand outwards to meet similar cells from other glands or those migrating from any intact remaining epithelium (2, 12). Repair of damage to transverse sub-epithelial endometrial arterioles within the stroma and to spiral arterioles, which can be severely injured during tissue breakdown, occurs concomitant with re-epithelialization. However, full regeneration of the endometrium is primarily if not entirely from stem/progenitor cells present in the basalis layer (which is not shed). This regeneration requires estrogen action, and is complete by the time of ovulation, $\sim$ 14 days after the start of menstruation, and 9 days following cessation of bleeding and full re-epithelialization (13). Data has indicated that mesenchymal to epithelial cell differentiation (EMT) contributes to restoration of the luminal epithelium at least in mice (14, 15); however, recent *in vivo* cell fate-tracing studies in mice have found no evidence for EMT in endometrial repair [(16), reviewed in (13)]. Evidence for an EMT contributing to human endometrial repair should be further examined.

Most adult wounds repair with scar formation, which may impair function and inhibit further growth whereas repair of the endometrium (and of wounds in fetal tissue), is scar free. There are also differences between wounds in the adult oral cavity and elsewhere in the body (17), largely due to unique mediators in saliva. Wound healing in general involves a complex interplay between numerous cell types, cytokines, mediators and the vascular system. Wounding in all tissues is accompanied by an influx of inflammatory cells, starting with neutrophils and their local release of chemokines that attract other leukocytes to the wound site. These cells together release a range of mediators and cytokines that promote re-epithelialization, angiogenesis and thrombosis. The fibroblasts in turn secrete ECM components that provide scaffolding for

the cellular events (18). The scar tissue that forms in most adult tissues, results from the formation and extension of fibrous tissue (fibrogenesis) derived primarily from stromal cells (19). However, the healed endometrium is without obvious fibrosis. Furthermore, while repair of most wounds takes 4–6 weeks, repair of the endometrial surface is generally complete within 5 days.

Since in most tissues, stromal cells are the major effectors of scarring, it must be assumed that endometrial stromal cells derived from stem/progenitor cells during endometrial regeneration are differently programmed. Given that re-epithelialization to cover the endometrial surface is so rapid, the stem/progenitor cells are likely brought into play more quickly than in other tissues. Interestingly, transforming growth factor (TGF)β1, a factor that strongly promotes the myofibroblast phenotype, is elevated in menstrual fluid compared with peripheral blood and could theoretically act on endometrial stromal cells *in vitro* to differentiate them into myofibroblasts (19). Since this does not occur, it must be that *in vivo*, either the TGFβ1 must be non-functional, or other regulatory stimuli must prevent such differentiation to prevent scarring. Importantly, menstrual fluid, added to cultures of stromal cells of adipose and dermal origin, suppresses their transition to myofibroblasts as it does with endometrial stromal cell cultures, supporting this contention (19). However, the active suppressive factor/s remain to be identified.

Uterine Fluid and Menstrual Fluid

Given that endometrial repair occurs rapidly within a microenvironment of menstrual fluid, evidence is now emerging that menstrual fluid contains bioactive factors that promote scar-free repair. What is currently known of these components and their potential actions will be discussed below. However, it is important to set the scene by first considering the composition of uterine fluid which changes throughout the menstrual cycle.

Uterine Fluid

During the menstrual cycle, a small volume of fluid is always present within the uterine cavity and its components (both soluble and extracellular vesicles) vary between the proliferative and secretory phases and between fertile and infertile women. A number of major soluble components that appear in uterine fluid even outside of menses, are transudated from blood, although this is very selective. Differential protein composition between peripheral blood and uterine fluid was first shown clearly in the 1980's with the advent of two-dimensional gel (2D-DIGE) analyses (20, 21). These studies highlighted proteins specific to uterine fluid and identified differences in fluid composition between the proliferative and secretory phases. Subsequently, 2D-DIGE identified a number of major serum proteins in uterine fluid (human serum albumin, transferrin, immunoglobulins (Ig)G and A, antitrypsin, haptoglobin and hemoglobin), which could be removed prior to further analyses, improving sensitivity for subsequent analysis of the remaining soluble factors (22–24). Such depletion at least doubled the number of proteins that could be identified (24) and which differed between receptive and non-receptive states in fertile and infertile women (23).

Other soluble components of uterine fluid are contributed from peritoneal and tubal fluids, and from endometrial epithelial cell secretions, particularly those of the glands. Uterine fluid proteins, including cytokines and chemokines have been examined using protein array technologies applied to samples taken across the menstrual cycle and between fertile and infertile women (22, 23, 25–27), with >30 cytokines, chemokines and growth factors being identified. These include interleukin (IL)-1β, IL-6, IL-12, IL-17, IL-18, tumor necrosis factor (TNF)α, macrophage migration inhibitory factor (MIF), eotaxin, monocyte chemotactic protein (MCP) 1, interferon-gamma (IFNγ)-inducible protein-10, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF)-AA and chemokine (C-X-C motif) ligand 1–3, all of which are detectable in >90% of samples.

There is no correlation between amino acid concentrations in serum and uterine fluid: 18 amino acids have been identified in human uterine fluid, their concentrations being altered by maternal diet. These include asparagine, histidine, serine, glutamine, valine, isoleucine, and leucine (28). In addition, lipids, a range of metabolites [review; (29)], miRNAs [review; (30)], and small extracellular vesicles (sEV) previously known as exosomes (3, 31, 32), have been identified and/or harvested from uterine fluid obtained from cycling women. These sEVs contain a cohort of miRNAs and proteins, with changes in their proteomes being defined between cycle phases (3, 31).

Menstrual Fluid

Menstrual fluid (MF) is most often harvested into menstrual cups, a relatively non-invasive and convenient method that can be managed at home. The most common time of collection is on the second day of menstruation, when menstrual flow is maximal. Importantly, MF collection using a menstrual cup has proven to be highly reliable and reproducible between women and between cycles (33). Among the components of MF are debris from tissue breakdown, live cells or groups of cells (epithelial, stromal, vascular) released when the surrounding extracellular matrix (ECM) is degraded, activated leukocytes and their products, endometrial stem cells and extracellular vesicles.

Since menstruation is a controlled inflammatory event, resulting in tissue breakdown, MF would be expected to contain many more soluble components than the uterine fluid of the late secretory phase, which immediately precedes menstruation. The additional soluble molecules will be derived from many cellular sources, including endometrial and immune cells (particularly the neutrophils, macrophages, uterine NK and mast cells, that are abundant in the tissue during menstruation), along with intracellular components of degraded cells. These are likely also to contribute to endometrial repair (summarized in **Figure 1**), a concept supported by reduced uNK cell numbers in late secretory tissue in women with heavy menstrual bleeding (34).

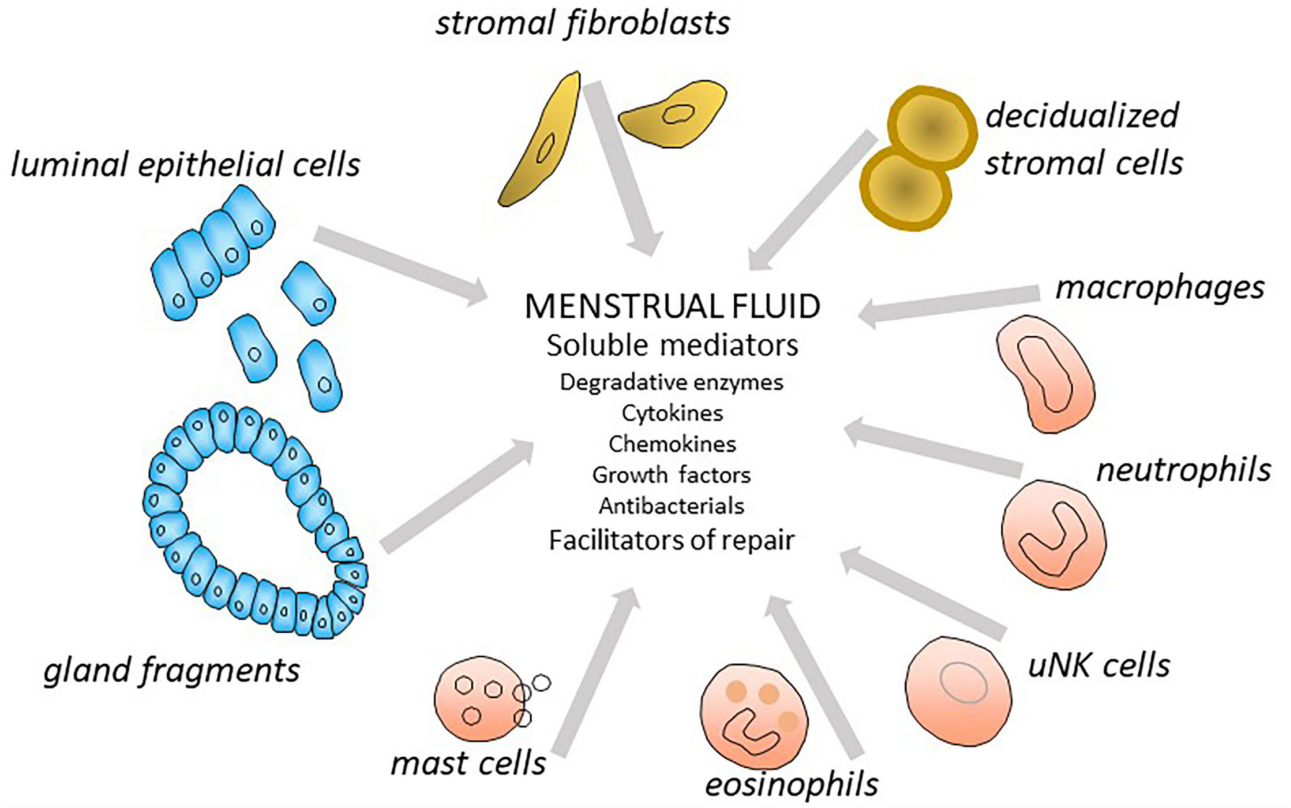

FIGURE 1 | The likely cellular origins of soluble components of menstrual fluid.

Soluble Components of Menstrual Fluid

Soluble Contributions From Non-migratory Endometrial Cells

Soluble components of menstrual fluid have been studied in much less detail than those of uterine fluid. Early studies arose from the need to understand how menstrual bleeding is regulated. Uterine haemostasis differs from that in other organs, in that the endometrial haemostatic plugs are morphologically different and very short lived (35, 36). Coagulation and fibrinolytic proteins measured in menstrual fluid supernatant on days 1 and 2 of normal menstruation, showed a virtual absence of thrombin-generating activity and very much higher levels of fibrin-related antigen, active plasmin and plasminogen activator than seen in normal serum, while functionally active α-2-antiplasmin was undetectable (37). Disappointingly this data did not provide a pointer to the mechanisms controlling menstrual blood loss. However, prostaglandins (PG) $F_2\alpha$ and E_2, are also present in menstrual fluid, and levels correlate directly with menstrual blood loss (38). Furthermore, a lack of detectable 9-ketoreductase or 9-hydroxydehydrogenase activity indicates there could be interconversion between the two PGs.

Most reproductive hormones do not show a difference between peripheral venous blood and menstrual plasma. However, while menstrual follicle stimulating hormone (FSH), estradiol-17β and progesterone are likely to arise entirely from the peripheral circulation, prolactin (PRL) levels are elevated only in menstrual blood (39), indicating release into the menstrual flow from mid-late secretory endometrium, where PRL is expressed both in the epithelium and in the decidualized stroma (40).

Recent application of state-of-the-art proteomics techniques (41, 42) to supernatant from centrifuged menstrual fluid (menstrual plasma) vs. matched peripheral plasma have provided extensive lists of proteins specific to menstrual fluid. While both investigators depleted the samples of abundant serum proteins, Evans additionally enriched samples using a combinatorial peptide ligand library (CPLL), along with capture of heparin and fibronectin binding proteins separately on the depleted samples. The most abundant identified proteins from these two studies are listed in **Table 1**. Many reflect the state of the endometrium in the late secretory phase when pre-decidual changes are evident with concomitant changes in protein production, including insulin growth factor binding protein (IGFBP)-1, matrix metalloproteinase (MMP)9, galectin 3, glycodelin A, glucose transporter (GLUT)1, IL1 and others (24). However, additional proteins not present before menstruation were identified, reflecting induced protease activity from epithelial and decidualized stromal cells, and factors derived from cells released upon tissue lysis (41). VEGF was also significantly elevated in menstrual fluid vs. peripheral plasma.

TABLE 1 | Proteins elevated in menstrual fluid vs. peripheral plasma and known to facilitate repair.

Protein name	Known as	Previously known actions in repair	References
Neutrophil gelatinase -associated lipocalin/lipocalin-2	NGAL	Promigratory in epithelial cells	*(43)*
Epidermal fatty acid binding protein-5	FABP-5	Augments peroxisome proliferator-activator receptor δ in promoting proliferation and survival	*(44)*
Follistatin-related protein 1	FSTL-1	See-saw regulation in wounded skin – inverse relationship with miRNA-198	*(45)*
Macrophage migration inhibitory factor	MIF	Proposed pro and anti-repair actions in skin, probably due to different skin models tested.	*(46, 47)*
Secretory leukocyte protease inhibitor	SLPI	Various roles in cell migration depending upon the system	*(24, 48)*
Human epididymis protein 4	HEP4	migration	*(49)*
S100 proteins	S100A8 S100A9 S100A11	Promote cell migration, but not proliferation Cell motility	*(50, 51)*
Lactotransferrin/lactoferrin	LTF	Promotes skin repair	*(52, 53)*
Stanniocalcin-1	STC1	Angiogenesis	*(54)*
Ninjurin-2	NINJ2	Adhesion protein, promotes cell growth	*(55)*
Neuroblast differentiation-associated protein	AHNAK	migration	*(56)*
Osteopontin	OPN	Cell survival, proliferation, migration	*(57)*
Galectin	Gal1 Gal3	Migration, proliferation Repair	*(58, 59)*
Macrophage inhibitory factor	MIF	Pro-inflammatory antibacterial	*(60)*
Interleukin 8	IL8	Attracts and activates neutrophils	*(60)*
Vascular endothelial growth factor A	VEGF-A	Neo-angiogenesis, Re-epithelialization	*(61)*

Data derived from (41).

More recently, using a custom magnetic Luminex assay, common inflammatory and repair proteins: secretory leukocyte protein inhibitor (SLPI); lipocalin-2 (NGAL); lactoferrin; follistatin-like 1 (FSTL1); and human epididymis protein-4 (HE4), were identified in >60% of menstrual fluid samples analyzed (62), reflecting their previous recognition (41). Interestingly, a negative association between menstrual fluid volume and abundance of some of these proteins (HE4, galectin-1, MIF, SLPI, NGAL, and FSTL1) was revealed following normalization for total menstrual fluid protein (ng/mg). It may be that as menstrual fluid volume increases, other endometrial- or peripheral-derived proteins may similarly increase, thus diluting the proteins of particular interest. Interestingly, many of the menstrual fluid factors listed in **Table 1**, positively cross reference with those in Senequest (https://Senequest.net/) which contains factors involved in cellular senescence.

Matrix degrading enzymes, including a number of matrix metalloproteinases (MMP), are major players at menstruation, and are released from endometrial epithelial and decidualized stromal cells specifically as progesterone levels fall and also from activated leukocytes (see below). These are accompanied by the release of potential activators and tissue inhibitors of MMPs (TIMPs) which are abundant in endometrial tissue and which together tightly control MMP actions (63, 64). Although only MMP9 was identified in a proteomic analysis of menstrual fluid (41), menstrual serum showed a pattern of MMP activity on zymography different from that of peritoneal fluid while both MMP-7 and MMP-9 were identified by Western blot uniquely in menstrual serum (65). While MMPs are very important for tissue breakdown, they also play roles in tissue repair largely due to their broad protease activities not related to matrix degradation. Some but not all actions on repair, have been validated in individual genetically-modified mouse models including those null for MMP1, MMP8, MMP9, MMP10, and MMP14 (66). In other repair situations, epithelial-derived MMPs facilitate cell migration by affecting cell-matrix adhesion. For example, in mucosal epithelia, MMP7 facilitates re-epithelialization by cleaving different ECM or ECM-associated proteins to affect integrin: matrix adhesion (66). Indeed MMP7-deficient mice have the most impairment of re-epithelialization of any MMP-null mice generated to date and show disturbance of the affinity of integrin $\alpha_2\beta_1$ cell-matrix interactions (67). In human endometrium MMP7 mRNA is highly increased in epithelial and decidual cells at menstruation and remains throughout the new proliferative phase indicating a role in repair and regeneration (68). Active MMP7 is recruited to the plasma membrane of epithelial cells, thus escaping TIMP inactivation and allowing processing of membrane-associated growth factors needed for epithelial repair and proliferation (69). The involvement of MMPs in endometrial repair remains to be determined.

Soluble Contributions From Immune Cells

Mononuclear cells isolated from menstrual blood samples are phenotypically similar to the reported phenotype for biopsy-derived endometrial cells, and distinct from peripheral blood mononuclear cells [PBMC: (33)] although percentages of NK cells are higher and those of *T* cells are lower.

Macrophages, neutrophils, mast cells, and eosinophils, all degranulate upon activation, releasing their soluble contents. Importantly, active forms have been identified during menstruation by virtue of the extracellular immunostaining of granular contents (70–72). These granulocytes have more than one type of granule and there are many similarities in granule morphology, granule content, stimulus for degranulation, and granule trafficking, most of which are not well-understood, particularly in the context of the endometrium. However, it is clear that there is considerable overlap between contents of granules from different sources; for example, eosinophils, neutrophils and macrophages all release matrix metalloproteinase 9 at menstruation (70).

While there is a paucity of information on immune cell products in menstrual fluid that may be relevant to endometrial repair, elsewhere, eosinophils produce a number of growth factors, including TGF-α and -β, fibroblast growth factor (FGF), EGF, PDGF, and VEGF, which participate in angiogenesis and myocardial repair (73). Eosinophils also produce cytokines, in particular IL5 and IL4 which have roles in wound healing and macrophage differentiation. Indeed, mice overexpressing IL5 displayed insufficient production of ECM components and had impaired wound healing. IL4 is essential for differentiation of macrophages toward an M2 phenotype, and regulating myocardial tissue regeneration (74). Uterine NK cells from menstrual fluid produce IFNγ, granzyme B, and perforin, upon stimulation with IL2 and IL15 (33).

Neutrophils contribute to physiological tissue repair, and seem to be necessary for normal healing at least in part by promoting angiogenesis (75). Furthermore, apoptosis of neutrophils after degranulation provides a powerful stimulus for macrophage differentiation into the anti-inflammatory M2 phenotype, through their production of annexin A1, lipocalin, lactoferrin, and cathelicidin. Neutrophil-derived MMP12 also possesses potent pro-resolving properties (74). Anti-bacterial agents within menstrual fluid including lactotransferrin and NGAL may also play a role in post-menstrual endometrial repair.

Mast cell actions are likewise realized through degranulation and secretion of the granules' content of cytokines or production of lipid mediators, depending on the nature of the stimuli received during activation. Relevant to wound repair, during cardiac tissue re-modeling their main function appears to be associated with regulation of fibrous tissue metabolism (76), and they may both enhance and inhibit post-myocardial fibrosis. Their pro-fibrotic properties are mediated primarily by chymase (present in the uterus only in myometrial mast cells) and tryptase (present in endometrial mast cells) (72), which are identified as activators of TGFβ and angiotensin II, well-known promoters of fibroblast activity. Mast cells also produce and secrete anti-fibrotic mediators such as IL10, IL13, CXCL10, and VEGFA (76).

Endometrial Stem Cells in Menstrual Fluid

Cells with mesenchymal stem cell properties have been identified in menstrual blood. Following depletion of red blood cells and $CD45^+$ leukocytes from menstrual fluid, endometrial stem/progenitor cells including clonogenic endometrial cells, sushi domain containing-2^+ ($SUSD2^+$) mesenchymal stem cells and N-cadherin$^+$ ($NCAD^+$) epithelial progenitor cells, have been isolated (62, 77, 78), with limited variability across menstrual cycles (62). These cells are not present in peripheral blood. They are generally retrieved from the menstrual fluid as plastic adherent cells and show differences in immunophenotype, proliferation and differentiation capacities from bone marrow-derived mesenchymal stem cells. Since these cells can be reliably purified from menstrual fluid, they may provide a useful non-invasive source of stem/progenitor cells for clinical application. However, isolation protocols and culture conditions must be standardized to maximize their potential. Importantly a serum-free culture protocol has been established that contains a TGFβ receptor inhibitor, that prevents spontaneous differentiation, apoptosis, and senescence of the clonogenic $SUSD2^+$ population and enhances their potency (77).

Extracellular Vesicles in Menstrual Fluid

Small extracellular vesicles (sEV) previously termed exosomes, are released from all cells. They act as carriers of "cargo" of bioactive molecules including miRNA, proteins and lipids, which they deliver to specific target cells: the phospholipid membranes of the sEV protect the "cargo" from extracellular degradation. Importantly the proteomes of endometrial epithelial cell sEV depend upon the hormonal environment of the cells of their origin (estrogen or estrogen plus progesterone), but are substantially different from those of the cellular proteomes (79). Endometrial-derived sEV are present in uterine fluid (3, 31, 32), although their role in endometrial repair has not yet been examined. A number of pre-clinical studies have evaluated effects of sEVs on the wound-healing process [review; (80)]. For example, in a mouse burn model, sEV derived from human menstrual blood -derived mesenchymal stem cells injected close to the site of injury, enhanced wound closure and increased neoangiogenesis was evident (81). Furthermore, sEV derived from human umbilical cord blood mesenchymal stem cells stimulate regenerative wound healing via TGFβ receptor inhibition (82). Likewise, EVs derived from normal resident lung epithelial cells, appear to possess anti-fibrotic properties, inhibit TGFβ-WNT cross talk and offer a promising anti-fibrotic treatment (83). If similar mediators are contained in sEV in menstrual fluid, this could provide an explanation for the lack of scarring during endometrial repair.

Functional Analyses of Menstrual Fluid and Its Soluble Components

The most difficult task following omics analyses is subsequent determination of the likely functions of the large number of identified molecules. In the context of menstrual fluid proteomics, Evans et al., (41), examined actions of the entire soluble fraction of menstrual fluid in a number of biologically relevant repair models. Subsequently, individual fluid molecules,

selected for their known function in wound repair were applied at concentrations selected from their measured concentrations in menstrual fluid. Endometrial cell cultures (ECC1 cell line) and keratinocyte cell cultures (HaCatT cell line) were chosen for these studies for their similarities to primary endometrial epithelial and primary keratinocytes. Classic wounding assays were applied to overconfluent cultures which were "wounded" by vacuum suction followed by imaging and analysis using imaging software daily for 3 days. Real-time label-free analysis of adhesion and proliferation using the xCelligence system (ACEA Biosciences, San Diego CA, USA) was also applied to cultures of similarly treated ECC-1 and HaCatT cells. In more physiological assays, *ex vivo* de-epidermized dermis (DED) pre-parations (84) were prepared and cultured for 4 or 8 days in the presence or absence of menstrual fluid. The area of migration of keratinocytes across the DED was quantified, then tissues were processed and embedded for histological examination and the thickness of both cornified and cellular layers were measured. Finally, *in vivo,* a porcine superficial wound model in juvenile pigs, in which the wounds were created by dermatome, was treated with wound dressings containing either peripheral plasma or menstrual fluid. Dressings were changed at days 3 and 5, with simultaneous imaging and quantification of re-epithelialization until day 7 when healing in such juvenile models is generally complete. By 5 days, re-epithelialization was significantly enhanced; wound area was slightly decreased; epidermal thickness was moderately increased; and number of hairs per-section was moderately increased. The latter three did not reach significance in the limited number of wounds approved by the ethics committee.

An important conclusion from these investigations is that factors in menstrual fluid advance the initial migratory phase of healing, a key difference from current skin repair treatments that stimulate epithelial proliferation, while vascular repair agents in menstrual fluid (including VEGF) will likely play roles in the initial repair of the vasculature and subsequent angiogenesis (41). Regrettably, this study did not include menstrual fluid factors with no known role in repair processes. These remain to be tested and may prove a potential "gold-mine" of new treatments for wound repair.

Endometrial Vascular Repair

Simultaneously with re-epithelialization, rapid repair of the open blood vessels must take place to stop the bleeding, some 5 days after menstruation starts. While experimental evidence for angiogenesis at this time of the cycle is lacking, circumstantial evidence indicates its likelihood. Menstrual fluid contains the potent angiogenic factor VEGF-A (85), (41), and this is markedly reduced in women with menorrhagia (85). Interestingly, in the rhesus macaque, a naturally menstruating primate, blockage of VEGF action with VEGF Trap, a potent VEGF blocker inhibited new blood vessel development and re-epithelialization of the denuded surface during menstruation (61). Further in a mouse model of menstruation, similar blockade of VEGF action delayed repair of the denuded endometrial surface and inhibited new blood vessel development (61). HIF-1α, a transcription factor known as the master regulator of the cellular response to hypoxia, can regulate VEGF by directly binding the VEGF pro-motor at least in macrophages (86) and thus hypoxia, may thus contribute to endometrial vascular repair. Indeed, women with prolonged menstrual bleeding have decreased endometrial HIF-1α during menstruation and the long bleeding period that follows. However, evidence for the presence of hypoxia during menstruation and repair is mixed (87), review:

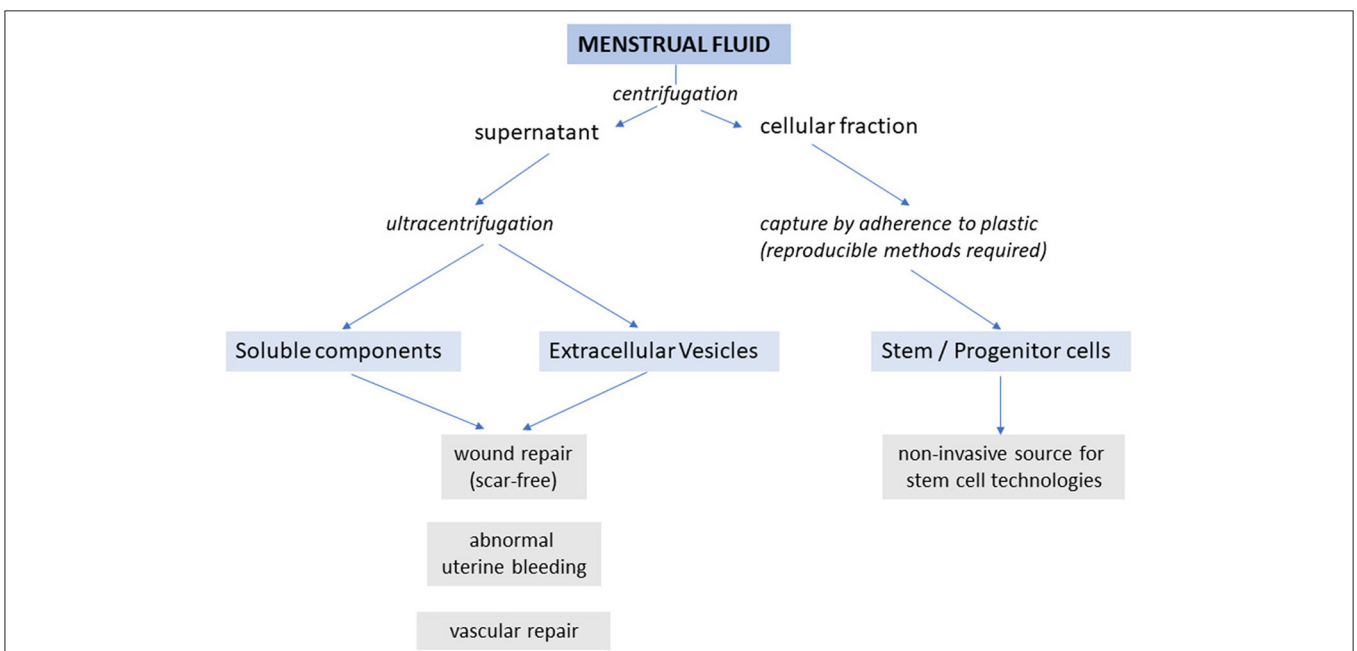

FIGURE 2 | Schematic showing potential applications of components of menstrual fluid.

(88), and any role of HIF-1 in endometrial repair needs to be confirmed.

Endometrial Regeneration After Menses

By the time menses has ceased, the wounded surface is essentially "repaired", covered by a new luminal epithelium with junctional complexes making a tight protective surface, that shields the underlying tissue from infection. During the next 10 or so days (cycle proliferative phase) and as estrogen levels rise, endometrial thickness and the full cohort of cellular structures, including glands, stroma, vascular structures and extracellular matrix (both interstitial and basal lamina) is regenerated through massive cellular proliferation in the growing functional layer. Current knowledge of this regeneration process has recently been detailed (13) and will not be further discussed here.

CONCLUSIONS

Rapid scar-free repair of the endometrium following menstruation is essential given that it occurs some 400 times during most women's reproductive lives and provides the basis for the subsequent regeneration and differentiation of the endometrium and its attainment of receptivity for embryo implantation in the new cycle. It is now indisputable that the microenvironment provided by menstrual fluid drives effective endometrial repair. Recent advances in analyses of menstrual fluid components has provided some insight but just which are the most important factors, soluble or those delivered to the damaged surface in extracellular vesicles, remains to be determined. Importantly menstrual fluid has the potential to provide factors for scar-free rapid wound repair, and to treat abnormal uterine bleeding (**Figure 2**).

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

FUNDING

LS and her laboratory have been funded extensively by Fellowships, Program and Project grants from the Australian National Health and Medical Research Council. Research at the Hudson Institute of Medical Research is funded in part by a State Government of Victoria Infrastructure support program.

ACKNOWLEDGMENTS

The author thanks all her colleagues and doctoral students over the past 30 years, who have contributed to building up the knowledge summarized herein. In particular, the ground-breaking work of Dr. Jemma Evans for proteomic analysis of menstrual fluid and functional studies of individual proteins, Dr. David Greening's collaboration on endometrial EVs, and Dr. David Woolley whose collaboration and insights first introduced me to the field of matrix metalloproteinases and leukocyte contributions to tissue re-modeling.

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frontiers in Reproductive Health

REVIEW
published: 23 December 2021
doi: 10.3389/frph.2021.804921

The Role of Decidual Subpopulations in Implantation, Menstruation and Miscarriage

Joanne Muter[1,2]*, *Chow-Seng Kong*[1] *and Jan J. Brosens*[1,2]

[1] *Division of Biomedicine, Warwick Medical School, University of Warwick, Coventry, United Kingdom,* [2] *Tommy's National Centre for Miscarriage Research, University Hospitals Coventry and Warwickshire NHS Trust, Coventry, United Kingdom*

In each menstrual cycle, the endometrium becomes receptive to embryo implantation while preparing for tissue breakdown and repair. Both pregnancy and menstruation are dependent on spontaneous decidualization of endometrial stromal cells, a progesterone-dependent process that follows rapid, oestrogen-dependent proliferation. During the implantation window, stromal cells mount an acute stress response, which leads to the emergence of functionally distinct decidual subsets, reflecting the level of replication stress incurred during the preceding proliferative phase. Progesterone-dependent, anti-inflammatory decidual cells (DeC) form a robust matrix that accommodates the conceptus whereas pro-inflammatory, progesterone-resistant stressed and senescent decidual cells (senDeC) control tissue remodelling and breakdown. To execute these functions, each decidual subset engages innate immune cells: DeC partner with uterine natural killer (uNK) cells to eliminate senDeC, while senDeC co-opt neutrophils and macrophages to assist with tissue breakdown and repair. Thus, successful transformation of cycling endometrium into the decidua of pregnancy not only requires continuous progesterone signalling but dominance of DeC over senDeC, aided by recruitment and differentiation of circulating NK cells and bone marrow-derived decidual progenitors. We discuss how the frequency of cycles resulting in imbalanced decidual subpopulations may determine the recurrence risk of miscarriage and highlight emerging therapeutic strategies.

Keywords: endometrium, implantation, miscarriage, decidualization, senescence, innate immunity, menstruation

OPEN ACCESS

Edited by:
Philippa Saunders,
University of Edinburgh,
United Kingdom

Reviewed by:
Gendie Lash,
Guangzhou Medical University, China
Ripla Arora,
Michigan State University,
United States

***Correspondence:**
Joanne Muter
j.muter@warwick.ac.uk

Specialty section:
This article was submitted to Gynecology, a section of the journal Frontiers in Reproductive Health

Received: *29 October 2021*
Accepted: *07 December 2021*
Published: *23 December 2021*

Citation:
Muter J, Kong C-S and Brosens JJ (2021) The Role of Decidual Subpopulations in Implantation, Menstruation and Miscarriage. Front. Reprod. Health 3:804921. doi: 10.3389/frph.2021.804921

INTRODUCTION

The human endometrium is defined by its ability to execute opposing functions, often simultaneously, and to transition seamlessly between different physiological states. For example, menstrual shedding occurs in parallel with activation of repair mechanisms (1, 2), optimal fertility depends on the receptive endometrium engaging in embryo selection and rejection (3), and pregnancy requires transformation of a cycling mucosa into a robust, semi-permanent matrix capable of accommodating the placenta throughout gestation (4). The remarkable capacity of the human endometrium to switch effortlessly between states and functions on a cyclical basis is shared only with a handful of other menstruating mammals, including higher primates, some species of bats, and the elephant shrew (5, 6). Menstruating mammals share several other reproductive characteristics, such as spontaneous ovulation, a hemochorial placenta characterised by deep invasion of the maternal arteries by placental trophoblast, and birth of only 1 or 2

well-developed offspring per pregnancy (6). Further, three distinct features set the non-pregnant endometrium of menstruating species apart from other mammals: rapid proliferation and tissue growth, accumulation of uterine natural killer (uNK) cells, and spontaneous decidualization of stromal cells (5).

Decidualization of endometrial stromal cells is a multistep differentiation programme that starts with an evolutionarily conserved acute cellular stress response (7), which results after several days in the emergence of specialist decidual cells (4, 8). In pregnancy, decidual cells cooperate with local immune cells to form a specialist matrix for controlled trophoblast invasion and placenta formation (9, 10). Decidualization occurs in all mammalian species where implantation involves breaching of the luminal endometrial epithelium by the conceptus, but the extent of the decidual reaction varies markedly and correlates to the depth of trophoblast invasion in each species (11). Importantly, decidualization and accumulation of uNK cells depend on signals emanating from the implanting embryo in most mammals (12). However, in menstruating species both processes are initiated in each cycle, irrespective of an implanting embryo (5, 6). As we will discuss later, this switch from embryonic to maternal control over the decidual process not only accounts for the evolution of menstruation but also bequeaths the endometrium with a robust mechanism to reject low-fitness embryos. In human endometrium, the initial decidual stress response coincides with the opening of the midluteal implantation window, whereas the emergence of morphologically differentiated decidual cells (DeC), characterised by abundant cytoplasm and enlarged nuclei, heralds the closure of the 4-day implantation window (4).

The endometrium is often viewed as an effector tissue solely under the control of the rise and fall in ovarian oestrogen and progesterone production and, consequently, capable of carbon-copying itself from cycle-to-cycle. The discovery that endometrial regeneration and tissue homeostasis are critically dependent on bone marrow-derived, non-hematopoietic progenitor cells and innate immune cells has all but torpedoed this historical misconception (13–17). Further, novel technologies, such as single-cell RNA-sequencing and endometrial organoid models, are revealing how oestrogen (E2)-dependent proliferation during the follicular phase controls the specification of endometrial epithelial and stromal cells into different subpopulations with distinct functions following ovulation (8, 18, 19). Here, we summarise recent insights into how distinct decidual subpopulations bestows the endometrium with the ability to become receptive, but also selective at implantation, to trigger menstrual breakdown while activating repair mechanisms, and to transition successfully into a gestational tissue. Furthermore, we highlight the importance of dyshomeostasis of decidual subpopulations in recurrent miscarriage and touch upon the ensuing therapeutic opportunities.

ONTOGENESIS OF SPONTANEOUS DECIDUALIZATION

Mammalian genes are highly conserved. Consequently, evolution relies largely on changes in non-coding, regulatory sequences that alter gene expression (20). A major mechanism driving reproductive diversity in mammals involves incorporation of transposable elements (TEs) in regulatory DNA sequences, which in turn leads to rewiring of signal transduction pathways and transcription factors (TFs) to drive expression of novel gene networks. TEs comprise a vast array of DNA sequences that can, or could, move to new sites in genomes, either by a "cut-and-paste" mechanism (transposons) or through "copy-and-paste" RNA intermediates (retrotransposons). In contrast to genes, TEs are highly variable and frequently species-specific (20). Colonisation of mammalian genomes with *MER20*, a 'cut-and-paste' DNA transposon, coincided with the emergence of decidualization and invasive placentas. *MER20* elements encode 13% of the putative enhancers of genes that gained expression in DeC of eutherian (placental) mammals, including higher primates and humans (21, 22). Further, emergence of menstruation in the primate lineage occurred in parallel with genomic integration of *Alu* retrotransposons harbouring a triple TF binding motif (oestrogen receptor-, basic leucine zipper-, and PAX domain-binding sequences) (23), indicating that TEs also governed the evolution of spontaneous decidualization. Based on comparative transcriptomics, hundreds of genes have now been identified in pregnant endometrium that were gained or lost in primate and human lineages (24). Emerging evidence suggests that decidual genes recruited recently in the human lineage play a disproportionate role in prevalent pregnancy disorders, including early pregnancy loss and preterm birth (24).

Despite these genomic adaptations, human endometrial stromal cells are not intrinsically capable of differentiating into DeC. Instead, spontaneous decidualization in response to hormonal signalling is an endometrial trait that emerges at some point after the menarche. For example, the endometrium in most term foetuses and neonates is only weakly proliferative, despite prolonged exposure to very high concentrations of unbound estrogens and progesterone *in utero*. While secretory changes in endometrial glands can be observed occasionally at birth, decidual or menstrual changes are rare (25). These findings from post-mortem studies are corroborated by the observation that overt neonatal uterine bleeding, defined as menstruation-like bleeding triggered by a rapid fall in circulating sex hormones of maternal origins, affects only 4–5% of female babies during the first week of life (26). Progesterone responsiveness of the endometrium becomes established after prolonged E2-dependent growth of the uterus, which starts before breast development in pre-pubertal girls and continuous after the menarche (27). The dependency of spontaneous decidualization on E2-dependent hyperproliferation is also apparent in the spatial organisation of this process in cycling endometrium. Following menstruation, proliferation of glandular epithelial and stromal cells accelerates with increasing distance from the endometrial-myometrial interface and peaks on cycle day 10 in the upper one third of the superficial endometrial layer (**Figure 1A**) (28). This positional proliferative response has been linked to presence of lymphoid aggregates residing in the basal endometrial layer (29–31), which purportedly secrete interferon gamma (IFN-γ), a potent inhibitor of steroid hormone responses (32). Thus, as the endometrium grows beyond the local IFN-γ gradient, cellular proliferation accelerates

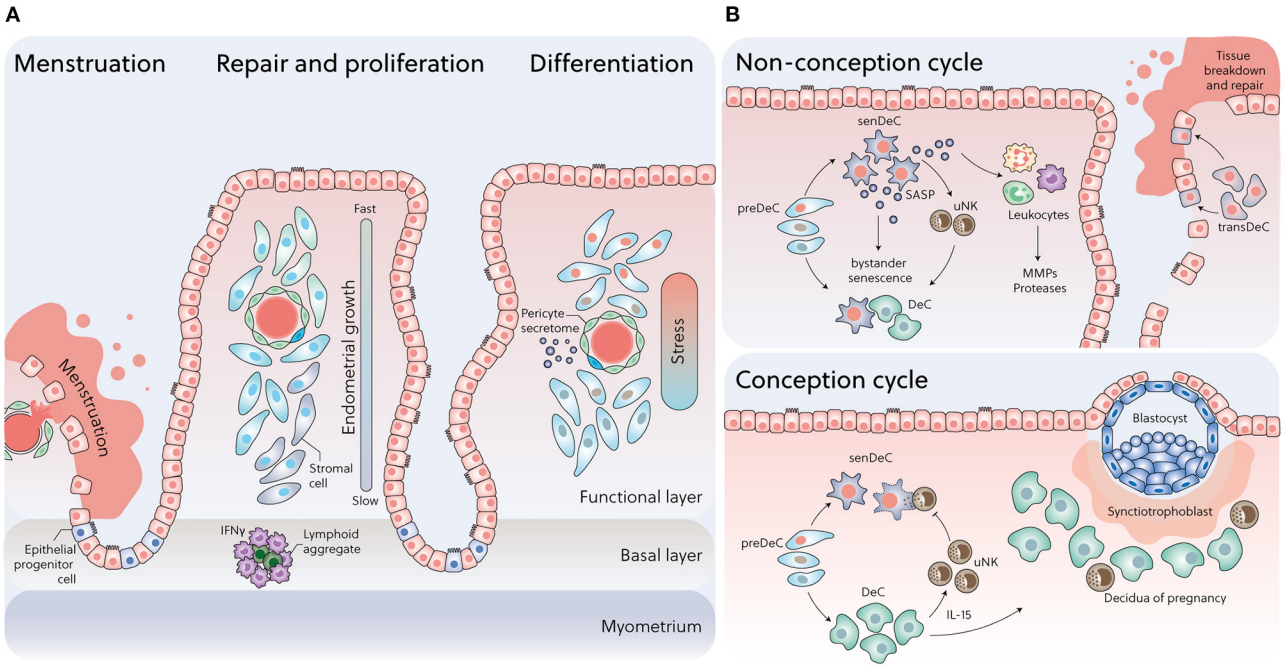

FIGURE 1 | Dynamic changes in cell states across the menstrual cycle. **(A)** Following menstruation, rapid re-epithelization of the endometrium may involve mesenchymal-epithelial transition (MET), proliferation of epithelial progenitors from unshed areas, or both. Positional oestrogen-dependent proliferation, reflecting an IFN-γ gradient generated by lymphoid aggregates residing in the endometrial-myometrial junction, imposes various levels of replication stress on individual cells during the follicular phase, which in turn drives the divergence in cell states upon decidualization. **(B)** Upper panel: In a non-conception cycle, falling progesterone levels promote senDeC/transDeC dominance and bystander senescence via prolonged SASP secretion, resulting in leukocyte recruitment and menstrual breakdown. Lower panel: Upon successful embryo implantation, progesterone-dependent DeC cells control rapid clearance of senDeC by recruiting and activating uNK cells.

quickly, thereby imposing various levels of replication stress on individual cells (**Figure 1A**). After the postovulatory rise in progesterone levels, proliferation of glandular epithelial cells first decreases and then ceases altogether in concert with the onset of apocrine glandular secretions, heralding the start of the midluteal window of implantation (33). Concurrently, proliferating uNK cells accumulate while stromal cells in the proximity of the luminal epithelial exit the cell cycle and start decidualizing (4). Pericytes surrounding the terminal spiral arterioles in the superficial layer also undergo morphological changes that are characteristic of a decidual response. Pericytes are not only biophysically and metabolically different from their stromal counterparts (34, 35), but produce a distinct decidual secretome, rich in chemokines and cytokines implicated in trophoblast migration and intravascular invasion (36) (**Figure 1A**).

Thus, across the menstrual cycle, the spatiotemporal responses of the endometrium to ovarian hormones are tightly controlled by changes in cell cycle status. How hyperproliferation of stromal cells is linked to spontaneous decidualization and subsequent endometrial fate decisions has been elusive until recently. As we will describe next, recent single-cell transcriptomic studies and modelling of the menstrual cycle in 3D cultures uncovered compelling evidence that human endometrium exploits rapid proliferation and replicative exhaustion to generate functionally distinct subpopulations that critically determine endometrial fate decisions following the implantation window (8, 18, 37).

SPECIFICATION OF DECIDUALIZING CELLS INTO FUNCTIONALLY DISTINCT SUBPOPULATIONS

Decidual States

Decidualization is not a binary differentiation response. Instead, it can be viewed as a triphasic process that starts with an acute inflammatory stress response, which is followed first by an anti-inflammatory phase and then a second, irreversible inflammatory state. As mentioned, the initial inflammatory decidual response maps to the midluteal implantation window (4). In non-conception cycles, the subsequent anti-inflammatory decidual phase is brief as falling progesterone levels promote rapid transition to the irreversible inflammatory state, which precedes menstrual breakdown (38). Upon successful embryo implantation, however, the anti-inflammatory decidual phase is massively prolonged and maintained for much of the pregnancy, although the decidua ultimately assumes a pro-inflammatory state prior to parturition (39, 40). The observation that DeC switch phenotype prior to the onset of labour in the absence of a discernible fall in circulating progesterone levels led to hypothesis that the timing of birth is determined by a "decidual

TABLE 1 | Mechanisms of DeC cellular defence and stress-resistance.

Pathway	Description	References
Cessation of circadian rhythms	Decidual loss of Period 2 (PER2) expression silences circadian gene expression in differentiating EnSCs, matching aperiodic gene expression in the implanting conceptus.	(50)
Oxidative stress resistance	Induction of various free radical scavengers upon decidualization including SOD2, monoamine oxidases A and B, thioredoxin, glutaredoxin and peroxiredoxin confer oxidative stress resistance to decidual cells. Upregulation of mitogen-activated protein phosphatase 1 (DUSP1) silences c-Jun NH-terminal kinase (JNK) stress signalling, and inhibits FOXO3a, a forkhead proteins implicated in oxidative cell death.	(51, 52)
Uncoupling of stress signals and SUMOylation	Decidualization imposes a stress resistant global cellular hypoSUMOylation state by modulation of various SUMO-specific ligases and proteases, preventing PGR transcriptional repression.	(53, 54)
Silencing of phospholipase C signalling	Induction of phospholipase C (PLC)-related catalytically inactive protein 1 (PRIP-1) uncouples PLC activation from intracellular Ca^{2+} release by attenuation of inositol triphosphate (IP3) signalling.	(55)
Resistance to microRNA mediated gene silencing	Downregulation of argonaut proteins (AGO1 and AGO2) upon decidualization renders the endometrium resistant to microRNA mediated gene silencing.	(56)
Downregulation of O-linked N-acetylglucosamine (O-GlcNAc) posttranslational modification	Decidualization of EnSCs results in reduced global O-GlcNAcylation, mediated by decreased expression of the metabolic stress enzyme O-GlcNAc transferase (OGT), without changes in its reciprocal mediator O-GlcNAcase (OGA)	(57)

clock" (41, 42). Thus, the term "decidualization," which is derived from the Latin verb "decidere" (to die, to fall off or to detach), aptly describes the physiological process that links the window of implantation to tissue destruction associated with menstruation and parturition.

It should be obvious that parsing the mechanisms that enable DeC to switch phenotypes is not merely of academic interest but fundamental for our understanding of the physiological processes that control embryo implantation, menstruation and parturition. Valuable insights into this process emerged from a simple reconstruction of the decidual pathway in cultured primary endometrial stromal cells using single-cell transcriptomics (8). This analysis revealed that differentiating stromal cells undergo extensive and coordinated transcriptional reprogramming during the initial inflammatory decidual phase, triggered by rising intracellular cyclic adenosine monophosphate (cAMP) and activation of decidua-specific TFs that interact with the liganded progesterone receptor (43–45). Transcriptional reprogramming of stromal cells involves an acute cellular stress response, which starts with a burst reactive oxygen production and secretion of inflammatory mediators and nuclear alarmins, including interleukin 33 (IL-33) and high mobility group box 1 (HMGB1) (13, 46, 47). In parallel, wholesale remodelling of the chromatin landscape, involving opening, as well as closure of numerous DNA loci, enables decidual TF complexes to gain access to promoter and enhancer regions that control the expression of specific decidual gene networks (23, 48, 49). Cells transitioning through this preparatory phase are denoted pre-decidual cells (preDeC). Most reprogrammed cells emerge as progesterone dependent, anti-inflammatory DeC. However, inflammatory reprogramming also compounds DNA damage already present stromal cells burdened by replication stress, which in turn gives rise to a discrete population of senescent decidual cells (senDeC; **Figure 1B**) (8, 13).

Anti-inflammatory Decidual Cells

Progesterone-dependent DeC are typified by activation of cellular defence mechanisms and selective silencing of stress-responsive signalling pathways (summarised in **Table 1**). Consequently, DeC are not only protected against oxidative and metabolic stress but also largely impervious to noxious environmental cues. Silencing of stress pathways is also critical for continuous progesterone signalling in DeC (51). Through tight intercellular connexions (58), DeC form a robust matrix, which in pregnancy accommodates invading extravillous trophoblast and local immune cell populations (9). Although the decidual secretome is largely devoid of inflammatory mediators, they produce an abundance of C-X-C motif chemokine ligand 14 (CXCL14) and IL-15, essential for uNK cell chemotaxis and activation, respectively (8, 59). By contrast, epigenetic silencing of other chemokines precludes infiltration of the decidual matrix by cytotoxic T lymphocytes (60).

Senescent Decidual Cells

In virtually all aspects, senDeC are the functional opposites of DeC. Senescence denotes a cellular stress response triggered by telomere shortening and replicative exhaustion as well as a myriad of other stressors that cause macromolecular damage (61). Activation of tumour suppressor pathways and upregulation of cyclin-dependent kinase inhibitors $p16^{INK4a}$ and $p21^{CIP1}$ lead to permanent cell cycle arrest, resistance to apoptosis, de-repression of retrotransposons, and production of a bioactive secretome, referred to as the senescence-associated secretory phenotype (SASP) (62). The composition of the SASP is tissue-specific and typically includes proinflammatory and immuno-modulatory cytokines, chemokines, growth modulators, angiogenic factors, and extracellular matrix (ECM) proteins and proteases (63). Senescence has been described as an evolutionarily conserved cellular programme with both

beneficial and detrimental effects (62). For example, acute senescence, characterised by transient SASP production and rapid immune-mediated clearance of senescent cells, is also widely implicated in processes involving physiological tissue remodelling, including during foetal development, placenta formation and wound healing (61, 64). By contrast, persisting senescent cells cause chronic, sterile inflammation, also known as "inflammaging" (63), a pathological state that underpins ageing and age-related disorders. In the endometrium, senDeC are characterised by their abounding capacity to initiate tissue remodelling, mediated by a SASP rich in ECM proteins and proteinases, angiogenic modulators, growth factors, and chemokines involved in neutrophil migration (18). Compared to undifferentiated stromal cells, DeC appear highly susceptible to bystander senescence, meaning that they acquire a senescent phenotype upon prolonged SASP exposure (13, 65, 66). When extrapolated to the *in vivo* situation, these observations suggest that in the absence of effective immune cell surveillance, cellular senescence is poised to propagate across the susceptible superficial endometrial layer, rendering tissue breakdown in a piecemeal fashion inevitable (1, 67).

Transitional Decidual Cells

Reconstruction of the decidual pathway in endometrial assembloids, consisting of gland-like organoids and primary stromal cells, not only confirmed the divergence of differentiating stromal cells into anti-inflammatory DeC and pro-inflammatory senDeC but also revealed a third decidual subpopulation with hallmarks of mesenchymal-epithelial transition (MET) (18). Computation predictions based on gene expression indicate that these cells, termed transitional DeC (transDeC), are highly autonomous, i.e., largely devoid of receptors and ligands that mediate interactions with other decidual subpopulations. Enrichment of gene ontology categories, such as "regulation of stem cell proliferation," "blood vessel development," and "wound healing," further suggests that transDeC are poised to effect tissue repair (18), which is in line with experimental evidence that MET drives re-epithelisation of the endometrium following menstruation and parturition (68, 69).

Pharmacological elimination of pre-stressed stromal cells in 3D assembloids blunts the initial decidual inflammatory response, which in turn massively accelerates the emergence of DeC at the expense of senDeC and, to a lesser extent, transDeC (18). This observation is pivotal as it demonstrates the importance of E2-dependent hyperproliferation and replicative exhaustion during the proliferative phase in determining the amplitude of the pre-decidual inflammatory response and the subsequent balance between DeC, and senDeC/transDeC. Thus, the *in vitro* data suggest that following rapid E2-dependent proliferation, the default trajectory of the decidual pathway is inevitably towards tissue destruction and repair. As DeC are sensitive to bystander senescence, they can escape this fate during a narrow window only by engaging innate immune cells to eliminate their senescent counterparts (**Figure 1B**) (8).

ENDOMETRIAL HOMEOSTASIS DURING THE LUTEAL PHASE

Although 2D and even 3D cultures are highly reductionist models to study *in vivo* events, several aspects of the *in vitro* decidual pathway are recapitulated *in vivo*. Tissular cAMP levels increase markedly in the endometrium following the postovulatory rise in progesterone levels (70). While the nature of the *in vivo* ligand(s) responsible for adenylyl cyclase activation in stromal cells has been debated for years, recent evidence firmly implicates prostaglandin E2 (PGE2) as the ancestral deciduogenic signal (71). As a consequence of cAMP-dependent protein kinase A activation and progesterone signalling, proliferation of epithelial and stromal cells in the superficial endometrial layer ceases and differentiation is initiated (72). In parallel, senescence-associated b-galactosidase (SAbG) activity in whole endometrial biopsies increases sharply following ovulation and levels continue to rise upon progression from the early- to late-luteal phase (13). SAbG activity, reflecting lysosomal mass (73), is a widely used biomarker of senescent cells, although it lacks specificity (62, 74). However, transition from proliferative to secretory phase also coincides with the emergence of other canonical senescence markers in the endometrium, including loss of lamin B1, induction of the tumour suppressor p53, the cyclin-dependent kinase inhibitor $p16^{INK4a}$, and senescence-associated histone modifications (13, 75). Spatiotemporal profiling of $p16^{INK4a}$-positive cells in 308 timed endometrial biopsies demonstrated that senescent cells are much more abundant in luminal when compared to glandular epithelium during the midluteal window of implantation. In the stroma, ∼1% of cells are $p16^{INK4a}$-positive in the early-luteal phase. Their abundance rises transiently during the window of implantation, which is followed by a much steeper increase in premenstrual endometrium (13).

The temporal profile $p16^{INK4a}$-positive cells in the endometrium highlights the important role for continuous progesterone signalling in constraining cellular senescence. Perhaps not surprisingly, this task is executed primarily by uNK cells, the most abundant immune cells in luteal phase endometrium and the decidua of pregnancy (**Figure 1B**) (13, 19, 37, 76). Both glandular epithelial cells and DeC secrete chemokines, most prominently CXCL14, involved in recruiting circulating NK cells into the endometrium (77, 78). This multifaceted chemokine also plays an important role in immunosurveillance for bacterial and viral infections (79). Once recruited, NK cells are subjected to progressive differentiation, a process under the control of DeC through secretion of IL-15 (80). Maturation of uNK is characterised by sequential acquisition of killer cell immunoglobulin-like receptors (KIRs) and CD39 on the cell surface. Immature uNK ($KIR^{-}CD39^{-}$) display higher proliferative capacity in response to IL-15, which diminishes with increasing maturity (80, 81). The mature uNK ($KIR^{+}CD39^{+}$) subset is characterised by increased production of cytotoxic granzyme-A (80). Single-cell analyses confirmed the presence of three transcriptionally distinct uNK subsets in both luteal endometrium and the maternal-foetal interface in early pregnancy (8, 10).

In primary cultures, uNK cells target and kill senDeC with exquisite precision and efficacy (13, 37). Clearance of senDec is achieved primarily through granule exocytosis, in which the uNK cells physically engage with target cells and deliver cytolytic granules containing perforin, granzyme A and granzyme B (13, 82). How uNK cell selectively target senDeC is not fully understood, although experimental evidence implicates activation of killer cell lectin like receptor K1 [KLRK1, also known Natural Killer Group 2 member D (NKG2D)] (83). This activating uNK cell receptor binds stress-induced ligands present on the surface of stressed and senescent cells. These ligands belong to the MHC class I chain-related protein (MIC) and unique-long 16 binding protein (ULBP) families of proteins (84, 85). SASP metalloproteinases, such as ADAM metallopeptidase domain 9 (ADAM9), ADAM10, and ADAM17, can cleave stress-induced ligands from the cell surface, thereby enabling senescent cells to evade immune recognition (86, 87). However, preDeC and DeC firmly block senDeC from activating this escape mechanism by secreting an abundance of TIMP metallopeptidase inhibitor 3 (TIMP3), a potent inhibitor of metalloproteinases (88). IL15, CXCL14 and TIMP3 are already highly expressed by preDeC during the midluteal phase, meaning that immune surveillance of damaged and senescent cells is operational during the implantation window.

The uNK cell-DeC partnership is critically dependent on continuous progesterone signalling, which explains, at least in part, why the endometrium switches dramatically to a pro-inflammatory state prior to menstruation. In a conception cycle, however, sustained uNK cell-DeC cooperation occurs alongside recruitment of circulating bone marrow-derived decidual progenitor cells (8, 14). Decidual progenitor cells in luteal phase endometrium are clonogenic cells poised for rapid proliferative expansion in early pregnancy. Unlike resident endometrial stromal cells, decidual progenitors highly express *PRL*, which encodes the canonical *in vitro* decidual marker prolactin (14, 16). Thus, endometrial homeostasis upon interstitial embryo implantation and subsequent transformation into the decidua of pregnancy are critically dependent upon successful recruitment of non-uterine cells; i.e., circulating NK cells and non-hematopoietic bone marrow-derived mesenchymal progenitor cells (8, 13, 14, 16, 37).

IMPLANTATION: ENDOMETRIAL RECEPTIVITY AND SELECTIVITY

The Implantation Paradigm

It is widely assumed that breaching of the endometrial surface (luminal) epithelium by the blastocyst is the critical, rate-limiting step during human implantation. This implantation paradigm, which is based on studies in mice and other animal models (89–91), assumes that the luminal epithelium is a robust barrier that only transiently expresses the machinery needed for embryo apposition, attachment and invasion. In other words, transient changes in luminal epithelium are believed to define the boundaries of the implantation window. A potential problem with this paradigm is that it glosses over distinct inter-species differences and reproductive challenges. Mice are litter-bearing mammals and the barrier function of the luminal epithelial is critical for synchronised implantation of multiple blastocysts. Murine embryos in the uterine cavity can temporarily arrest in development (diapause) while awaiting a transient surge in circulating oestrogen levels, which simultaneously renders the endometrium receptive and activates dormant embryos for implantation (92). Oestrogen levels also rise transiently during the midluteal phase of the menstrual cycle but there is no evidence that it serves as a nidation signal (93, 94). In contrast to mice, human conception involves a single blastocyst, which often harbours complex chromosomal errors and lacks the ability to enter diapause (95, 96). Further, the luminal epithelium during the midluteal phase consists of a patchwork of $p16^{INK4a}$-positive and -negative epithelial cells (13). While yet untested, $p16^{INK4a}$-positive senescent epithelial cells plausibly create areas of little or no resistance to embryo implantation. It is indeed notable that apposition and attachment of blastocysts to luminal epithelium have been observed in many species, but histological evidence of this implantation stage has not yet been documented in humans (97). Our assertion that the barrier function of the human endometrium is degraded does not imply that luminal epithelial cells are dispensable for implantation. For example, the luminal epithelium mediates progesterone-dependent absorption of uterine fluid (98, 99), which critically ensures "closure" of the uterine cavity during the implantation window (100). In co-cultures, contact between human blastocysts and endometrial epithelial cells induces an embryonic transcriptional response that may promote further implantation (101).

Amongst primates, only humans and apes exhibit primary interstitial implantation, where the entire conceptus is drawn into the endometrium (97). Rather than reflecting the intrinsic invasiveness of embryos, deep interstitial implantation depends on active migration of preDeC and encapsulation of the conceptus (102–104). In 2D and 3D co-culture experiments, migratory preDeC first home in and then attach to the polar trophectoderm before "dragging" the conceptus into the stromal matrix (18, 104). This process is remarkable in several aspects. First, it is time sensitive as subsequent differentiation of preDeC into DeC leads to complete loss of directed migration and attachment to the conceptus. When extrapolated to the *in vivo* situation, these observations indicate that lack of senDeC, which accelerates the emergence of DeC, may lead to entrapment of the conceptus in a largely static matrix and implantation failure (18, 105). Second, high-quality human embryos stimulate migration of preDeC whereas low-quality embryos fail to do so. Conversely, migration of undifferentiated stromal cells is actively inhibited by high-quality embryos, but not low-quality embryos (104, 106, 107). Taken together, these observations suggest that the initial steps in the implantation process evolved in fundamental aspects across Eutherian (placental) mammalian species, likely reflecting maternal adaptations to different challenges imposed by rapidly evolving embryos (108). By relaxing the barrier function of the luminal epithelium, the implantation process arguably becomes primarily under control of preDeC cells, which first engage in

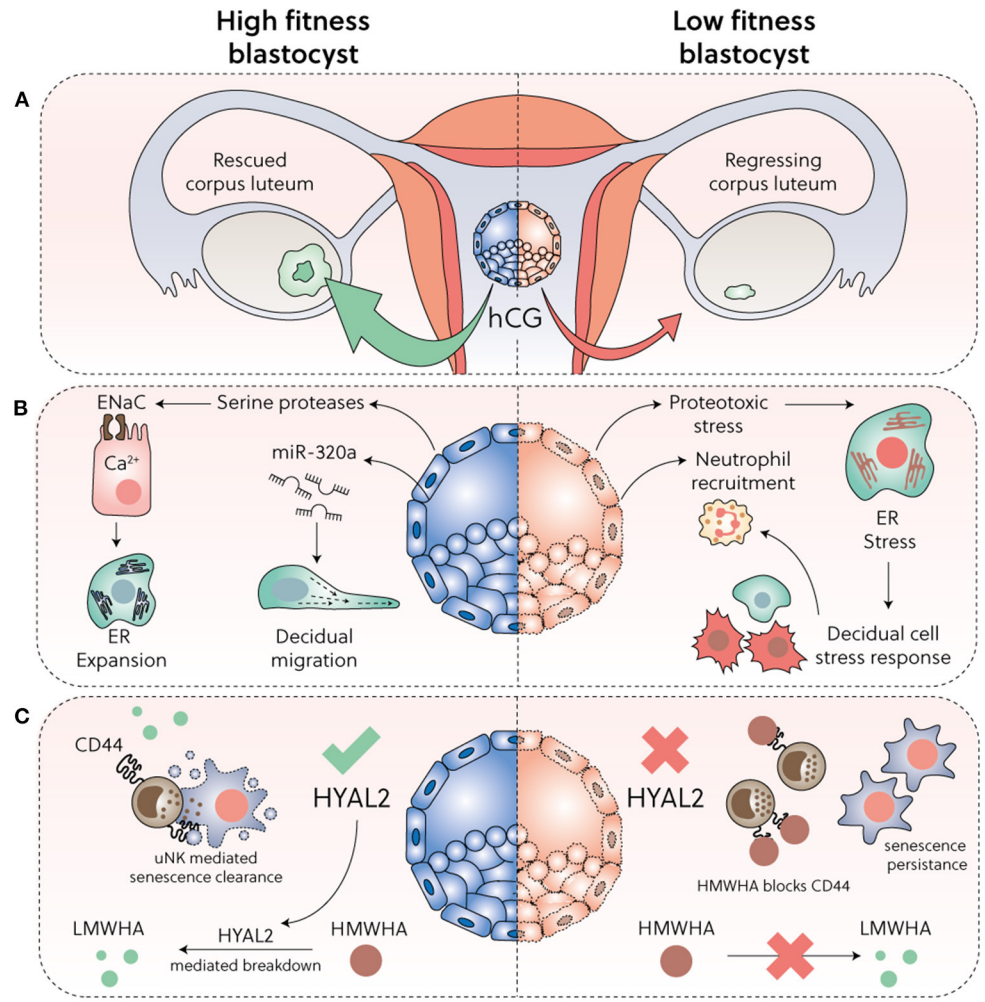

FIGURE 2 | Embryo biosensing and selection. **(A)** Sufficient hCG secretion from implanting blastocysts is required to prevent corpus luteum involution. **(B)** High-fitness human blastocysts elicit a supportive decidual response, including secretion of evolutionary conserved serine proteases that activate epithelial Na^+ channel (ENaC) expressed on luminal epithelial cells, triggering Ca^{2+} signalling, ER expansion and subsequent induction of implantation specific genes. Further, by secretion of microRNA miR-320a, competent blastocysts promote migration of pre-decidualizing cells. Conversely, low fitness embryos trigger a decidual ER stress response, repression of key implantation factors, and secretion of CXCL12 and CXCL8 leading to neutrophil recruitment and activation. **(C)** HYAL2 production in developmentally competent blastocysts cleaves HMWHA into LMWHA, which supports further development. Loss or reduction of HYAL2 in low-fitness embryos promotes HMWHA accumulation, which binds to CD44 expressed on uNK cells and disables selective killing of senDeC.

embryo biosensing before actively encapsulating the conceptus or not.

Embryo Biosensing and Selection

Figure 2 summarises the various mechanisms implicated in embryo biosensing by preDeC and DeC. While it is increasingly incontrovertible that spontaneous decidualization bequeaths the endometrium the ability to decode embryonic fitness signals (3, 109), how this process leads to menstruation-like disposal of developmentally compromised embryos is less obvious. Clearly, if the conceptus fails to secrete sufficient human chorionic gonadotropin (hCG), the corpus luteum involutes and falling progesterone levels trigger endometrial breakdown (**Figure 2A**). However, several prospective cohort studies in young, healthy women reported that 30% of pregnancies will fail after an initial rise in hCG levels (110–113). Most of these failures occur soon after implantation and therefore remain undetected. In a substantial number of early pregnancy losses, hCG concentrations only diverge from those in healthy pregnancies when the miscarriage is in progress (114), suggesting that an alternative mechanism of tissue breakdown is activated (**Figures 2B,C**). A recent study highlighted that embryonic fitness signals modulate the ability of uNK cells to target and clear senDeC. In this study, spent media from IVF embryos that subsequently failed to implant completely abrogated uNK cell-mediated killing of senDeC *in vitro* (37). Loss of embryonic hyaluronidase 2 (HYAL2) production was shown to be responsible for uNK cell inhibition (**Figure 2C**). HYAL2

cleaves high molecular weight hyaluronic acid (HMWHA) into low molecular weight hyaluronic acid (LMWHA) (115, 116). Hyaluronic acid, a ubiquitous ECM glycosaminoglycan, exerts distinct biological effects dependent on its molecular weight. In pre-implantation embryos, high HYAL2 activity and LMWHA production promotes development, whereas HMWHA does the opposite (116). Upon implantation, binding of HMWHA to CD44 expressed on uNK cells abrogates targeted killing of senDeC, whereas LMWHA has no inhibitory effect. Addition of recombinant HYAL2 to the spent medium of low-fitness human blastocysts was sufficient to restore uNK cell-mediated clearance of senDeC, at least *in vitro* (37). On the other hand, hCG promotes proliferation uNK cells (117), which presumably enhances immune surveillance of senDeC in conception cycles. Thus, the timing of embryo disposal after implantation is likely determined by the balance of opposing fitness signals as well as the ability of the endometrium to decode these signals, a function that resides with DeC and uNK cells.

ENDOMETRIAL BREAKDOWN AND REPAIR

In non-conception cycles, falling progesterone levels disable cooperation between DeC and uNK cells, which steers the decidual pathway to senDeC and transDeC involved in tissue breakdown and repair, respectively. Notably, senescent cells also co-opt innate immune cells, foremost neutrophils and macrophages, which upon activation and degranulation reinforces cellular senescence and ECM breakdown (2, 118). As outlined above, low-fitness embryos also disrupt uNK cell-DeC interactions, thereby engineering their own demise by triggering menstruation-like breakdown (**Figures 2B,C**). Thus, a switch in decidual state towards senDeC and transDeC may be the common pathway underpinning tissue breakdown and repair in menstruation and early pregnancy loss.

During menstruation, areas of shed endometrium are found alongside areas of unshed and repaired tissue (1, 67). This appearance of the endometrium during menstruation reinforces our conjecture that promulgation of cellular senescence primes the superficial endometrial layers for tissue breakdown, which upon recruitment of leucocytes becomes irrevocable. Arguably, a piecemeal approach to menstrual shedding abates the risk of infection and excessive haemorrhage, although rapid repair of the luminal epithelial remains critical (2). The mechanism driving re-epithelization of the endometrium during menstruation is, however, contentious. Based on scanning electron microscopy (SEM) studies, re-epithelization was initially attributed to proliferating or migratory epithelial cells arising from exposed gland stumps in the basal layer or from residual intact epithelium near the cornual and isthmic regions of the uterus (67, 119). Subsequent studies refuted this interpretation as there is no evidence of cellular proliferation during menstrual repair (120). Further, the discovery of isolated or small islands of immature epithelial cells with a smooth surface and a low cuboidal shape by SEM indicated that re-epithelization is primarily driven by differentiation of stromal cells via MET (1, 120). More recently, identification of ambiguous cells that express both epithelial and mesenchymal marker genes in luteal phase endometrium and decidualizing assembloids by single-cell transcriptomics provides additional credence to the assertion that MET (i.e., transDeC) play an important role in endometrial re-epithelization following menstrual desquamation (**Figure 1B**) (8, 18). Of course, both mechanisms are not mutually exclusive.

The mechanism that protects the regenerative basal layer from menstrual shedding is not entirely clear but presumably reflects the lack of hormone responses in this layer. Further, endometrial glands form a horizontal network in the basal layer, which may confer protection against menstrual destruction and aid regeneration (121). Intriguingly, transient but not prolonged exposure to SASP promotes tissue rejuvenation by reprogramming committed cells into stem-like cells (122). In fact, dedifferentiation of resident cells into stem cells is now recognised as the dominant mechanism for tissue regeneration in multiple organs (123). These observations raise the intriguing possibility that premenstrual senescence determines the regenerative potential of the basal layer following menstruation. In other words, the capacity of cells to cope with replication stress during oestrogen-dependent hyperproliferation may already be determined by the level of premenstrual senescence in the preceding cycle. Thus, while premenstrual senescence may be important for inter-cycle homeostasis, prolonged exposure to SASP, for example associated with clinical miscarriages, is predicted to impact adversely on stemness of the basal layer and to increase the likelihood of endometrial dyshomeostasis in subsequent cycles.

DECIDUAL DYSHOMEOSTASIS AND RECURRENT MISCARRIAGE

The Recurrence Risk of Miscarriage

Recurrent miscarriage is a devastating disorder and a sentinel risk factor for obstetrical disorders in future pregnancies (124). Approximately 15% of all clinically recognised pregnancies end in miscarriage, mostly before 12 weeks of gestation. The population prevalence of women with one, two or three or more previous miscarriages is 10.8, 1.9, and 0.7%, respectively. Apart from the physical trauma (pain, bleeding, and infection), each miscarriage compounds the risk of significant psychological morbidity (depression, post-traumatic stress disorder, and suicide) and obstetrical complications in a future ongoing pregnancy (preterm birth, foetal growth restriction, placental abruption, and stillbirth) (124).

Two independent risk factors, maternal age and the number of previous pregnancy losses, have disproportional effects on miscarriage rates (125, 126). The age-related risk is driven by meiotic errors in oocytes leading to foetal aneuploidy and increases sharply after the age of 34-years. Lack of geographic or ancestry-related variation indicates that the age-related risk is "hardwired" in human reproduction (127). Miscarriage rates also increase stepwise by ∼10% with each additional loss (125, 126). This recurrence risk is age-independent and therefore not driven by chromosomal errors. To date, there is no epidemiological

evidence that the recurrence risk of miscarriage has changed in recent decades nor does it differ substantially between populations (128–130). Thus, as is the case for age-related risk of miscarriage, the recurrence risk may also be grounded in a fundamental (patho-)physiological process, plausibly triggered by the miscarriage itself.

Clinically, recurrent miscarriage is defined by an arbitrary number of previous, consecutive or non-consecutive, pregnancy losses (124, 131). There is no consensus on precise criteria, although both the American Society for Reproductive Medicine (ASRM) and the European Society of Human Reproduction and Embryology (ESHRE) now define recurrent miscarriage as two previous pregnancy losses. The losses do not have to be consecutive, but the ESHRE definition includes preclinical (biochemical) losses whereas the ASRM definition does not (124). Importantly, there is no pathophysiological rationale for these definitions as the risk of miscarriage increases stepwise, even after a single loss, in women of all ages (125, 126, 128–130). Further, arbitrary definitions of recurrent miscarriage increase the risk of amalgamation of patients with wildly different prognoses under a single disease umbrella. For example, after two consecutive pregnancy losses, recurrent miscarriage patients aged 30 or 40 years will have ∼80 and ∼53% chance, respectively, of a successful next pregnancy (125). Hence, even over this age range, it is more likely than not that a subsequent pregnancy will be successful in these recurrent miscarriage patients, although women aged 40 will also have to content with reduced fertility. After 5 consecutive losses, however, the likelihood of a pregnancy resulting in live birth drops to ∼55 and ∼25%, respectively, in women aged 30 and 40 years (125). Thus, binary definitions of recurrent miscarriage are a major confounder in research and clinical trials as the likelihood of reproductive success and the effectiveness of therapeutic interventions differ markedly between study populations (132).

It is standard practise to attribute the recurrence risk of miscarriage to a host of subclinical disorders, ranging from subtle clotting, endocrine and immunological perturbations to vitamin deficiency and lifestyle factors. Apart from progesterone support, this disease paradigm has not resulted in effective interventions that prevent miscarriages, even after decades of research and numerous clinical trials (131–134). Further, two large cohort studies reported no difference in the likelihood of a successful pregnancy in women with "explained" vs. "unexplained" ("idiopathic") recurrent miscarriage (135, 136). Despite the astonishing lack of evidence, clinical practise remains grounded in a historical, and arguably patriarchal, misconception that early pregnancy represents an exceptionally precarious physiological state, easily disrupted by "perturbations" that otherwise do not impact overtly on health and well-being outside pregnancy (137). This disease paradigm does not explain high cumulative live birth rates in miscarriage patients, nor is it easily reconciled with the incontrovertible fact that all our ancestors, over millennia, reproduced successfully despite harsher environments and poorer health conditions. Of course, overt disease, such as uncontrolled diabetes, can cause early pregnancy loss but cases are rare. Further, our criticism of current clinical practise does not challenge the notion that certain risk factors, such as obesity, impact adversely on the prognosis of miscarriage patients by aggravating the underlying pathophysiology (124, 131, 138).

The Peri-implantation Endometrium in Recurrent Miscarriage

Surprisingly little attention has been paid to implantation biology in the context of recurrent miscarriage. The discovery that nidation is critically controlled by decidual subsets with opposing functions under the homeostatic control of extra-uterine innate immune cells and bone marrow derived decidual progenitors point towards a novel disease dimension. Recurrent miscarriage is associated with loss of clonogenicity in midluteal endometrium (76), reflecting lack of decidual progenitors (14). Based on computational modelling, decidual progenitors are predicted to give rise in early pregnancy to a distinct subset of cells present the superficial compact layer of the decidua (decidua compacta), that is, the site of initial trophoblast invasion (14). Importantly, the level of stem cell depletion in midluteal endometrium correlates inversely with the number of previous pregnancy losses and, hence, the recurrence risk of miscarriage (76). Several studies have focused on the abundance of uNK cells in luteal phase endometrium of recurrent miscarriage patients (139), invariably motivated by the questionable assumption that high levels signal a pending immune attack on the semi-allogenic conceptus (140). However, the abundance of uNK cells varies naturally throughout the luteal phase and between cycles (13), which is entirely in keeping with the homeostatic role of these innate immune cells. Lack of standardised protocols and failure to normalise uNK cell levels for cycle day further accounts for inconsistent findings in the literature (139). Nevertheless, there is evidence that lower uNK cell levels and activity in the endometrium, as well as peripheral blood, associates with higher miscarriage rates (8, 141–143). Together, the data suggest that key homeostatic mechanisms that regulate the transformation of the cycling endometrium into the decidua of pregnancy are relaxed in recurrent miscarriage patients. Loss of stringency is predicted to increase the likelihood of embryo implantation in an endometrium that is destined for breakdown in early pregnancy and, by extension, the recurrence risk of miscarriage. For example, obesity adversely impact on decidual progenitor cells in peri-implantation endometrium, exemplified by a significant inverse correlation between increased body mass index and the level of clonogenic endometrial cells (144). Obesity is further associated with uNK cell depletion and dysfunction in pregnancy (145), which may explain why it is a major risk factor for higher-order miscarriages (146).

Perturbations in the peri-implantation endometrium can potentially be exploited to identify clinically useful biomarkers for screening of women at increased risk of miscarriage before pregnancy. Such biomarkers may also be useful in assessing pre-pregnancy interventions aimed at optimising the uterine implantation environment. Accurate assessment of endometrial clonogenicity is possible but cumbersome as it relies on colony-forming unit assays that take 10 days to complete (76, 144, 147). Measurements of uNK cell levels are also fraught

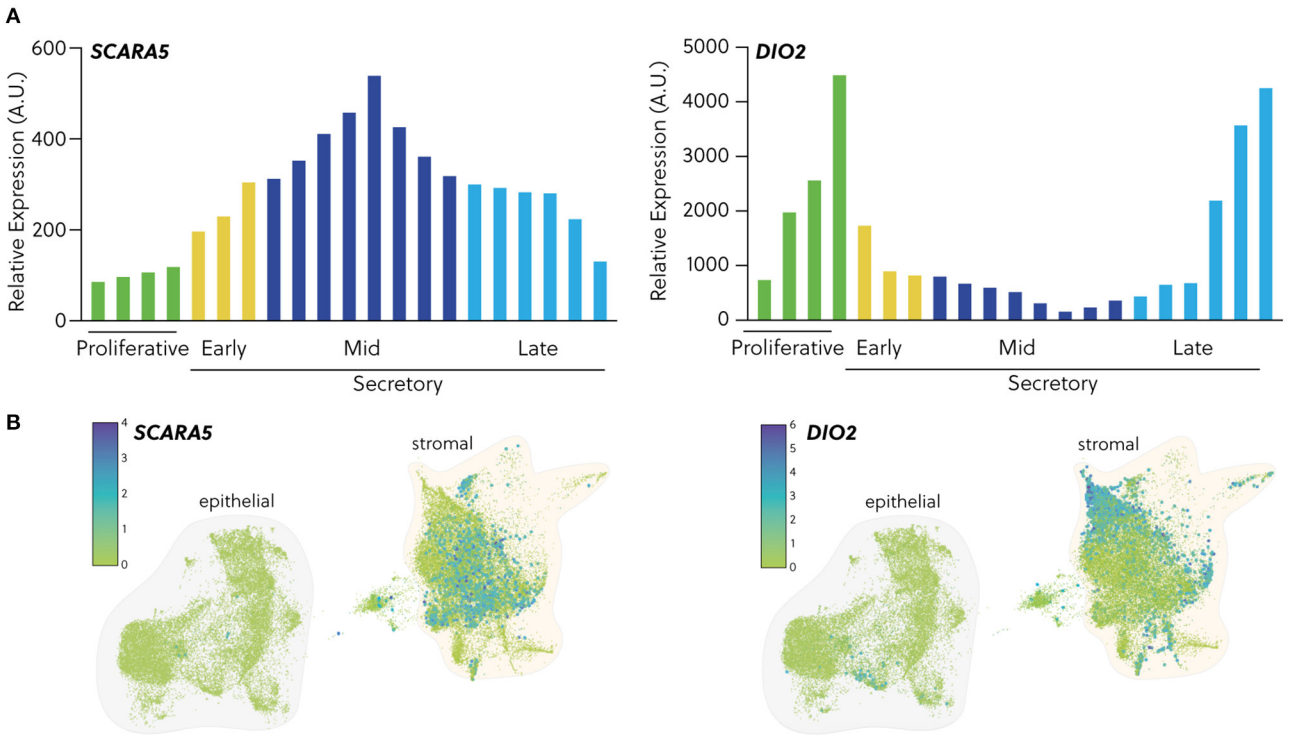

FIGURE 3 | Expression of cell fate biomarkers. **(A)** *In silico* analysis of GDS2052 microarray data showing transcript expression of the DeC biomarker *SCARA5* and the senDeC biomarker *DIO2* in proliferative, early-, mid- and late-secretory endometrium expressed in arbitrary units. **(B)** Temporal expression of *SCARA5* and *DIO2* transcripts in human endometrium from single cell profiling of endometrial biopsies obtained across the cycle ($n = 15$). Data were extracted from Garcia-Alonso et al. (148) and available on reproductivecellatlas.org.

because of intrinsic intra- and inter-cycle variations (13). An alternative approach is to measure the relative abundance of different decidual subsets in midluteal biopsies. An early sign of stromal cells earmarked for cellular senescence in pregnancy is lack or loss of progesterone-responsiveness, defined here as expression of genes firmly repressed by progesterone. For example, progesterone strongly represses *DIO2*, a stromal cell-specific gene in the endometrium that encodes iodothyronine deiodinase 2, the enzyme that catalyses the conversion of prohormone thyroxine (T4) to the bioactive thyroid hormone (T3) (8). As shown in **Figure 3A**, *DIO2* expression is high during the proliferative phase and the late-secretory phase, i.e., when progesterone levels are low and stromal cells are highly metabolically active. *SCARA5*, encoding the ferritin receptor, is a stromal cell-specific, progesterone-responsive gene (8). Not unexpectedly, the temporal expression profile of *SCARA5* across the menstrual cycle is the inverse of that of *DIO2*. As cells cannot be simultaneously progesterone-responsive and -resistant, *SCARA5* and *DIO2 a priori* mark distinct stromal subpopulations in peri-implantation endometrium (**Figure 3B**). Interestingly, recurrent pregnancy loss is associated with increased frequency of cycles with low *SCARA5* and high *DIO2* expression in midluteal biopsies (8), indicating that lack of DeC at implantation predisposes for senescence-mediated breakdown of the placental-decidual interface in pregnancy. In agreement with this conjecture, a recent single-cell transcriptomic study reported a striking senescence-associated gene signature in stromal and decidual cells at the maternal-foetal interface upon the diagnosis of missed miscarriage (149), indicating that decidual senescence precedes the physical disintegration of pregnancy.

SUMMARY AND THERAPEUTIC PERSPECTIVE

Single-cell "omics" approaches are rapidly transforming our understanding of the cellular dynamics underpinning key endometrial functions, including embryo implantation, menstrual shedding and repair, and the spectacular transformation of a short-lived uterine mucosa into a robust matrix that accommodates the placenta throughout pregnancy. Underpinning all these functions is spontaneous decidualization, an iterative process that follows positional E2-dependent endometrial hyperproliferation and leads to emergence of subsets of cells with specialised functions that control endometrial fate decisions at implantation. Balancing decidual subsets and states from one cycle to the next is under control of non-uterine cells. On the one hand, uNK cells engender selective elimination of senDeC, *de facto* rejuvenating the endometrium following embryo implantation (37). On the other, recruitment and engraftment of bone marrow-derived decidual progenitor cells may impart tissue plasticity to accommodate a rapidly growing conceptus and invading trophoblast (14).

Recent insights in the cellular dynamics during the peri-implantation window are poised to lead to bespoke therapeutic interventions for intractable reproductive disorders, such as recurrent miscarriage. Based on our current understanding, two therapeutic "windows" in the menstrual cycle are predicted to have maximal impact on an ensuing pregnancy. First, interventions could focus on enhancing the stringency of homeostatic control in peri-implantation endometrium. For example, a recent study demonstrated that bone marrow transplants from wild-type mice to mice carrying a heterozygous deletion of *Hoxa11*, a pivotal decidual transcription factor, not only restore the decidual response but also prevent pregnancy loss in these animals (16). Further, sitagliptin, a dipeptidyl-peptidase IV (DPP4) inhibitor used in the management of diabetes, was found in a randomised, double-blind placebo-controlled feasibility trial to increase the abundance of decidual progenitor cells by almost 70% when given over 3 consecutive menstrual cycles to recurrent miscarriage patients (147). Notably, increased engraftment of bone marrow-derived progenitors coincided with a marked reduction in *DIO2* expression in peri-implantation endometrium, suggesting amelioration of the pro-senescence endometrial state. There is yet no information on whether the endometrial effects of sitagliptin are transient or durable. Recruitment of decidual progenitors and uNK cells likely continues at the maternal-foetal interface in early gestation, at least until the second trimester of pregnancy when transformation of maternal spiral arteries into large fibrinoid vessels by endovascular trophoblast is complete (11, 150). Hence, it seems sensible to maintain treatment after embryo implantation, but sitagliptin is not licenced for use in pregnancy, at least in the UK, because of insufficient safety data. An alternative approach to balance decidual subpopulations is to target E2-dependent hyperproliferation. Therapeutic interventions confined to the proliferative phase would go a long way in abating justifiable fears of embryo/foetal toxicity of novel drugs. This strategy is not only appealing but increasingly realistic, especially in view of current explosion in the development of drugs that target stressed and senescent cells for the treatment of age-related disorders (63, 151). These drugs can be broadly categorised into two categories: pharmacological agents termed "senolytics," which eliminate senescent cells, and "senomorphics," which prevent the detrimental cell-extrinsic effects of senescent cells and include SASP inhibitors (63, 151). Further, organoid and assembloid technologies now enable rapid screening of the effectiveness of drugs in restoring or enhancing the implantation environment (18, 152–154). Altogether, a new age of non-hormonal "endometrial therapeutics" appears just around the corner.

AUTHOR CONTRIBUTIONS

JB conceptualised the article. JB, JM, and C-SK drafted the article. JM prepared the figures. All authors approved the final version.

FUNDING

The Tommy's Charity funds the Tommy's National Centre for Miscarriage Research. JJB is a holder of a Wellcome Trust Investigator Award (Grant/Award Number: 212233/Z/18/Z), University Hospital Coventry and Warwickshire (UHCW) NHS Trust provided further financial support.

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frontiers in Reproductive Health

REVIEW
published: 03 January 2022
doi: 10.3389/frph.2021.795464

Genetic Regulation of Transcription in the Endometrium in Health and Disease

*Sally Mortlock, Brett McKinnon and Grant W. Montgomery**

Institute for Molecular Bioscience, The University of Queensland, Brisbane, QLD, Australia

The endometrium is a complex and dynamic tissue essential for fertility and implicated in many reproductive disorders. The tissue consists of glandular epithelium and vascularised stroma and is unique because it is constantly shed and regrown with each menstrual cycle, generating up to 10 mm of new mucosa. Consequently, there are marked changes in cell composition and gene expression across the menstrual cycle. Recent evidence shows expression of many genes is influenced by genetic variation between individuals. We and others have reported evidence for genetic effects on hundreds of genes in endometrium. The genetic factors influencing endometrial gene expression are highly correlated with the genetic effects on expression in other reproductive (e.g., in uterus and ovary) and digestive tissues (e.g., salivary gland and stomach), supporting a shared genetic regulation of gene expression in biologically similar tissues. There is also increasing evidence for cell specific genetic effects for some genes. Sample size for studies in endometrium are modest and results from the larger studies of gene expression in blood report genetic effects for a much higher proportion of genes than currently reported for endometrium. There is also emerging evidence for the importance of genetic variation on RNA splicing. Gene mapping studies for common disease, including diseases associated with endometrium, show most variation maps to intergenic regulatory regions. It is likely that genetic risk factors for disease function through modifying the program of cell specific gene expression. The emerging evidence from our gene mapping studies coupled with tissue specific studies, and the GTEx, eQTLGen and EpiMap projects, show we need to expand our understanding of the complex regulation of gene expression. These data also help to link disease genetic risk factors to specific target genes. Combining our data on genetic regulation of gene expression in endometrium, and cell types within the endometrium with gene mapping data for endometriosis and related diseases is beginning to uncover the specific genes and pathways responsible for increased risk of these diseases.

Keywords: endometrium, transcription, gene expression, disease, genetic regulation, hormones, menstrual cycle

OPEN ACCESS

Edited by:
Philippa Saunders, University of Edinburgh, United Kingdom

Reviewed by:
Deepak Modi, National Institute for Research in Reproductive Health (ICMR), India
Silvia Vannuccini, University of Florence, Italy

****Correspondence:***
Grant W. Montgomery
g.montgomery1@uq.edu.au

Specialty section:
This article was submitted to Gynecology, a section of the journal Frontiers in Reproductive Health

Received: *15 October 2021*
Accepted: *06 December 2021*
Published: *03 January 2022*

Citation:
Mortlock S, McKinnon B and Montgomery GW (2022) Genetic Regulation of Transcription in the Endometrium in Health and Disease. Front. Reprod. Health 3:795464. doi: 10.3389/frph.2021.795464

INTRODUCTION

Human endometrium lines the inner surface of the uterus and plays a vital role in female reproduction and maintenance of pregnancy, providing the receptive microenvironment for embryo implantation and placental development. Endometrium is composed of several cell types including luminal and glandular epithelial cells, endometrial stromal cells, vascular cells and immune cells (1). In preparation for embryo implantation endometrial stromal fibroblasts (ESCs) terminally differentiate to secretory decidual stromal fibroblast cells (DSCs) and in the absence of conception the tissue undergoes controlled shedding, tissue repair, re-epithelialisation, regeneration and remodelling (2). This process is cyclical, averaging 25–30 days in length, and is controlled by ovarian steroid hormones (2, 3). During the menstrual cycle the endometrium is continuously undergoing cellular proliferation, differentiation and structural remodelling in response to circulating steroid hormones. These changes in cellular function and composition reflect the changing roles of this dynamic tissue and can be broadly defined into stages of endometrial development. An initial proliferative phase is characterised by endometrial tissue regeneration and cellular proliferation that prepares for embryo implantation and preceds ovulation. It is followed by the secretory phase with development of more complex glands, spiral arteries and stromal oedema designed to support a developing embryo in response to progesterone secreted by the corpus luteum (4, 5). In the absence of pregnancy the functional layer is shed during the menstrual phase before repair and regeneration commences again (1).

It is important to understand the complex regulatory processes influencing gene expression in the endometrium and relationships to endometrial structure and function, fertility, and reproductive pathologies. Gene expression in the endometrium is dominated by events across the menstrual cycle and influenced by hormonal regulation and changing cellular composition (**Figure 1**). Patterns of expression for individual genes show marked variation (6, 7), with expression of some genes high in the proliferative phase and then decreasing in the secretory phase or the reverse patten with low expression in the proliferative phase and increasing later during the secretory phase. Some genes are on for only a few days and others show variable patterns of expression detected only in a proportion of individuals (7). In addition to the cycle changes observed in most individuals, epigenetic signatures (8, 9) and the expression of many genes is under genetic control in endometrium (**Figure 1**) and other tissues contributing to variation between individuals (6, 7, 10).

The purpose of this review is to discuss the regulation of gene expression in endometrium. It is not intended to provide

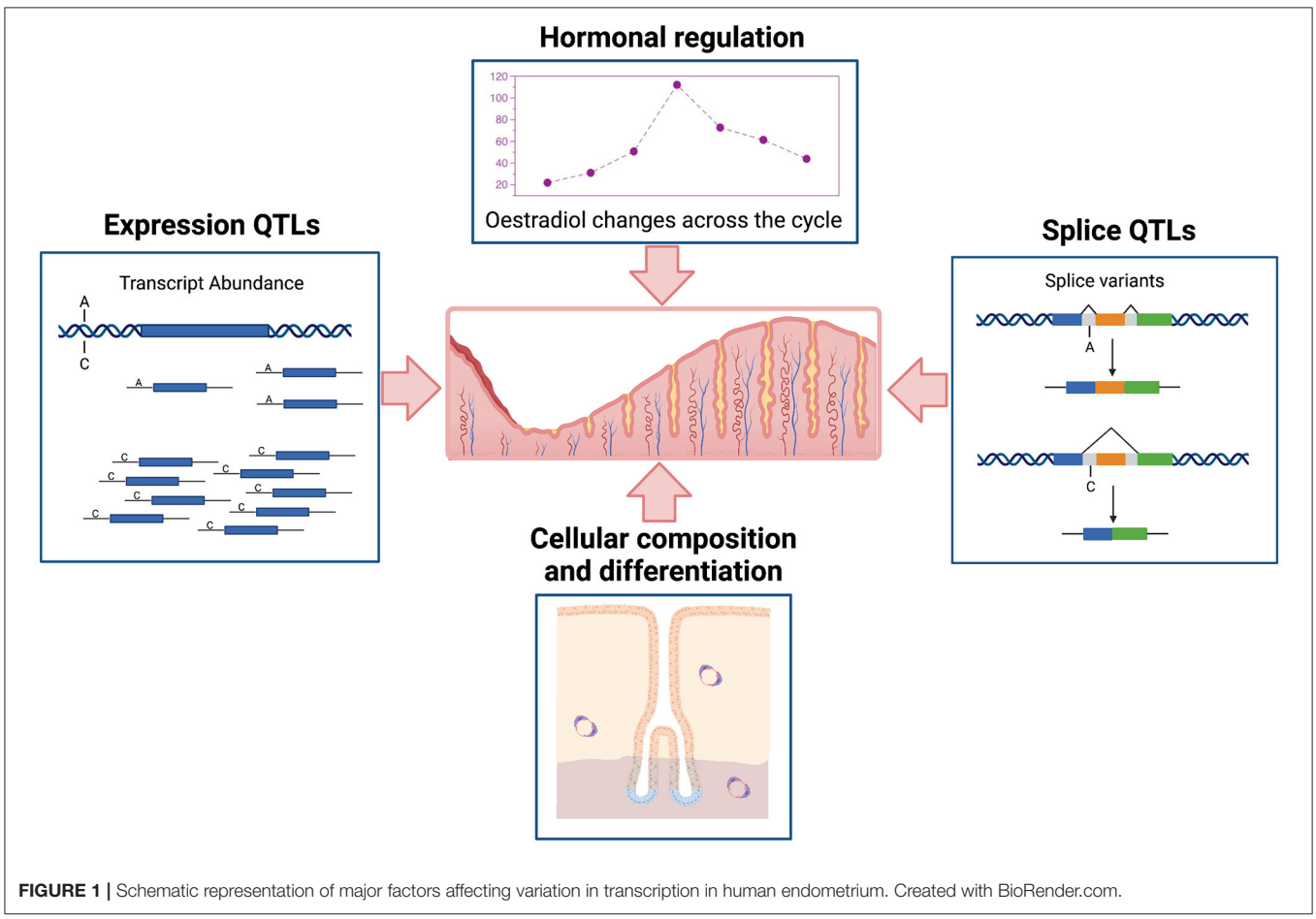

FIGURE 1 | Schematic representation of major factors affecting variation in transcription in human endometrium. Created with BioRender.com.

a systematic review of the endometrial gene expression literature, but to review major factors influencing the expression of genes in the endometrium and lessons from recent studies in other tissues.

HORMONE REGULATED ENDOMETRIAL GENE EXPRESSION

The complex cellular anatomy and diverse functions of human endometrium are reflected in the dynamic nature of endometrial gene expression. Analysis of gene expression measured in eutopic endometrial samples using microarray and RNA-seq technologies has shown significant differences in the expression of thousands of genes across the menstrual cycle (6, 7, 11–14). Studies show that these differences in gene expression occur both at the level of mean expression and differences in gene activation i.e., the proportion of samples expressing individual genes. An average of 62–66% of genes expressed in endometrium were expressed in >90% of samples however, the remaining 34–38% of genes were transcribed in varying proportions of samples (6, 7). Evidence suggests that the expression of more than 30% of genes in the endometrium differ significantly in mean expression or in the proportion of samples expressing each gene across the menstrual cycle. These genes are enriched in hormone response pathways, transcription factor targets and epithelial mesenchymal transition pathway (6).

The biggest differences in gene expression are observed between the proliferative and secretory phases of the menstrual cycle and between stages (early, mid and late) within the secretory phase of the cycle (6, 12). Transition between the proliferative to the early secretory stages has been characterised by the upregulation of genes involved in metabolic processes, negative regulation of cell proliferation, hormone response and secretion. Down-regulated genes are enriched in cell cycle regulation and cellular mitosis and division pathways (6, 12, 13). Subtle changes have also been reported within the proliferative phase which is characterised by healing and cell proliferation (6, 15). Genes upregulated in the proliferative phase have roles related to cell proliferation, differentiation, tissue remodelling, immunomodulation and angiogenesis (14, 15). Differences in expression between the early and mid-secretary phases likely reflect the cellular and molecular events governing endometrial receptivity and preparation for implantation. Upregulated genes are involved in cell adhesion, motility and communication, growth factor and cytokine binding and signalling, the immune and inflammatory responses and hormone response. Down-regulated genes are involved in cell division (6, 13, 14). Finally the transition between the receptive mid-secretary to late-secretary phase is characterised by preparation of the tissue for desquamation and menstruation reflected in changes in expression of genes involved in alterations of the extracellular matrix, the cytoskeleton, cell motility, communication and adhesion, vasoconstriction, immune response, wound healing and inflammatory mediation (6, 13, 14).

Menstrual cycle phase is a major source of variability in endometrial datasets and consideration must be given to apply appropriate corrections for cycle phase when analysing data generated from endometrial samples. Observed changes in gene expression across the menstrual cycle are likely mediated by a combination of changes in cell composition and response to changing levels of circulating hormones. The expression of some genes in the endometrium is reported to change in response to fluctuating levels of steroid hormones oestrogen and progesterone (12, 13, 16, 17). Response to circulating hormones, oestrogen and progesterone, is mediated through several hormone responsive genes, regulators and mediators and has been reviewed in detail elsewhere (1, 18). Oestrogen receptor (*ESR1*) and progesterone receptor (*PGR*) have been shown to be vital to maintaining healthy gene regulatory networks in the endometrium (18). *PGR* plays an important role regulating cell differentiation and proliferation through extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) and Protein Kinase B (AKT) pathways, and its target genes (e.g., *IHH, HOXA10, IGFBP1, STAT3, FOXO1, SOX17*) are required for successful implantation and decidualisation (18, 19). *ESR1* regulates endometrial epithelial proliferation, promotes stromal cell differentiation and is critical for endometrial receptivity and decidualisation through its induction of cytokines, IGF1 signalling, Wnt/β-catenin signalling, FGF signalling, ERK-MAPK signalling and PGR signalling (18, 20). The importance of hormonally driven regulatory pathways in healthy endometrial function is reflected by their dysregulation in endometrial pathologies including endometrial cancer (21) and endometriosis (18). However, not all genes with variable expression across the menstrual cycle are correlated with hormone levels or hormone receptors (*ESR1, PGR*) suggesting hormonal regulation alone cannot explain all the variation in gene expression (6, 22). Inter-individual variation in gene expression not explained by menstrual cycle stage is likely the result of a combination of cell type composition, genetic regulation and environmental effects.

Regulation of genes in the endometrium plays a vital role in female fertility. The Human Gene Expression Endometrial Receptivity database (HGEx-ERdb) is a compilation of datasets that provides information about the expression of >19 k genes in human endometrium during various phases of the menstrual cycle and in other conditions (23). Genes with consistent patterns of differential expression in endometrium during the receptive phase have been classified as receptivity associated genes (RAGs). RAGs play a role in the regulation of pathways that facilitate the structural and functional modifications required for successful embryo implantation (23). Several RAGs have been identified as potential biomarkers for endometrial receptivity (23–25). Biomarkers for endometrial receptivity can be used as diagnostic tools in reproductive medicine. One such tool, the endometrial receptivity array (ERA), can be used to diagnose receptivity in women with recurrent implantation failure (RIF) to guide decisions around personalised embryo transfer as a therapy (26). Receptivity signatures continue to be refined further to define different transcriptomic signatures within the receptive phase that are associated with clinical outcomes such as successful pregnancy (27). There are also extensive changes in endometrial gene expression profiles in response to embryonic signals and physiological changes during pregnancy (28, 29). *In vitro* studies in human endometrial stromal cells have shown changes in

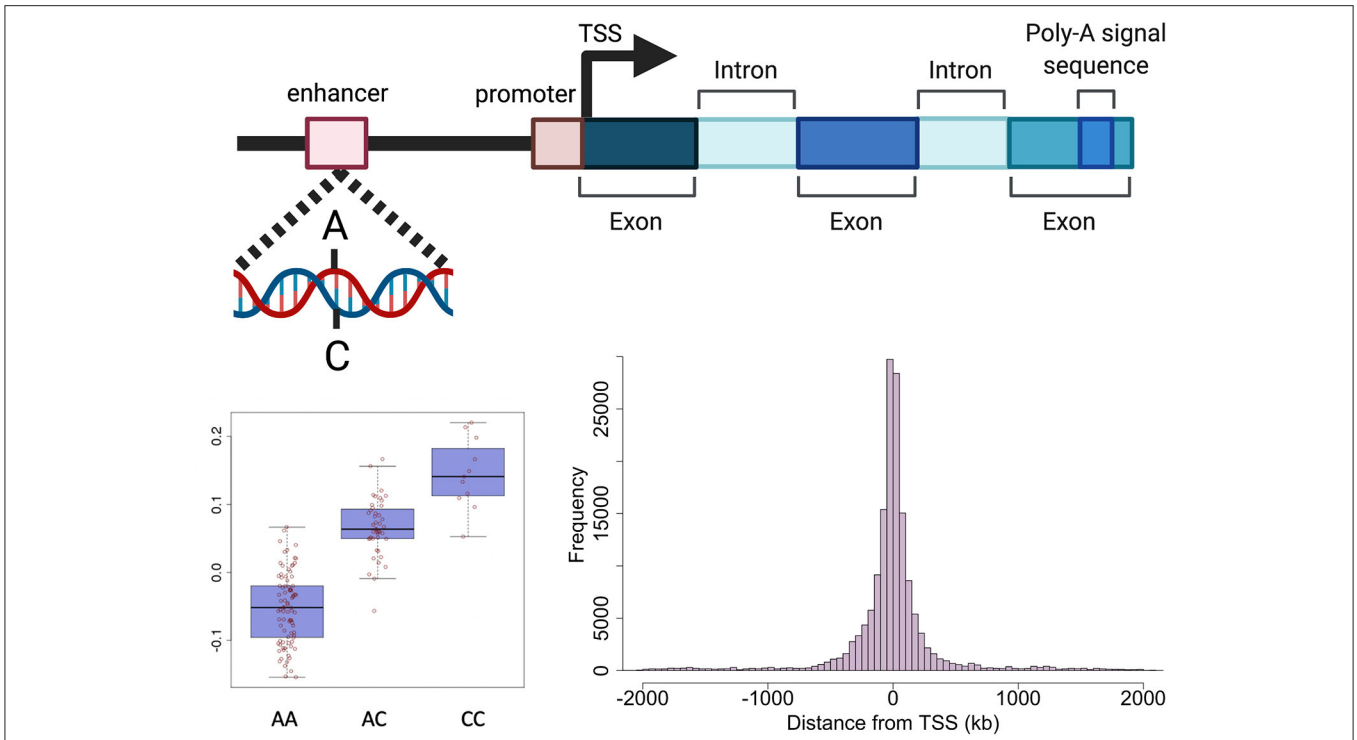

FIGURE 2 | Schematic representation of genetic regulation of gene expression. Expression quantitative trait loci (eQTL) describe the association between genetic variation and variation in the level of gene expression. The top panel depicts how a genetic variant located in a regulatory region (e.g., enhancer) can effect the level of expression of a nearby gene. The presence of a particular allele can be associated with an increase or decrease in expression of the gene as shown in the boxplot in the bottom left panel. The histogram in the bottom right panel shows the distance between variants effecting gene expression and the target gene in human endometrium. Created with BioRender.com.

expression of thousands of genes in response to trophoblast cells including induction of immune and angiogenic pathways (30, 31).

GENETIC REGULATION OF TRANSCRIPTION

Expression Levels

Genetic variants can effect transcription through various mechanisms including, but not limited to, altering promoters, transcription factor (TF) binding sites, enhancers, regulatory ncRNAs, RNA splicing and UTRs (important for post-translational regulation) (32). The expression of a large proportion (>80%) of genes expressed in tissues is regulated by genetic variation defined as expression quantitative trait loci (eQTLs) (10, 33). An eQTL denotes the association between a genetic variant (eSNP) and expression levels of mRNA transcripts of either a nearby gene (*cis*) or distant gene (*trans*) (**Figure 2**). cis-eQTLs are commonly located close to transcription sites (**Figure 2**) with 70% of eSNPs within 300 kb of the gene transcription start sites (6, 10, 34, 35). Consortium efforts have generated large eQTL datasets in multiple human tissues including 49 tissues from GTEx (33) and in blood (10). The majority of eQTLs (>70%) observed in smaller eQTL studies are shared between tissues. The recent large eQTL meta-analysis for blood (eQTLGen) derived gene expression analysis from 31,684 individuals and identified *cis*-eQTLs for 88% of autosomal genes expressed in blood (10). The replication rate for these eQTL in GTEx tissues excluding blood was much lower (15%) than previous studies. This may be a power issue, but could suggest eQTLs with smaller effects may be more tissue specific. The median pairwise correlation of eQTL effect sizes (r_b) between tissues is estimated as 0.55 across GTEx tissues and higher within biologically similar tissues such as skin tissues ($r_b = 0.80$), arterial tissues ($r_b = 0.74$) (36, 37) and between brain tissues ($r_b = 0.94$) (38).

Cis-eQTLs for 627 genes have been identified in endometrium using microarray and RNA-seq technologies (6, 7, 13). The most recent endometrial eQTL study was conducted using RNA-seq data generated from the endometrial samples of 206 European women identifying significant genetic effects on the expression of 444 genes (7). Mapping of endometrial eQTLs was performed independent of menstrual cycle phase, with cycle phase included as a covariate in the analysis. Compared to larger eQTL datasets like eQTLGen which identified >6,000 *trans*-eQTL genes, smaller endometrial eQTL datasets have had limited power to detect distal genetic effects on gene expression in the form of *trans*-eQTLs. *Trans*-eQTLs for only 28 genes have been validated between studies (6, 7). Subsequent context specific analyses did not detect any eQTLs with effects that differed between menstrual cycle phases or pathologies. Importantly, several genes used to assess endometrial receptivity on the ERA

had evidence of genetic regulation suggesting an individual's genetic background may also influence the appropriate time for embryo transfer (6).

The majority (85%) of cis-eQTLs in endometrium were also reported in other tissues including ∼72% detected in blood however, eQTLs for 61 genes appear to be specific to endometrium (7). Endometrial eQTLs were highly correlated with other reproductive tissues such as ovary and uterus (7). Interestingly, the effects of eQTLs in endometrium were also highly correlated with digestive tissues ($r_b > 0.67$), possibly reflecting similarities in tissue structure, cell composition, and functions between the tissues (7). Overlap in expression profiles and genetic regulation between endometrium and other tissues may underpin some comorbid relationships between endometrial disorders and other diseases through shared genetic risk loci. Studies have reported epidemiological associations and genetic correlation between subfertility and gastrointestinal disease (39) and between endometriosis and abnormalities in gastric mucosa (40, 41), uterine fibroids (42) and ovarian cancer (43).

Splicing

Genetic variants can also regulate splicing of mRNA transcripts in addition to regulation of gene expression level. Splicing is a process whereby pre-mRNA is spliced at different sites to produce multiple mRNA isoforms that include or exclude different exonic sequences (44). Alternative splicing (AS) has been estimated to occur in 95–100% of human mRNA that contain >1 exon (45). Comparisons of AS between tissues have shown that 47–74% of splicing events show variation between tissues and 10–30% show individual-specific variation (46). The ability to map transcripts using RNA-seq data and correlate splicing events with genetic variants has allowed identification of splicing quantitative trait loci (sQTLs) (47, 48). *Cis*-sQTLs have been identified in multiple tissues in the GTEx data, 210,485 sQTLs affecting 6,963 genes (sGenes) were identified across 48 tissues averaging 1,158 genes per tissue (49). Overall 44% of protein coding genes had an sQTL compared to eQTLs identified in 95% of protein coding genes (49). In the GTEx data, there is a high correlation of sQTL effect sizes between biologically similar tissues and overall 66% of sGenes are shared between tissues, similar to that observed for eQTLs (49). sQTL sharing analysis has shown that reproductive tissues (uterus, ovary, vagina) cluster together alongside arterial and gastrointestinal tissues. eQTLs are only observed for 52% of genes with sQTLs and in particular, tissue specific sQTLs do not necessarily have tissue-specific gene level expression highlighting the importance of characterising regulation of gene expression at the different levels and in relevant tissues (49). To date there has been no comprehensive analysis of sQTLs in endometrial tissue. Data available from GTEx shows a total of 182,070 sQTLs have been identified in uterus for 12,800 sGenes, 94% of these sGenes also had sQTLs in ovary or vagina compared to 66% in blood. sQTL mapping in endometrium using available RNA-seq and genotype data is ongoing.

Methylation

Epigenetic mechanisms such as DNA methylation can regulate transcription by recruiting methyl-CpG-binding proteins involved in gene repression or by inhibiting the binding of specific transcription factors (50). Variation in DNA methylation (DNAm) profiles in the endometrium across the menstrual cycle have been reported (8, 51–53), although these changes are less marked than observed changes in gene expression. Differentially methylated sites have been shown to correlate with expression of nearby genes in endometrium and are enriched for genes also reported as differentially expressed across the menstrual cycle (8, 51, 52). Genetic variation has been associated with methylation at 4,546 CpG sites in endometrium, defined as methylation quantitative trait loci (mQTLs) (8). 414 endometrial mQTLs were associated with the expression of 186 genes suggesting genetic regulation of gene expression in endometrium can also be mediated through methylation. Potential endometrial specific mQTLs have been annotated to genes with roles in hormone responsive proliferation, maintenance of cell structural integrity and adhesion and endometrial receptivity (8). Genetic regulation of methylation in endometrium has also been associated with reproductive traits including endometriosis, age at menopause and ovarian cancer (8). One example on chromosome 2 features a variant (rs11674184) associated with both endometriosis and methylation at cg16908938, a CpG site located in an intron of *GREB1* (8). This same variant is associated with alternative splicing of *GREB1* in GTEx data (49).

CELL SPECIFIC REGULATION OF TRANSCRIPTION

The functions of complex tissues, such as the endometrium, are facilitated by the interaction of multiple cells with divergent roles. Each cell has a unique life cycle from maturation to programmed cell death and are often categorised based on their developmental pathway, degree of maturation and resulting form and function, all of which are driven by a programmed course of gene expression that will underpin their individual role within the tissue. Expression levels for each gene in the endometrium therefore reflects not only the range and proportions of different cell types present within the sample but also their degree of differentiation, maturation and current state of activation (**Figure 3**). Genetic regulation of gene expression occurs in a cell type specific manner (54–56) and evidence is emerging that this is also the case with cell state (57). Characterising the changing cellular composition, as well as the developing cellular state and identifying how this interacts with the genetic influence on gene expression will be required to understand endometrial transcription, endometrial function and how perturbations in this mechanism contribute to endometrial disease susceptibility (**Figure 3**).

The endometrium functions through contributions from epithelial, stromal, immune and vascular cells. The dynamic nature of the tissue means that the relative composition and state of these cells is in a constant state of flux. Endometrial stromal and epithelial cells form the majority of the 5–7 mm thick multi-functional tissue (58). In response to changing progesterone concentrations endometrial stromal cells undergo transcriptional reprogramming, potentially through the regulation of promyelocytic leukaemia zinc finger (PLZF) to

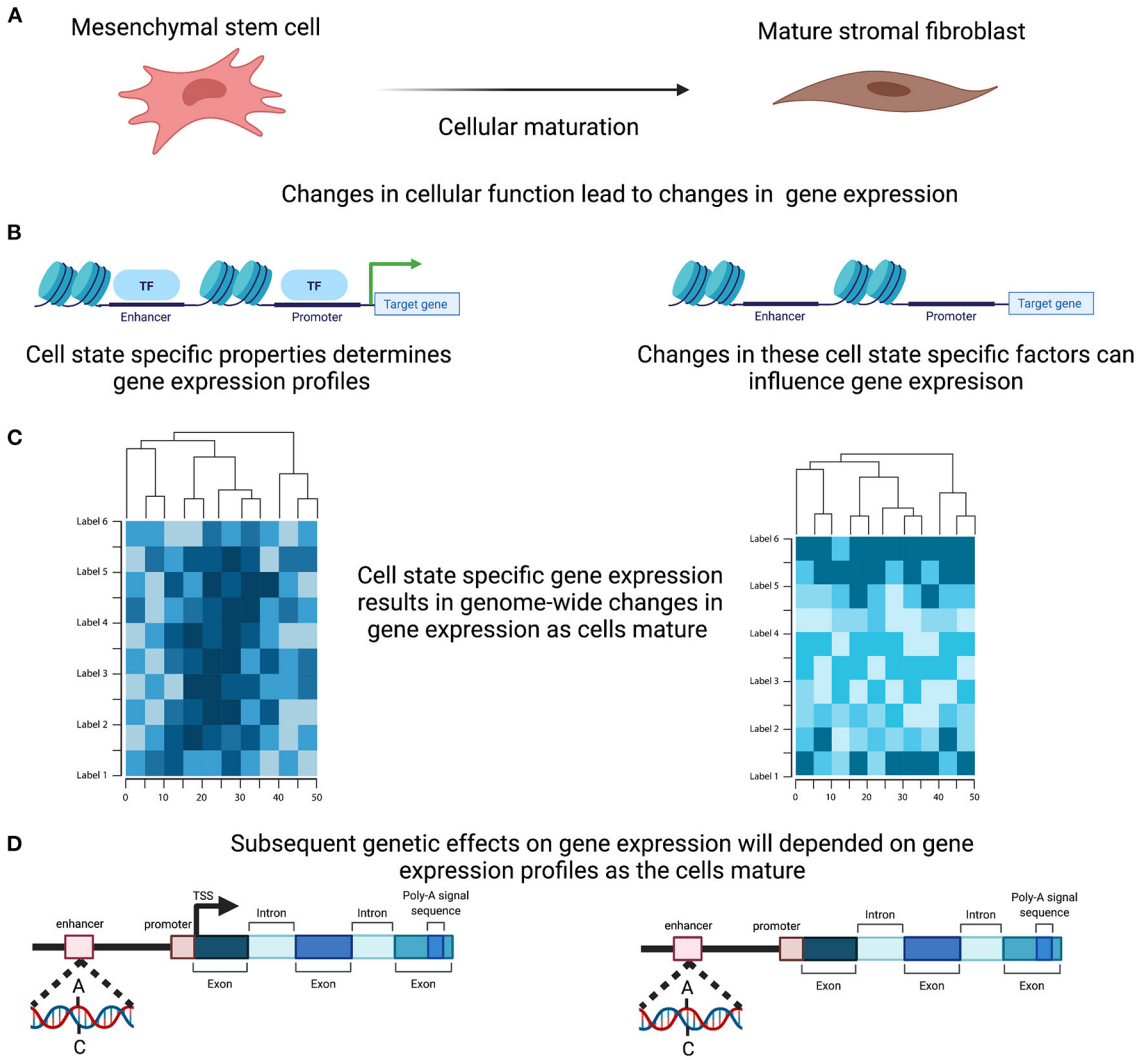

FIGURE 3 | Cell Specific genetic effects on gene expression. **(A)** Cells within the endometrium, such as the mesenchymal stem cells, undergo proliferation and maturation each menstrual cycle to generate a mature cell. **(B)** Maturation is controlled by a programmed course of gene expression that results in cell specific properties and are reflected in alteration in the active transcription. **(C)** In combination and across the genome these active change in expression will induce distinct genome wide gene expression profiles. **(D)** Genetic effects on transcription therefore will ultimately induce differing effects as the cells mature. Created with BioRender.com.

drive decidualisation, altering both gene expression signature and consequent function (59). The process of decidualisation encompasses a complex network of regulatory processes involving hormonal, biochemical, immunological and molecular factors recently reviewed by Ng et al. (60). Important molecular regulators of decidualization include Homeobox A10 (*HOXA10*), Wnt Family Member 4 (*WNT4*) and Forkhead box O1 (*FOXO1*) which targets transcription of decidual genes Prolactin (*PRL*) and insulin-like growth factor-binding protein 1 (*IGFBP1*) (60–62). Similarly, epithelial cells can be directed toward luminal, or glandular, ciliated or non-ciliated epithelial cells, mediated by divergent differentiation (63). Both tissue resident immune cells and immune cells that are derived from the transient infiltration from systemic circulation in response to changes in oestrogen and progesterone concentrations (64) will also influence endometrial cellular composition. Endometrial regeneration is also accompanied by a restoration of vascular integrity and angiogenesis (65), expanding the endothelial component. Cellular composition and the proportions of different cell types can be altered significantly by different pathologies (66).

The dynamic nature of the endometrial tissue means that these divergent cell types also present with a broad continuum of cell states. Endometrial regeneration is initiated from just a few endometrial mesenchymal stem and epithelial progenitor cells that remain in the basalis layer after menstruation (67–69). The eMSC are derived from perivascular locations (69) representing a subset of CD140+/CD146+ pericytes (70) with

a gene expression signature that is highly dependent on the microenvironment (71) and may contribute essential cytokines and growth factors to the stem cell niche (72). Additionally, within this niche there is emerging evidence of the presence of additional pluripotent, very small embryonic-like (VSEL) stem cells, identified in reproductive tissue of humans such as testis (73), as well as the endometrium of mice that may contribute to the regeneration of functional endometrium, as well as the regeneration of their damaged endometrial tissue (72).

In response to secreted molecules and extracellular matrix signals from the stem cell niche environment (74), regulatory changes are initiated that facilitate gene activation and stimulate cellular differentiation toward a cascading progression of cell states. Differentiation and maturation of each cell from their progenitor is therefore, both driven and accompanied by, changes in their gene expression profile. Genetic variants that influence the ability of these regulatory mechanisms of gene expression, through the mechanisms discussed above, will have significant influence on the transcriptomic signatures, timing of cellular maturation and may contribute to subtle variations that lead to endometrial pathologies. Better understanding the interaction between genetic variants and cellular development will be vital to understand the regulation of gene transcription in tissues.

Unravelling the composition and contribution of individual cells within complex tissue is however challenging. To study the genetic influence on cells individually requires the ability to focus on individual cells within a complex mixture. For endometrium we are yet to establish a comprehensive cell atlas to examine cell composition across the changing stages of the menstrual cycle. Although admiral efforts are being made these still require more than single patients to capture the natural variability of the population (75). Using whole excised endometrial tissue inhibits the possibility of directly studying the contribution of each cell to the tissue gene expression signature. Additionally, the site of tissue biopsy may contribute to variation in the cellular composition of each sample and/or variation between individual participants in a study. Conversely, physical dissociation of individual cell types and subsequent gene expression analysis result in the removal of the niche environmental factors that modulate cell state and its associated gene expression signatures. It is also a labour intensive process that requires significant work to collect the sample numbers required for studying the effects mediated by genetic regulation. A number of experimental designs and novel techniques are however being utilised to overcome these challenges.

Single-Cell Transcriptomics

Single-cell RNA-sequencing offers a new avenue to investigate cellular heterogeneity of endometrial tissue and assess the influence of genetic variants in individual cells and cell types. Early studies using single-cell transcriptomics to profile endometrial cell populations across the menstrual cycle identified seven main cell types based on clustering and expression of canonical markers and differentially expressed genes. These cell types included stromal fibroblasts, endothelial cells, macrophages, lymphocytes, ciliated and unciliated epithelial cells and smooth muscle cells with mesenchymal stem cell characteristics (75). Different expression profiles within each cell type were detected across the cycle including epithelial and stromal profiles likely to characterise the transition between early and late proliferative and early secretory endometrium, as well as the transition into the window of implantation with the upregulation of known receptivity genes (75). Sub-populations of stromal and epithelial cells have also been identified (76), some of which have been associated with endometrial pathologies (77, 78). Ma et al. (78) observed that characteristics of the eutopic endometrium between women with and without endometriosis were generally similar, however there was evidence of differences in the cell subtypes reflected by gene expression (78), suggesting that some inconsistencies in observed differences in gene transcription in bulk endometrial tissue may be confounded by cellular heterogeneity. Single-cell transcriptomic studies in endometrium have been limited by small sample sizes and the type of endometrial sample used which may not fully capture all relevant endometrial cell types.

Combining single-cell transcriptomic data with genome-wide genotyping information also provides the opportunity to assess the genetic regulation of gene expression in individual cells, cell types and the impact on changes in cell state. Recent evidence in skin fibroblasts from 79 donors found the majority of eQTLs were specific to cell subtypes and reprogramming these cells into iPSC resulted in almost all eQTL disappearing entirely (57), suggesting genetic regulation is dynamic across both individual cells and during cell maturation. This is an emerging opportunity to be investigated in endometrial tissue. While providing powerful new insights, the depth of sequencing produced by single cell sequencing limits the data to only the highly expressed genes, often missing genes that only require low expression to initiate cascading events, thereby limiting the potential to identify important changes. Both the 5' and 3' amplification procedure also limits the potential to examine the genetic influence on splicing. Techniques that can perform high throughput are yet to be developed.

Bioinformatic Deconvolution and Cell Type Enrichment

Computational methods have been developed in the last 10 years to dissect cellular heterogeneity and account for its influence on tissue gene expression profiles (66, 79, 80). Two main approaches include, deconvoluting cellular composition and enumeration of cell subsets, and assessing the enrichment of indivual cell types. These methods use additional data from a "reference" gene set from purified cell types or single cell RNA sequencing to define cell-type specific gene signiatures. Computational methods to deconvolute bulk gene expression data enable some cell type specific inferences, but their accuracy depends on the availability of expression profiles for relevant cell types. In addition, several sources of variation influence the use and interpretation of methods of cellular decomposition of bulk RNA-seq data containing mixed cell types (81). The performance of deconvolution methods varies with cell type, source laboratory and tissue. The methods are strongly influenced when individual cell profiles and mixed tissue sample originate from different

laboratories and when profiles are generated from single cell sequencing (81). Careful consideration is needed when applying these approaches to endometrial data without well-characterised endometrial cell type specific gene signatures. Studies are underway to dissect the cellular heterogeneity in endometrium by adapting existing approaches and incorporating signatures from endometrial cell-types using sorted cell expression data and single-cell RNA-seq (82).

Multiplexing Spatial Transcriptomics

Spatial transcriptomics offers the potential to identify transcriptome expression at a single cell level while maintaining spatial resolution and capturing the niche influences. Currently it is limited because of the expense for individual slides. However, methods are being developed that will allow multiple samples to be included on individual slides. Accompanying this data with genome-wide genotype information will map changes in cell state associated with changes in genetic regulation.

ASSOCIATION BETWEEN GENETIC RISK FACTORS FOR DISEASE AND TRANSCRIPTION

Gene expression is an indicator of cellular state and misregulation of gene expression can be indicative of disease. Gene expression signatures in the endometrium have been associated with endometrial traits and disease. Evaluation of gene expression in the receptive phase has identified signatures for recurrent implantation failure characterised by downregulation of genes involved in cell cycle regulation and cell division and cytoskeleton and cilia formation (83). Obesity has also been associated with significant transcriptional changes during window of implantation (WOI) which may contribute to lower implation rates seen in obese women (84). Transcriptional dysregulation in proliferative-to-secretory transition and during the WOI in endometrium of women with moderate/severe endometriosis has also been reported (85, 86). Differences in gene expression in eutopic endometrium between women with and without endometriosis have also been reported however, candidate endometriosis susceptibility genes have failed to replicate between studies. In larger studies with greater power, significant differences in expression between women with and without endometriosis are not detected following correction for menstrual cycle stage and appropriate correction for testing multiple gene signatures (6, 7, 53). Allowing for variation induced by the individual genetic background could explain and be applied to reduce the inconsistencies.

Genetic variants regulating transcription in endometrium have been associated with several reproductive traits and diseases. SNPs regulating gene expression have been associated with age at menopause, age at menarche, endometriosis, polycystic ovarian syndrome (PCOS), endometrial cancer and ovarian cancer (6, 7). Formal statistical tests should be used to determine if the same causal SNP effects both endometrial gene expression and the disease trait. Methods include Bayesian colocalization analyses such as COLOC (87) and transcriptome-wide association analyses (TWAS) such as Summary-data-based Mendelian Randomisation (SMR) (88), PrediXcan (89) and TWAS-Fusion (90).

Transcriptome-wide association analyses (TWAS) assess the association between the expression of each gene and a trait. In the absence of gene expression data from a large sample the expression of genes can be predicted using eQTL information or a reference set containing expression data and its association with genetic variants (91). SMR is a Mendelian randomisation approach that integrates eQTL and GWAS summary statistics to identify associations between gene expression and complex traits and also applies a heterogeneity test to distinguish pleiotropy from linkage (88). Endometrial eQTLs have been used in SMR analyses alongside summary statistics from a range of reproductive traits and diseases identifying several genetic variants regulating both expression of genes in endometrium and traits. Integration of GWA summary statistics for endometriosis (92) and endometrial eQTLs using SMR identified significant associations between the expression of three genes in endometrium, Long Intergenic Non-Protein Coding RNA 339 (*LINC00339)*, Vezatin (*VEZT)* and FYVE, RhoGEF And PH Domain Containing 6 (*FGD6)* and risk of endometriosis (6, 7, 93). Given the large overlap of eQTLs between endometrium and blood, large blood eQTL datasets can also be used as a proxy for genetic regulation of expression in endometrium, such approaches identifying association between genetic regulation of expression of *VEZT*, Cell Division Cycle 42 (*CDC42)*, *LINC00339* and endometriosis (7, 92). Similar analyses identified genetic regulation of transcription in endometrium is associated with other reproductive traits and pathologies including expression of Neighbour of BRCA1 LncRNA 2 (NBR2) and Copine 1 (CPNE1) and age at menopause and expression of Leucine Rich Repeat Containing 37A (LRRC37A), Leucine Rich Repeat Containing 37 Member A2 (LRRC37A2) and Charged Multivesicular Body Protein 4C (CHMP4C) and epithelial ovarian cancer (7).

Other TWAS methods predict genome-wide expression into a GWAS dataset using the weighted effect of each SNP on each cis-gene from a reference set and then test the association between levels of expression and the trait (91). A TWAS performed using estimates of the genetic effects of gene expression in endometrium and endometriosis GWA summary statistics identified 252 genes in 33 loci associated with endometriosis including 28 loci that had not previously been identified as genome-wide significant (7). Many loci identified by the TWAS contained several genes whose expression was correlated highlighting that not all risk loci may have a single target gene. This was also shown in a recent analysis of the chromosome 6q25 risk locus near *ESR1* which showed that expression of genes in this region were highly correlated and that these genes are likely co-regulated (22). This approach has the potential to identify target genes for a range of other endometrial pathologies and fertility traits for which GWA summary statistics are available.

To better understand the mechanisms by which genetic variants are effecting gene expression and disease, analytic approaches have been developed to integrate GWAS and eQTL

data with other molecular traits including protein expression, splicing, methylation and various epigenic marks (94). Risk variants associated with both expression and disease can also be functionally annotated using epigenic databases, such as EpiMap (95), RoadMap (96) and ENCODE (97) however, these databases lack epigenetic data from endometrial relevant tissues and cell-types. Previous investigations of the interactions between endometriosis risk SNPs in the 1p36.12 locus and candidate target genes using chromosome conformation capture (3C) in Ishikawa cell lines suggest that endometriosis risk SNPs interact with the promoters of both *LINC00339* and *CDC42.* Subsequent luciferase reporter assays suggested the risk SNP rs12038474 was located in a transcriptional silencer for *CDC42* and the risk allele increases expression of *CDC42* in blood (93). More recently, promoter associated chromatin looping from HiChIP analysis in an endometrial cancer cell line provided evidence of an interaction between a variant in the 1p36.12 locus associated with endometrial cancer, endometriosis and pelvic organ prolapse, and promoter regions of *CDC42* and *WNT4* (98). Expression of *LINC00339* has also been reported in endometriotic lesions and perturbation of *LINC00339* expression in endometrial stromal cells was shown to alter expression of genes in immune defence pathways (99).

Identification of genes associated with disease risk in endometrium can also provide insight into putative pathogenetic pathways that can be targeted for disease prevention, management and treatment. Genes functionally annotated to endometriosis risk loci have roles in hormone metabolism, cell cycle regulation, proliferation and adhesion (92). Oestrogen-responsive growth regulation by oestrogen in breast cancer 1 (*GREB1*) is an essential component of the oestrogen receptor transcription complex (100), risk variants have been associated with *GREB1* splicing, epigenic regulation and TF binding sites (8, 101). Risk variants for endometriosis on chromosome 6p25.1 are located in regulatory regions near oestrogen receptor 1 (*ESR1)* and both VEZT and FGD6 on chromosome 12q22 have roles in cell adhesion (102, 103). *CDC42* is involved in cell cycle regulation with evidence suggesting that *FGD6* activates *CDC42* to coordinate cell adhesion (104). Genetic regulation of genes involved in maintaining the endometrial environment via regulation of cell proliferation and immune response (*PAEP, SPP1, IL15, TSPAN8, OLFM1, MMP7* and *CXXC1*) have also been associated with endometrial receptivity, fecundity and implantation failure (6, 105).

Formal overlap between GWAS signals and eQTLs identifies some likely candidate genes, but the proportion of clear relationships between genetic risk factors and functional candidates has been disappointing. There are several possible explanations. As noted above, a high proportion of eQTLs for the small endometrial studies, and in GTEx, are common to many tissues (6, 7, 33). Some argue that eQTLs of large effect, and common to many tissues, may be neutral or have limited functional effects under steady state conditions (106). These eQTLs are rarely associated with genes that are intolerant to loss of function mutations (10, 106). Consequently, genes regulated by these eQTLs can vary in expression level, or in protein-coding sequence, with limited functional effects and are therefore not subject to negative genetic selection. Umans et al. (106) suggest we may have more success using a dynamic regulatory approach mapping eQTLs in model systems subject to experimental stimulation. This will uncover tissue or cell specific eQTLs more likely to have functional effects associated with disease risk. Studies in endometrium may satisfy this experimental approach because of the highly variable gene expression across the menstrual cycle (6, 7). While our studies are still relatively small, we found few context-specific eQTL, where genetic effects on gene expression were identified at only one stage of the menstrual cycle and we did not see evidence to support the dynamic regulatory approach.

It is estimated that, compared to *cis*-eQTLs, *trans*-eQTLs explain the majority of heritability in gene expression (10, 107). The large eQTLGen study had greater power to detect *trans*-eQTL and tested overlap between 10,317 GWAS signals for complex traits and *trans*-eQTLs. A high proportion (37%) of GWAS signals were associated with *trans*-eQTL effects. *Trans*-eQTLs are considered to be more specific for individual tissues and cell types and analysis of available single-cell data sets for blood showed nominal replication of 84% of the disease associated *trans*-eQTL effects (10). Taken together, results suggest we need to identify more tissue and cell-type specific *cis*- and *trans*-eQTLs and splice variant QTLs to understand how genetic risk factors change gene regulation and increase disease risk.

SUMMARY AND CONCLUSIONS

Gene expression is influenced by the external and internal environment through neuronal, hormonal and other signalling pathways and by genetic and epigenetic factors. In addition to the dynamic cyclical changes in cellular structure and function, the human endometrium has complex mechanisms regulating gene transcription. Understanding and controlling for sources of variation in endometrial transcription is critical. Critical sources of variation include accurate determination of stage of the menstrual cycle, genetic effects on expression level and splicing and variable cell composition and heterogeniety.

Genome-wide gene expression studies have characterised significant changes in endometrial gene expression and activation of genes, across the menstrual cycle. These strong effects of menstrual cycle stage on transcription in the endometrium likely reflect changes in cell composition and response to circulating steroid hormones. Independent of menstrual cycle phase, genetic variation between individuals is also associated with the level of expression of >600 genes in endometrium of which the majority of effects are shared between tissues and are most highly correlated with biologically similar tissues. Epigenomic analyses in endometrium indicate transcriptional variation can also be mediated by genetic regulation of methylation. The ability to identify more subtle, tissue-specific, genetic effects on regulation is limited by the power and size of studies, sample heterogeneity and context.

Endometrium is made up of multiple cell types and changes in cell composition and activity change across the menstrual

cycle. Understanding cell-type specific genetic effects on gene expression is challenging and functional effects may require intercellular communication between more than one cell types. Methods of cell-type deconvolution and single-cell sequencing are being adapted and applied to studies in endometrium to investigate cell-type specific regulation.

Evidence suggests that genetic regulation of endometrial gene expression (eQTL) contributes to reproductive traits and diseases. Effects of genetic variation on RNA splicing (sQTL) and distal genes (*trans*-eQTL), and cell-type specific genetic effects, are also enriched for variants associated with complex traits and diseases highlighting new directions for investigation. Understanding which genes and pathways should be targeted in which cell type can be used to improve fertility and disease management.

A comprehensive understanding of factors affecting regulation of transcription in the endometrium and endometrial cell-types is vital for accurate analysis and interpretation of data from endometrium across biological and disease contexts. Hormonal, genetic, epigenetic and cell-type specific regulation of gene expression can influence menstruation, fertility and endometrial pathologies making it vital for researchers and clinicians to consider an individual's genetic background and hormonal influences when investigating, assessing and managing fertility and disease.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

This work was supported by the National Health and Medical Research Council of Australia (grants GNT1026033, GNT1049472, GNT1105321, GNT1078399, GNT1147846, and GNT1177194) and Medical Research Future Fund Research (Grant MRF1199785).

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frontiers in Reproductive Health

REVIEW
published: 06 January 2022
doi: 10.3389/frph.2021.801843

Mechanisms of Scarless Repair at Time of Menstruation: Insights From Mouse Models

*Phoebe M. Kirkwood, Isaac W. Shaw and Philippa T. K. Saunders**

Centre for Inflammation Research, The University of Edinburgh, Edinburgh, United Kingdom

The human endometrium is a remarkable tissue which may experience up to 400 cycles of hormone-driven proliferation, differentiation and breakdown during a woman's reproductive lifetime. During menstruation, when the luminal portion of tissue breaks down, it resembles a bloody wound with piecemeal shedding, exposure of underlying stroma and a strong inflammatory reaction. In the absence of pathology within a few days the integrity of the tissue is restored without formation of a scar and the endometrium is able to respond appropriately to subsequent endocrine signals in preparation for establishment of pregnancy if fertilization occurs. Understanding mechanisms regulating scarless repair of the endometrium is important both for design of therapies which can treat conditions where this is aberrant (heavy menstrual bleeding, fibroids, endometriosis, Asherman's syndrome) as well as to provide new information that might allow us to reduce fibrosis and scar formation in other tissues. Menstruation only occurs naturally in species that exhibit spontaneous stromal cell decidualization during the fertile cycle such as primates (including women) and the Spiny mouse. To take advantage of genetic models and detailed time course analysis, mouse models of endometrial shedding/repair involving hormonal manipulation, artificial induction of decidualization and hormone withdrawal have been developed and refined. These models are useful in modeling dynamic changes across the time course of repair and have recapitulated key features of endometrial repair in women including local hypoxia and immune cell recruitment. In this review we will consider the evidence that scarless repair of endometrial tissue involves changes in stromal cell function including mesenchyme to epithelial transition, epithelial cell proliferation and multiple populations of immune cells. Processes contributing to endometrial fibrosis (Asherman's syndrome) as well as scarless repair of other tissues including skin and oral mucosa are compared to that of menstrual repair.

Keywords: hypoxia, endometrium, mesenchyme to epithelial transition (MET), inflammation, cytokine, angiogenesis, scarless

OPEN ACCESS

Edited by:
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Reviewed by:
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Gregory Burns, Michigan State University, United States

****Correspondence:***
Philippa T. K. Saunders
p.saunders@ed.ac.uk

Specialty section:
This article was submitted to Gynecology, a section of the journal Frontiers in Reproductive Health

Received: *25 October 2021*
Accepted: *08 December 2021*
Published: *06 January 2022*

Citation:
Kirkwood PM, Shaw IW and Saunders PTK (2022) Mechanisms of Scarless Repair at Time of Menstruation: Insights From Mouse Models. Front. Reprod. Health 3:801843. doi: 10.3389/frph.2021.801843

INTRODUCTION

The endometrium is unusual amongst adult tissue in that it exhibits an unparalleled capacity for rapid scar-free repair, which occurs at the end of each non-fertile cycle during the phase known as menstruation. Menstruation is the culmination of vascular, cellular and inflammatory changes which leaves the luminal surface in a "wounded" state (1). In order to limit blood loss and regain tissue function for the subsequent cycle, rapid re-epithelialisation and structural re-organization is

required and occurs without the accumulation of any functional damage or fibrotic scar tissue (1–3). Although the human endometrium is the only adult tissue that undergoes regular and repeated cycles of destruction and repair under normal physiological conditions (4) parallels may be drawn between mechanisms of post menstrual endometrial repair and the wound healing response of the oral mucosa which also heals without a scar (5–7).

Whilst morphological and cellular changes that occur during the various phases of the human menstrual cycle have been well documented and extensively studied much less is known about the temporal and spatial changes in tissue function that occur during the menstrual phase due to the challenge of timing collection of human tissue during this phase (8). One of the most revealing studies to document the appearance of the endometrium at the time of menses used a combination of a hysteroscopic, histological and scanning electron microscopy. Examination of the surface of the endometrium during initial phases of menses revealed that tissue shedding and repair was not uniform but rather a "piecemeal process" which occurred simultaneously in regions throughout the uterine cavity with the authors suggesting the stromal compartment played an important role (9). One of the most well established mechanisms triggering tissue breakdown is the rapid fall in progesterone which occurs with involution of the corpus luteum in a non-fertile cycle (8). Progesterone also plays a pivotal role in stimulating changes in gene expression and cell function resulting in transformation of the stromal cells so that they secrete factors essential for successful implantation–a process collectively known as decidualization (10). During the normal cycle decidualization is limited to the luminal (functional) layer of the endometrium and this is also the region of tissue shed at menstruation. The occurrence of menstruation is associated with spontaneous decidualization, as opposed to decidualization induced by a fertilization event, and is limited to the higher-order primates including humans, four species of bat, the elephant shrew (11) and a Spiny mouse species (*Acomys cahirinus* (12, 13).

Studies in animal models have included those in primates that spontaneously menstruate such as the baboon (14) as well as species such as the macaque where menstruation can be induced by hormonal manipulation (15, 16). These models have been a valuable complement to studies on human tissue providing an opportunity to harvest samples that include the full thickness of the endometrium at defined timepoints during progesterone withdrawal to explore differences between gene expression in basal and functional zones (17). The classic studies undertaken by Markee (18) used rhesus tissue grafted into the ocular cavity allowing for direct observation of vasoconstriction in the spiral arteries. Notable results from primate studies have included time-dependent expression of metalloproteinases in endometrium (15) (14) and endometrial grafts (19), studies on hypoxia and expression of angiogenic factors such as VEGF (20, 21). Other models have included transplantation of human endometrial tissue into immunocompromised mice and *in vitro* culture of human tissue explants (16).

In addition to the clear intrinsic benefits of improved understanding of the mechanisms that regulate endometrial shedding and repair, this understanding is the basis for development of improved therapies for disorders such as heavy menstrual bleeding and endometriosis (1, 22). The endometrium may also serve as an exemplar of scarless repair with the potential to inform comparative studies and improve our understanding of chronic disease processes such as fibrosis.

WHAT ARE THE ADVANTAGES AND BARRIERS TO USING MICE FOR STUDIES ON ENDOMETRIAL REPAIR?

Whilst the uterus of rodents and women share a common architecture (luminal and glandular epithelial layers, complex stroma, myometrial muscle layers) the common, inbred species of laboratory mice and rats have relatively short "oestrus" cycles with four phases (proestrus, oestrus, met-oetrus, dioestrus) without spontaneous decidualization or cyclical tissue breakdown.

One of the incentives to develop and use mice in studies on endometrial function is the availability of a wide range of genetically modified animals including those using fluorescent protein to identify active promoters, to identify specific cell populations, and targeted deletion of genes either ubiquitously, in a cell-specific manner or following timed induction (23, 24). For example, in *Pdgfrb*-BAC-eGFP mice GFP is expressed under the control of the PDGFRbeta promoter (25) and a recent study has shown that the GFP is expressed in the cells of mesenchymal origin in the mouse endometrium mirroring the expression of the endogenous protein (26). Single cell gene expression analysis of GFP+ cells recovered from cycling endometrium of *Pdgfrb*-BAC-eGFP mice has identified five different populations of cells in the stroma including three transcriptionally distinct populations of fibroblast (26). Targeted deletion of the estrogen receptor alpha gene (*Esr1*) has been a powerful technique which when applied to studies on the mouse endometrium has provided novel insights into the importance of the stromal compartment in estrogen receptor dependent control of epithelial cell proliferation (27). Likewise our understanding of the pivotal role of progesterone in decidualization, fertility and regulation of downstream genes including those of the Wnt pathway has been illuminated by genetic manipulations involving the progesterone receptor gene (28).

DEVELOPMENT AND REFINEMENT OF MOUSE MODELS OF ENDOMETRIAL REPAIR (MENSTRUATION)

To overcome the critical limitation that mice lack a spontaneous decidualization response and provide a platform for improved understanding of the mechanisms regulating human menstruation mouse models based on hormonal manipulation have been developed: the most widely used involves ovariectomy of mice and was first reported by Finn and Pope in the 1980's (29), the second relies on induction of pseudopregnancy (30).

Ovariectomy Model of Endometrial Repair

Briefly, adult female mice are ovariectomised, allowed to recover for 7 days to deplete endogenous hormones, primed with a hormone schedule to mimic the fluctuating hormones experienced by women during the menstrual cycle (estrogen priming and progesterone administration) and the endometrium artificially stimulated to induce decidualization, a process normally initiated following the arrival of a blastocyst in this species. A number of variations on this model have been reported but in all cases tissue breakdown was, as in women, triggered by cessation of progesterone stimulation (simulating CL demise).

In the original model reported in the 1980s 7 days after ovariectomy mice received daily injections of oestradiol (E2, 100 ng/100 μl, 2 days); 3 days of rest, 3 days of injections of E2 (20 ng/100 μl) and progesterone (1 mg/100 μl) followed 4–6 h later by exposure of the uterus and intraluminal injection of peanut oil (29, 31). One problem with this model was variation in the extent of decidualization, however when it did occur removal of progesterone resulted in tissue breakdown accompanied by tissue necrosis, inflammation and luminal shedding (29). Notably the authors recorded changes in the stromal compartment which started with the congestion of dilated blood vessels followed by breakdown of the vessel walls and extravasation of blood. The basal area (outer ring) of the stroma proximal to the myometrium did not take part in the degenerative process but a central core of blood cells and degenerating decidual cells became detached and was shed into the lumen (29). Animals treated in exactly the same way but with the omission of the decidual stimulus did not show such changes in the stroma consistent with data from menstruating species which highlight the importance of stromal cell differentiation as a pre-requisite for the process of menstrual shedding (10).

The model was updated by the Salamonsen Group who modified the protocol to use inbred mice and to include a silastic progesterone-secreting pellet to replace progesterone injections thus ensuring a steadily increasing concentration of circulating progesterone, considered to be more comparable to what happens in women (32). Using this model decidualization was successfully induced in the uterine horns and endometrial breakdown was initiated 12–16 h following progesterone withdrawal. The entire decidua was detached and shed at 24 h and re-epithelialisation of the luminal surface was almost complete by 36 h. Notably this study was the first to define the endometrial breakdown and repair phase as being complete 48 h following withdrawal of progesterone (32). Wang and colleagues (33) investigated the critical time window for progesterone withdrawal using the Salmonsen group model with induction of decidualization by injection of acarchis oil into the lumen of one uterine horn on day 9 and removal of the pellet 49 h later. They reported that replacement of progesterone at 8 and 12 h after pellet removal blocked menstrual-like bleeding while replacement at 16–24 h had no effect and tissue shedding still occurred (33).

A further refinement to this model was reported by researchers in Edinburgh (**Figure 1**): specific changes included induction of decidualization by transvaginal injection of sesame oil directly into the uterine cavity thereby avoiding an additional abdominal surgery as well as an increase in the duration of progesterone administration (pellet in place) from 2 to 4 days after oil exposure ensuring a more robust and reproducible decidualization response (3, 34). In common with other reports shedding was maximal at 24 h after progesterone withdrawal, the luminal epithelial layer was typically intact at 48 h and tissue architecture resembled control intact mice by 72 h. Overt vaginal bleeding was recorded.

Menning and colleagues also reported overt bleeding following induction of decidualization by injection of sesame oil into the uterus followed by removal of a P4 implant 4 days later (35): they recorded time dependent bleeding which was maximal at 24 h and used a tampon collection system to quantify the amount of blood providing a platform for testing drugs including those that modulate angiogenesis (35).

Peterse et al. (36) conducted a study to see if they could maximize the amount of decidual tissue that could be generated for use in a syngenetic model of endometriosis (37) they compared the response when the intrauterine oil stimulus was delivered via the vagina or via surgical laparotomy with the idea that the latter might be more "traumatic" to the tissue and therefore potentially elicit a more robust response (36). Decidualization was achieved in more than 83% of mice with significantly higher rates of bicornate decidualization in the laparotomic group (89%) compared with vaginal administration (38%) suggesting the former was useful if the priority of the study was to maximize decidual material but at the cost of extra surgical intervention.

Pseudopregnancy Model

Pseudopregnancy can be achieved by mating intact female mice to vasectomised males combined with artificial induction of decidualization. For example, Fan et al. mated adult female CD-1 mice with vasectomized males of the same strain combined with direct injection of 20 μl sesame oil into each uterine lumen on day 4 of pseudopregnancy. On day 6 (2 days after oil injection), bilateral ovariectomy was performed to remove steroids and induce endometrial breakdown (30). When this pseudopregnancy model was used in combination with inhibition of Wnt7a endometrial repair was not normal with a failure of re-epithelialisation and degradation of the basal gland noted (38). In a modified version of the pseudopregnancy mouse model Rudolph *et al* administered the potent progesterone receptor antagonist mifepristone 2 days after induced decidualization instead of performing ovariectomy. This blockade mimicked progesterone withdrawal and stimulated decidual tissue breakdown. Bleeding was evaluated by vaginal lavage and was also visible at the opening of the vagina (39). Recently Wang et al used a pseudopregnancy model to investigate the impact of stress signals on menstrual breakdown (40) reporting that acute stress resulted in an increase in corticosterone contributing to more rapid breakdown and shedding of the endometrium.

In general the ovariectomy model is more widely used as it is very well established and usually considered to provide a more

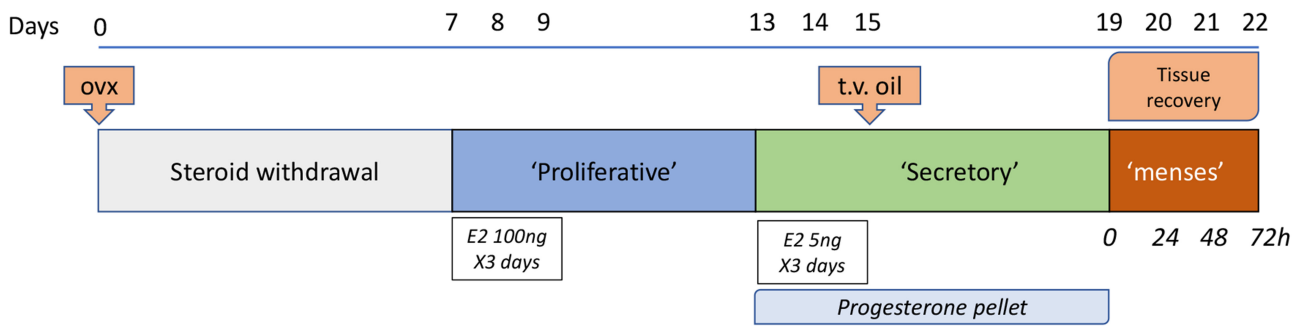

FIGURE 1 | Edinburgh Mouse model of menstruation. This model is based on the pioneering work of Finn and Pope (29) with several refinements including the use of a progesterone pellet (32) and induction of decidualization via a trans-vaginal [t.v.; (3)] route. Samples are recovered on day of pellet removal (time 0, full decidualization) and at times thereafter between 4 and 72 h. Twenty four hours after pellet withdrawal has been characterized as a time of maximal tissue breakdown but by 48 h the epithelial layer surrounding the uterine lumen is typically fully restored.

reliable and reproducible timeframe for endometrial breakdown and repair.

MECHANISMS IMPLICATED IN ENDOMETRIAL REPAIR IDENTIFIED IN MOUSE MODELS

Hormonal Regulation

In women the menstrual phase of the cycle is characterized by low circulating concentrations of ovarian derived oestrogens and progesterone suggesting that endometrial repair processes are steroid independent. This question has also been addressed in the mouse models of menstruation. The standard ovx+ mouse model of menstruation (**Figure 1**) is characterized by depletion of ovarian hormones with both shedding and repair occurring in the absence of endogenous oestrogens (3, 8). Kaitu'u-Lino and colleagues argued that other sources of oestrogens, including phytoestrogens in the diet and local metabolism in fat, might be available and the model could not be considered completely steroid-depleted (41). They therefore conducted the model using mice maintained on a soya-free diet and complementing this with administration of aromatase inhibitor letrozole (41). Importantly, no significant difference in the rate of endometrial repair was observed in the complete absence of estrogen, suggesting that this steroid was not essential for complete endometrial restoration in their model.

The presence of abundant androgen receptors in the stromal cells of the basal compartment, which remains intact during menses (42), and evidence for intracrine generation of bioactive androgens within endometrial tissue in response to decidualization (43) led Cousins et al to hypothesize that androgens could modulate the repair process even if the concentrations in blood were low (44). They administered a single injection of the potent bioactive, non-aromatizable androgen, dihydrotestosterone, in parallel with removal of the progesterone pellet. They reported that this treatment increased the duration of vaginal bleeding and delayed restoration of the luminal epithelium with striking spatial and temporal impacts on immunoexpression of MMPs 3 and 9 (44). These results may partially explain why women who have high androgen levels as a result of polycystic ovarian disease sometimes report heavy or extended bleeding during menses (45). Further investigation is required to pin down the precise role of androgens in the endometrial repair process.

Hypoxia and Angiogensis

Studies on human tissues and in primates have highlighted a role for hypoxia in regulation of endometrial repair processes and angiogenesis (46, 47). Withdrawal of progesterone is associated with an a marked increase in the synthesis of prostaglandins, increased arteriole vasoconstriction and a reduction in oxygen tension within the tissue (47). A key factor in sensing of oxygen tension in tissue is the transcription factor HIF1a (hypoxia inducible factor one alpha) (48, 49). Stabilization of HIF1α in human endometrial tissue has been detected during the secretory and menstrual phase and implicated in regulation of expression of genes involved in angiogenesis including IL8 (46, 50). In an *in vitro* model using human endometrial biopsies it has been shown that P4 withdrawal increased IL8 secretion but only in the presence of hypoxia (50). Coudyzer et al. published contrasting data from a xenograft model where fragments of human endometrium were engrafted to ovariectomised immunodeficient mice: in this model they could not detect evidence for increased HIF1α and concluded that hypoxia is not required to trigger menstrual-like tissue breakdown or repair in human endometrium (51).

The results from studies in the mouse models of menstruation have demonstrated that hypoxia occurs following progesterone withdrawal and that this is also associated with levels of HIF1a and changes in expression of angiogenic genes. For example, Cousins et al. used hypoxyprobe^{TM} to detect low oxygen levels and demonstrated dynamic changes in staining that were consistent with a striking increase in hypoxic conditions during the repair phase and time dependent changes in expression of angiogenesis-associated mRNAs encoded by *Vegfa, Cxcl12, Flt1,* and *Kdr* (34). These results have been complemented by investigations into the role of HIF which can have a dramatic impact on gene expression in low oxygen

tissue environments (52). Notably using genetic targeting of *Hif* and pharmacological intervention in combination with the Edinburgh mouse model of endometrial repair Maybin and collaborators were able to manipulate the duration of endometrial shedding simulating heavy menstrual bleeding in women (52) with data supporting manipulation of HIF as a therapeutic target for this prevalent disorder.

The importance of angiogenesis was also confirmed by Menning et al (35) who administered Cediranib, a potent VEGF receptor signaling inhibitor, to mice on days 8 to 15 of their protocol (from day of decidualization to pellet removal) showing a drastic 85% reduction in menstrual like bleeding in treated animals compared with controls.

Inflammation

The human endometrium hosts a diverse population of immune cells, the abundance and composition of which changes throughout the menstrual cycle. Menstruation has been classified as an inflammatory event because the mechanisms and cellular changes involved are similar to those observed during other physiological inflammatory responses including the increase in the expression of prostaglandins, cytokines and chemokines which are secreted by the decidual cells in response to progesterone withdrawal (50, 53–55). The production of these factors is believed to stimulate the influx of inflammatory cells such as neutrophils and macrophage/monocyte populations (56, 57). Notably induction of excess inflammation in model systems has been shown to be associated with dysregulated repair and fibrosis (58) and may underlie some endometrial pathologies including heavy menstrual bleeding (8). Studies using the mouse models of menstruation have facilitated detailed time-dependent and spatial analysis of the inflammatory process and how it relates to both initiation and resolution of the endometrial menstrual "wound" with some of them highlighted below. In the 1980's Finn and Pope reported that one of the first changes in the decidualized mouse endometrium following cessation of progesterone was infiltration of leukocytes into the stroma (31). Subsequent studies have used a wide variety of methods to study the inflammatory response including immunohistochemistry and flow cytometry (3, 35, 56), GFP-labeling of monocyte lineages (23) as well as antibody-dependent cell depletion (59). For example, in their 2003 paper Brasted et al (32) used an antibody directed against CD45 (leukocyte common antigen) to interrogate uterine tissue recovered 0, 12, 16, 20, 24, 36 and 48 h after removal of the progesterone pellet (P withdrawal). Their analysis identified leukocytes in decidualized tissue often in close association with the luminal epithelium, throughout the basal zone and close to the newly regenerated epithelium at later time points. Notably they identified some of these cells as macrophages based on their morphology (32). Manning and colleagues used flow cytometry to analyse tissue digests recovered at 0, 24 and 72 h time points. They reported a massive increase in of CD45+ cells so that they comprised ∼10% of the decidua at time zero (mostly NK cells, macrophages and granulocytes) with a striking increase in granulocytes (Gr1+/F480) making up 90% of immune cells during maximal tissue shedding (24 h). Armstrong et al compared tissue sections from human and mouse stained with antibodies directed against neutrophil elastase or GR1 respectively to focus on the neutrophil subtype of granulocytes demonstrating they increased at 8 hours after progesterone withdrawal and at 24 h (56) mimicking results in women and in agreement with other data from ovx models (23, 35, 56). Cousins et al used transgenic "Macgreen" mice in which enhanced green fluorescent protein (EGFP) is expressed under the control of the c-fms promoter (encodes CSF-1R) expressed in the monocyte phagocytic lineage in the mouse (60) as well as some neutrophilic granulocytes (60). Using this lineage marker they were able to shed new light on the dynamic changes in monocyte derived immune cells over the course of tissue breakdown and repair [**Figure 2** (23)]. One of the main findings from their study was that distinct populations of "classical" monocytes (GFP+F4/80–), monocyte-derived macrophages (GFP+F4/80+) and a population of putative tissue-resident macrophages (GFP–F4/80+) that became localized to different regions within the tissue during breakdown, repair and remodeling suggesting cells of the monocyte lineage may play distinct roles in these processes (23). The recent application of single cell sequencing analysis of human endometrial tissue is likely to complement these findings by identifying immune cell subpopulations although datasets have not had sufficient depth of read to enable this (61).

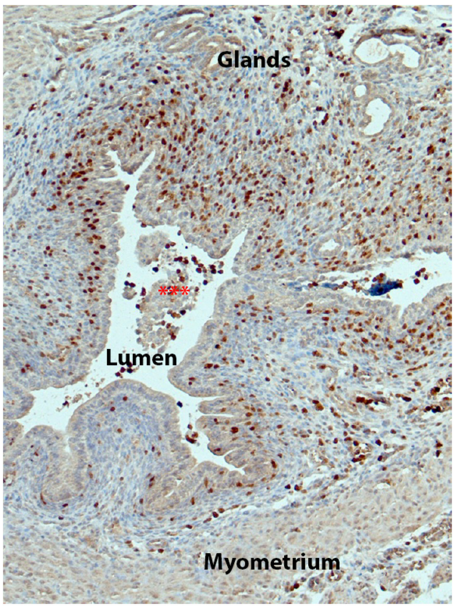

FIGURE 2 | Immune cells of the monocyte/macrophage lineage increase in the mouse endometrium during tissue breakdown. Figure shows endometrium from a Macgreen mouse 24 h after progesterone withdrawal with immune cells identified by immunostaining of GFP (brown, fluorescent images of similar tissues are shown in (23)). Note that there are abundant GFP+ cells in the stromal compartment with many adjacent to the newly intact luminal epithelium.

The role(s) of immune cell populations have also been investigated using antibody depletion. For example, using the anti-mouse GR-1 antibody Menning and colleagues reported that cells positive for this marker (assumed to be neutrophils) played

a role in regulating the expression of matrix modifying enzymes such as MMP3, 9 and 10 and their depletion impaired tissue repair (35). In another study cells were depleted using the anti-GR1 (clone RB6-8C5) antibody and a delay in endometrial repair reported that was concluded to be a consequence of neutrophil depletion (59). The anti-GR1 (clone RB6-8C5) antibody binds to both Ly6G expressed solely on neutrophils and Ly6C expressed on neutrophils, monocytes and subsets of CD8 T cells (62) and therefore cannot be considered to be specific to one of these cell types. Notably Cousins et al (23) used the same RB6-8C5 clone in their studies and reported that many of the GFP+(Ly6G-) monocytes they detected were also GR1+ hence depletion with this antibody is likely to target cells in addition to neutrophils and the immunostaining performed by Armstrong may need to be reevaluated (56).

In women there are well-documented increases in inflammatory chemokines and cytokines that coincide with the withdrawal of progesterone including CCL2 (MCP-1), CXCL8 (IL-8), IL-6, TNF, and COX-2 all showing increased expression in the late secretory and menstrual phases of the menstrual cycle (reviewed in (8)). Complementary studies in the mouse models have extended these findings. For example, Menning et al highlighted the very rapid and transient increase in expression of *Cxcl2*, *Ccl3*, *Tnf*, *Il6* and *Ccl2* (35). Other studies reported similar findings for *Ccl2*, *Il6* and *Cxcl8* (56).

Increased prostaglandin biosynthesis is also an important regulator of inflammatory processes during menstruation (8) that has been explored in the mouse "menstrual" models. In mice as in women induction of a menstrual like event is associated with increased expression of COX-2, an inducible enzyme that acts as a key regulator of the biosynthesis of prostaglandins from arachidonic acid (35, 63). Xu and colleagues used the mouse ovx model to demonstrate that administration of either a nonspecific COX inhibitor (indomethacin) or the COX-2 selective inhibitor DuP-697 led to less influx of leukocytes and inhibition of the menstrual-like process (63).

Tissue Breakdown

Studies in human tissue have highlighted a critical role for enzymes including matrix metalloproteinases in the destruction of the extracellular matrix (ECM) which is an essential step in tissue breakdown and shedding (64, 65). Historically, elegant studies in rhesus monkeys demonstrated a rapid rise in MMPs (stromelysins/matrilysin; MMP7, MMP3, MMP10) in the luminal portion of the endometrium in response to progesterone withdrawal (19).

Using their mouse model Kaitu'u-Lino *et al* examined the distribution of MMPs revealing an important role for MMP7 and MMP9 during endometrial tissue breakdown, and MMP3 and MMP7 during re-epithelialisation (66). However treatment of mice with the MMP inhibitors doxycycline and batimistat, both of which effectively reduced MMP activity, did not appear to have significant effects on endometrial breakdown or repair (66). The mouse model has been further used to demonstrate dynamic expression and functional importance of ECM interactions (67) and the expression of activin A in specific epithelial and stromal cell populations which may have a role in regulating re-epithelialisation (68). In their 2012 paper Menning et al documented dynamic time-dependent changes in mRNAs encoding Mmp1, 2, 3, 7, 9, 10, and 11. Cousins et al identified changes in the spatial and temporal expression of both MMP9 and MMP3 during the breakdown and repair phases (3, 12) which appeared consistent with the influx of immune cells known to produce MMPs highlighting the ability of the models to recapitulate changes seen in human tissue.

Epithelial Migration and Proliferation

Kaitu'u-Lino *et al* also used the mouse model to explore the role(s) of epithelial proliferation and progenitor cells in endometrial repair (69, 70). In one study newborn mouse pups were pulse-labeled with bromodeoxyuridine (BrdU) and chased for 5 week before decidualization, endometrial breakdown, and repair were induced (70). In the second study adult mice were also pulse labeled with BrdU immediately after induction of the same model. They reported that very rapid dilution of bromodeoxyuridine label was observed in the luminal epithelium consistent with rapid proliferation, whereas label within the glandular epithelium remained constant. In contrast during the later repair phase glandular epithelial cells had a decrease in detectable BrdU. The authors concluded that a population of epithelial progenitor cells may reside in the basal glands and contribute to postmenstrual repair (69).

In the studies by Cousins et al. they also reported rapid proliferation of epithelial cells including those remaining at the un-denuded surface of the luminal epithelium as well as some stromal cells and epithelial cells surrounding glands (3). In the conclusion of their paper they suggested that re-epithelialisation involves epithelial cell proliferation, epithelial cell migration and transformation of a subpopulation of stromal cells into those with epithelial characteristics in areas where the surface was denuded of epithelial cells (3). These studies provide new ideas about the mechanisms that might operate in parallel to ensure rapid repair of the luminal epithelial cell layer but require further interrogation and testing.

Mesenchyme to Epithelial Transition (MET)

One of the most striking features of endometrial shedding in women is the piecemeal loss of epithelium resulting in areas of denuded stroma (9). In mice the shedding of the decidual mass is not as piecemeal but it does result in areas of denuded stroma and it was in this region of the endometrium that Cousins and colleagues detected stromal cells which co-expressed vimentin and cytokeratin during the most active phase of endometrial repair (24 h after progesterone withdrawal) (3). These authors also analyzed the expression of genes implicated in mesenchymal-to-epithelial transition across the time course of the repair process with evidence of changes in expression of regulatory genes including *Wt1* and members of the snail/slug family that are known to play a role in regulation of MET (3). Whether this is a transient change in the stromal population or is part of their differentiation into a functioning epithelium required further investigation.

Studies on postpartum endometrial repair have also provided evidence that MET occurs (71, 72). Specifically, the authors used

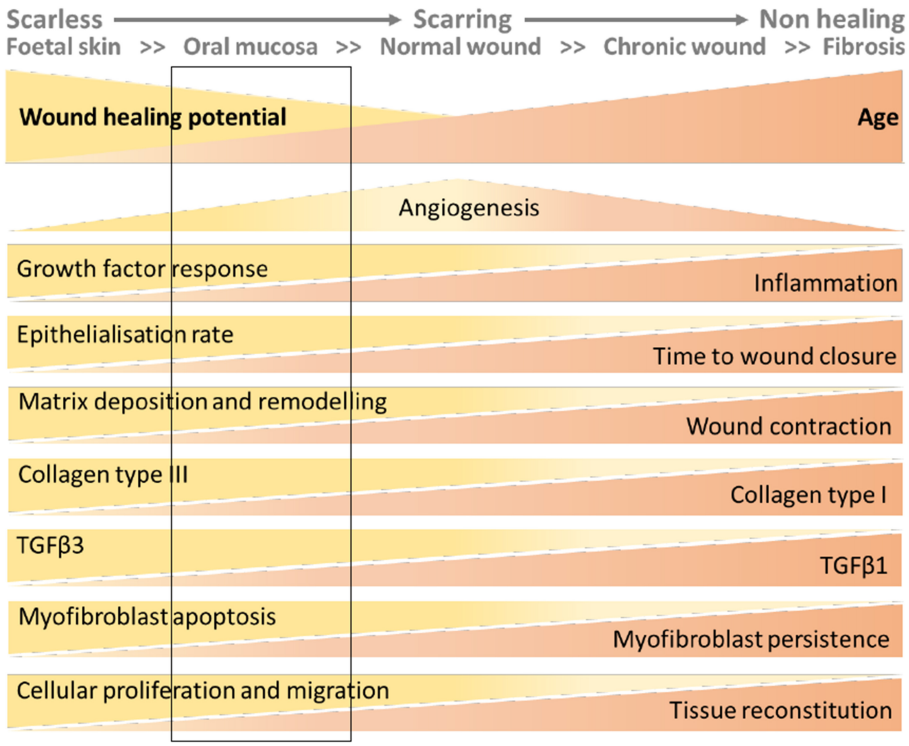

FIGURE 3 | Wound healing continuum with inclusion of putative location of endometrial tissue based on data from the mouse models. The figure has been adapted from that published in (100). The rectangular box added to the figure represents the characteristics of endometrial wound repair based on the interrogation of the mouse models described in this review.

genetic manipulation to allow for fate mapping of uterine cells expressing *Amhr2* using beta-galactosidase (71) or EGFP (72). In both studies positive signal (blue/EGFP) was restricted to stromal cells and myometrium in normal cycling mice but following parturition when there is extensive damage to the endometrial tissue, some of the labeled cells transformed into cells with epithelial characteristics, including expression of cytokeratin, and became incorporated into the luminal and glandular epithelial cell layers (71). In their 2013 study Patterson *et al* also used the mice in combination with the pseudopregnancy menses model described above and reported co-localization of vimentin (stromal marker) and cytokeratin (epithelial marker) in cells within the basal zone close to the myometrial border that peaked at 48 h post-ovx (72). Despite the location of these putative MET cells being different to that reported by Cousins (3) likely reflecting differences between the two models, these data further support a role for MET in post-menstrual repair.

A recent paper by Ghosh et al. (73) challenged the idea that MET was involved in maintenance and regeneration of the epithelium of the endometrium and oviduct. Specifically, they conducted a comprehensive examination of embryonic and adult reproductive tracts using LacZ reporter lines driven by promoters for *Amhr2, Sm22, Cspg4, Thy1,* and *Pdgfrβ* to explore whether epithelial cells expressing reporter protein arose in adulthood from MET or had an embryonic origin because they were induced at a time when cells had meso-epithelial characteristics. In all cases they attributed epithelial expression of the reporter protein in adulthood to activation of the promoters during embryonic life ruling out MET in adult cycling mice (73).

Some of the findings summarized above are consistent with endometrial stromal cells having an inherent "plasticity" to change their phenotype from that of mesenchyme to one more consistent with epithelium. In addition to the studies on the menstrual models it is notable that decidualization might be considered as a form of hormone-induced MET with endometrial stromal fibroblasts acquiring epithelioid characteristics, such as expanded cytoplasm, rough endoplasmic reticulum, and a reorganized actin cytoskeleton (30). We postulate that this feature of endometrial mesenchymal cells may be an important contributor to the resilience of the endometrium to acute insults such as the breakdown and shedding of endometrium at the end of each menstrual cycle but further studies including those using lineage tracing are required to confirm this.

Progenitor/Stem Cells

Cells with stem cell-like properties, such as high proliferative potential, multilineage differentiation ability *in vitro* (adipo-, osteo- and myo-genic), and expression of stem cell-associated markers, have been identified in the human endometrium [basal compartment, perivascular location, PDGFRβ+CD146+, SUSD2+; (74, 75)], but the precise contribution of these cells to cyclical endometrial repair mechanisms remains the

subject of intense investigation. Recent progress has included use of specific surface markers for isolation of progenitor/stem populations from tissue samples and menstrual effluent with novel applications proposed for regenerative medicine and tissue repair (76, 77). The role of stem/progenitor cells has also been investigated in the mouse model of menstruation although this has been challenging due to the lack of a specific lineage marker. A study by Kaitu'u-Lino *et al* using the LRC technique reported results suggesting that a population of epithelial progenitor cells might reside in the basal glands and that stromal LRC, located in a perivascular location could have an active role to play in endometrial repair (70). Despite evidence for the presence of multiple lineage-restricted stem/progenitor cell populations within the human/mouse uterus, the exact contribution to endometrial tissue repair remains elusive in part due to a lack of definitive markers. A recent study by Kirkwood et al identified an equivalent population of perivascular PDGFRβ+CD146+ cells in the mouse endometrium and demonstrated exclusive expression of NG2 (Cspg4) (26). The emergence of such novel identification markers will allow for their specific role in endometrial repair and regeneration to be interrogated.

CAN WE TRANSLATE KNOWLEDGE GAINED FROM STUDIES ON ENDOMETRIAL REPAIR TO TREAT ENDOMETRIAL FIBROSIS?

Endometrium repair is not always scar-free and intrauterine adhesions can occur as a result of a fibrotic response within the basal layer and is associated with poor pregnancy outcomes (78). The existence of these intrauterine adhesions is usually referred to as "Asherman's syndrome" (AS) with risk reported to be increased by repeated miscarriage, cesarean section and surgical removal of uterine contents [curatage; (79)]. Mouse models of AS have been developed by inducing a fibrotic response within the uterus by repeated "wounding" with a needle (80, 81). These models have been used to the test the ability of cell-based therapies to improve fertility, the rationale being that stem/progenitor cells may be involved in endometrial regeneration (82) and have been successfully applied for tissue repair in models of prolapse (77). One paper reported the use of human perivascular stem cells (hPVSCs) from umbilical cords was able to rescue the poor pregnancy outcome in AS mice via HIF1-dependent angiogenesis (83). Other studies have used mesenchyme cells derived from cultured human pluripotent stem cells (81) or from bone-marrow derived stem cells also with some promising results (84). A recent review considered a wider range of different sources of mesenchyme stem/stromal cells including menstrual blood [as discussed above, (76)] as well as evidence that extracellular vesicles secreted by these cells might also be considered as a cell free therapy for AS (85) which, given the logistical challenges of cell therapy, deserves further investigation.

Recently the importance of inflammatory pathways in the etiology of AS has gained more prominence (86) and this would be in agreement with their central role in endometrial repair (discussed above) as well as in the development of fibrosis in other tissues such as the liver (87). In a recent study immunostaining of endometrial tissue from 10 patients with AS identified not only increased amounts of fibrosis within the stromal compartment (collagen fibers and smooth muscle actin) but also alterations in macrophage phenotype (88). Changes in macrophage phenotype and pro-fibrotic cell changes are have also been identified in a mouse model of endometriosis (89), and in both disorders there appears to be potential for targeting macrophage phenotype/function as a novel therapy. Further insights from the mouse models of menstruation and comparison to those of AS may help refine the type(s) of immune and cell based therapies that can treat patients and improve their fertility.

COMPARISONS BETWEEN MECHANISMS OF TISSUE REPAIR IN THE ENDOMETRIUM, FETAL SKIN AND ORAL MUCOSA

Unchecked inflammation, fibrosis and scaring in response to tissue injury can result in significant tissue damage and associated morbidity (90). A number of studies have contributed to a greater understanding of the plasticity and heterogeneity of fibroblasts and their role in fibrosis (90). Whilst to date there has been little cross-over between studies using single cell analysis methods to explore fibroblast heterogeneity in fibrosis-prone tissues (90) and those using similar methods to interrogate endometrium in human (91) or mouse (26) this is clearly a topic that could be explored using existing data and bioinformatics to see if any of the endometrial cell subtypes have unique gene signatures. As the new single cell datasets have only recently been generated to date most attention has been paid to considering mechanisms that might explain scar-free healing of skin in the fetus and (92, 93) and lining of the mouth (94, 95) with a strong focus on exploring mechanisms that might be manipulated therapeutically in other sites (96).

A recent review summarized information obtained from studies using mice which have identified significant differences between gene expression in fibroblasts, deposition of extracellular matrix, the numbers of immune cells, expression of inflammatory regulators (IL33, prostaglandins) and metalloproteinases (MMPs) in fetuses where skin repair is rapid and scar-free (E15) and when scars are formed (E18/19) (93). In a detailed study using single cell fate mapping and 3D confocal imaging Jiang and colleagues identified two different fibroblast lineages that are responsible for the transition from scarless to skin scaring, again highlighting the importance of this cell type (92). Consistent with the results reported in fetuses repair of the oral mucosa also heals more rapidly than adult skin. In a recent study using nude mice, fibroblasts from the oral mucosa were shown to improve healing rates of adult skin wounds (97).

The inflammatory component of wound healing in the oral mucosa is associated with lower numbers of immune cells including macrophages, when compared to wounds of equivalent size in the adult skin, as well as decreased expression of the pro-inflammatory cytokines IL-6 and TGFβ1 (98). An animal model

that can augment our understanding of skin repair is the African Spiny mice (Acomys) where cutaneous repair in adults closely resembles that of fetal stages of laboratory mice. Notably in this species skin repair is also associated with less inflammation, reduced collagen secretion and reduced numbers of macrophages mirroring findings in fetal mice (99). These results appear at odds with the wound response of the endometrium in which progesterone withdrawal triggers increased expression of inflammatory cytokines as well as a rapid increase in the numbers of immune cells including macrophages (8) but this may reflect the difference in the time scale and tissue response involved with endometrium breaking down and shedding over days whereas studies on skin wounding have focused on acute, usually incisional insults. Further comparisons between the inflammatory responses in skin and endometrium will be useful in finding both similarities and differences.

In summary, endometrium, fetal skin and oral mucosa all heal more rapidly than adult skin. Fibroblasts play a key role in regulating the efficiency of the repair processes in all these tissues. If we represent wounding of the skin as a continuum from scar-free in the fetus to the non-resolving wounds associated with aging (100) the endometrium would appear to most closely align with that of oral mucosa with rapid repair but potential for fibrosis (**Figure 3**).

SUMMARY AND CONCLUSIONS

The endometrium is a remarkable tissue which may experience 400 cycles of repeated breakdown, shedding and repair during a woman's lifetime with restoration of tissue architecture so that it is able regenerate and transform into a receptive state to receive the blastocyst during the next menstrual cycle. Endometrial repair is tightly regulated both temporally and spatially and mal-adaptations to the mechanisms responsible result in disorders including heavy menstrual bleeding (inefficient repair?) and Asherman's syndrome (intrauterine fibrosis/excess repair?) (1, 8). Whilst the common laboratory species of mouse do not naturally experience menstrual cycles protocols based on manipulation of hormones, artificial induction of stromal cell decidualization, and acute withdrawal of progesterone have led to the development of robust and reproducible induction of a "menses-like" event in the mouse endometrium. Comparison with human tissue samples shows that these models recapitulate the key physiological changes associated with menstruation. Specifically local/focal hypoxia, spatial and temporal expression of metalloproteinases, increased expression of angiogenic factors and inflammatory mediators, epithelial cell proliferation and the influx of large numbers of immune cells. An intact luminal epithelial layer is rapidly restored and the tissue appears "unwounded" within 48–72 h of progesterone withdrawal. Studies in mice have provided the platform for testing drugs and cell depletion to better inform new therapeutic opportunities for women's health disorders.

It is anticipated that further studies on the mouse models of menstruation, including more extensive comparison to regeneration and repair mechanisms in other tissues will continue to inform both our understanding of the normal physiology of menstruation but also an important platform for development of new therapies to treat conditions such as heavy menstrual bleeding, endometriosis and Asherman's syndrome.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

The University of Edinburgh provides funding to cover costs of open access publishing. Research in the author's group on endometrial biology has been supported by MRC MR/N024524/1.

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frontiers in Reproductive Health

REVIEW
published: 20 January 2022
doi: 10.3389/frph.2021.811537

Endometrial Stem/Progenitor Cells–Their Role in Endometrial Repair and Regeneration

Fiona L. Cousins[1,2]*, *Caitlin E. Filby*[1,2] *and Caroline E. Gargett*[1,2]

[1] *The Ritchie Centre, Hudson Institute of Medical Research, Clayton, VIC, Australia,* [2] *Department of Obstetrics and Gynecology, Monash University, Clayton, VIC, Australia*

The human endometrium is a remarkable tissue, undergoing ∼450 cycles of proliferation, differentiation, shedding (menstruation), repair, and regeneration over a woman's reproductive lifespan. Post-menstrual repair is an extremely rapid and scar-free process, with re-epithelialization of the luminal epithelium completed within 48 h of initiation of shedding. Following menstruation, the functionalis grows from the residual basalis layer during the proliferative phase under the influence of rising circulating estrogen levels. The regenerative capacity of the endometrium is attributed to stem/progenitor cells which reside in both the epithelial and stromal cell compartments of the basalis layer. Finding a definitive marker for endometrial epithelial progenitors (eEPCs) has proven difficult. A number of different markers have been suggested as putative progenitor markers including, N-cadherin, SSEA-1, AXIN2, SOX-9 and ALDH1A1, some of which show functional stem cell activity in *in vitro* assays. Each marker has a unique location(s) in the glandular epithelium, which has led to the suggestion that a differentiation hierarchy exists, from the base of epithelial glands in the basalis to the luminal epithelium lining the functionalis, where epithelial cells express different combinations of markers as they differentiate and move up the gland into the functionalis away from the basalis niche. Perivascular endometrial mesenchymal stem cells (eMSCs) can be identified by co-expression of PDGFRβ and CD146 or by a single marker, SUSD2. This review will detail the known endometrial stem/progenitor markers; their identity, location and known interactions and hierarchy across the menstrual cycle, in particular post-menstrual repair and estrogen-driven regeneration, as well as their possible contributions to menstruation-related disorders such as endometriosis and regeneration-related disorder Asherman's syndrome. We will also highlight new techniques that allow for a greater understanding of stem/progenitor cells' role in repair and regeneration, including 3D organoids, 3D slice cultures and gene sequencing at the single cell level. Since mouse models are commonly used to study menstruation, repair and regeneration we will also detail the mouse stem/progenitor markers that have been investigated *in vivo*.

Keywords: stem/progenitor cells, endometrium, menstruation, regeneration, repair, endometriosis

OPEN ACCESS

Edited by:
Jacqueline Maybin, University of Edinburgh, United Kingdom

Reviewed by:
Emma Shelley Lucas, University of Warwick, United Kingdom
Rachel W. S. Chan, The University of Hong Kong, Hong Kong SAR, China

***Correspondence:**
Fiona L. Cousins
fiona.cousins@hudson.org.au

Specialty section:
This article was submitted to Gynecology, a section of the journal Frontiers in Reproductive Health

Received: *09 November 2021*
Accepted: *23 December 2021*
Published: *20 January 2022*

Citation:
Cousins FL, Filby CE and Gargett CE (2022) Endometrial Stem/Progenitor Cells–Their Role in Endometrial Repair and Regeneration. Front. Reprod. Health 3:811537. doi: 10.3389/frph.2021.811537

INTRODUCTION

The Menstrual Cycle

The human endometrium undergoes ∼450 cycles of proliferation, differentiation, breakdown, shedding, and repair across a woman's reproductive lifespan. The endometrium is composed of two layers. The basalis is adjacent to the muscular myometrium and is not shed during menstruation, and it is from this layer that the upper layer of the endometrium, the functionalis, arises during each menstrual cycle (1). The functionalis undergoes the most structural changes throughout the menstrual cycle in response to ovarian-derived steroids 17β estradiol and progesterone (2).

The estradiol-dominant proliferative phase begins on approximately day 4 of an average cycle (3), stimulating proliferation of the glandular epithelium, the vasculature and stroma. Estradiol primes the endometrium for the structural changes that it will undergo during the secretory phase by inducing estrogen-dependent expression of the progesterone receptor (4). During the secretory phase, progesterone is secreted by the corpus luteum following ovulation. Epithelial cell proliferation decreases, stromal cells undergo cellular enlargement to become pre-decidual cells. During the mid-secretory phase decidualization of pre-decidual cells occurs under the luminal epithelium and around spiral arterioles. By the late secretory phase, the decidua is infiltrated by T cells, uterine natural killer cells and macrophages (5).

In the absence of an implanted blastocyst, the corpus luteum regresses resulting in a rapid decrease in ovarian-derived steroid production. Progesterone withdrawal initiates menstruation, a cascade of events that results in the piecemeal shedding (6) of the functionalis and expulsion of tissue via the vagina. Whilst outward bleeding may last for up to 5 days in some women, repair processes have been initiated from day 1. Scanning electron microscopy (SEM) studies show that re-epithelialization of the endometrium occurs within 48 h and in a piecemeal fashion (7). The endometrium is unique in that it displays unparalleled tissue remodeling following menstruation, resulting in a scar-free tissue (6). Furthermore, this process occurs in a steroid hormone-depleted environment, as evidenced in animal models of endometrial repair (8, 9).

Re-epithelialization of the endometrium is thought to occur by two mechanisms. The first was proposed by Novak and Te Linde in 1924, who suggested the new luminal epithelium arises from the residual basalis glands (10). SEM studies of menstrual phase endometrium show epithelial extensions attached to basal glands (7). The second mechanism is that of mesenchymal to epithelial transition (MET) where stromal cells in the basalis undergo cellular transformation to become new luminal epithelial cells. Three studies have reported low epithelial cell proliferation during post-menstrual repair, along with isolated epithelial cells on the surface of the endometrium, unassociated with the glandular epithelium (11–13). The role of MET has also been investigated in mouse models of menstruation/post-partum repair, which would indicate that the stroma does contribute in some part to the luminal epithelium (14–16) but it is likely the glandular epithelium is the main contributor to the new luminal surface.

Regeneration of the endometrium following repair is an estrogen-dependent process, whereby the endometrium grows from a post-menstrual depth of 0.5 to 7–8 mm during the mid-proliferative phase (17). This highly regenerative capacity is likely driven by stem/progenitor cell populations that reside in the basalis.

In this review we will focus on stem/progenitor populations that are likely involved in menstruation, repair, and early regeneration of the tissue as well as those populations which may contribute to menstrual/regeneration disorders such as endometriosis and Asherman's syndrome.

HUMAN STEM/PROGENITOR CELLS IN MENSTRUATION, ENDOMETRIAL REPAIR, AND REGENERATION

Adult stem cells are rare, undifferentiated cells found in most tissues and organs with the unique properties of self-renewal to maintain the stem cell pool and differentiation to generate the functional cells of the tissue in which they reside (18). Paradoxically, these stem/progenitor cells are often quiescent and rarely proliferate. Their transit amplifying daughter cells rapidly expand to ensure cellular replacement in regenerating tissues. It was initially hypothesized that endometrial stem/progenitor cells would be located in the basalis, as it remained during menstruation and provided a cellular source to regenerate the functionalis in the following cycle (19, 20). The epithelial cells of the basalis are quiescent and only proliferate occasionally, while functionalis glandular epithelium acts as the rapidly proliferating transit amplifying population in endometrial regeneration (20).

Endometrial Epithelial Progenitors

Human endometrial epithelial progenitors were first identified as rare clonogenic cells comprising 0.22% of the epithelial cell adhesion molecule positive ($EpCAM^+$) epithelial cell population from hysterectomy tissue which includes the basalis (21). Subsequently, the stem cell properties of self-renewal, high proliferative potential, and differentiation into large gland like structures in 3D cultures were demonstrated *in vitro* for individual large endometrial clonogenic epithelial cells (22). Specific markers of basalis epithelial cells were then identified; AXIN2 (23, 24), SSEA-1 and nuclear SOX9 (nSOX9) (25). The first specific surface marker enriching for clonogenic epithelial cells, N-cadherin encoded by *CDH2*, was identified using an unbiased gene profiling approach comparing $EpCAM^+$ endometrial epithelial cells from pre- and post-menopausal women (26). A potential epithelial hierarchy was also identified, based on the location (niche) of the N-$cadherin^+$ cells in the bases of the branching glands in the basalis adjacent to the myometrium. N-$cadherin^+$ SSEA-1^+ $nSOX9^+$ epithelial cells were proximal to N-$cadherin^+$SSEA-1^- cells and N-$cadherin^-$ SSEA-1^+ $nSOX9^+$ (**Figure 1**) more proximal again to occupy an ill-defined functionalis-basalis junction. The majority of the functionalis comprised epithelial cells negative for N-cadherin,

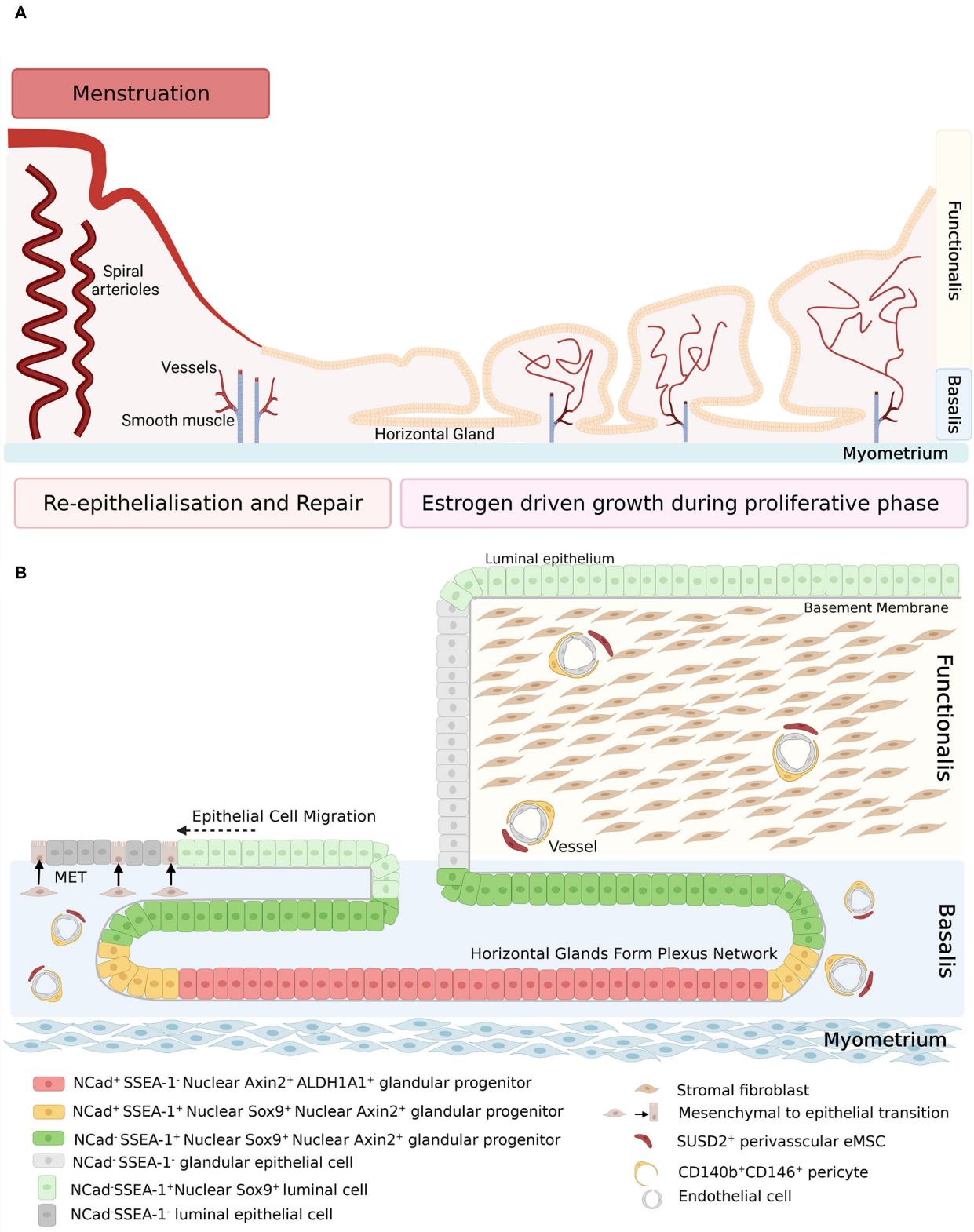

FIGURE 1 | (A) Schematic of human endometrium in menstrual and proliferative phases. **(B)** Schematic of human endometrial mesenchymal stem cell and epithelial stem/progenitor hierarchy in menstruating and proliferating endometrium. During re-epithelization, SSEA-1$^+$ epithelial cells (light green) migrate from the stumps of residual glands across the denuded surface, with some evidence of mesenchymal to epithelial transition of stromal fibroblasts (brown). During the proliferative phase,

(Continued)

FIGURE 1 | regeneration of the endometrium is initiated by clonogenic epithelial stem/progenitor cells that form a hierarchy in the basalis glands (red, yellow, and dark green cells), followed by rapid proliferation of functionalis epithelial cells as the vertical glands elongate. Endometrial mesenchymal stem cells are localized around blood vessels in the functionalis and basalis. Created with BioRender.com, adapted from (27).

SSEA-1 and nSOX9. However, the luminal epithelium is SSEA-1^+ nSOX9$^+$, most likely due to rapid re-epithelializing of the raw surface by these cells migrating from the remaining gland stumps during menstruation (**Figure 1**), indicating their role in endometrial repair (27, 28). The ALDH1A1 isoform of ALDH co-localizes with 78% of N-cadherin$^+$ epithelial cells by immunofluorescence and confocal microscopy (29), suggesting a role for retinoic acid signaling in the progenitor function of N-cadherin$^+$ epithelial cells. EpCAM$^+$ N-cadherin$^+$, EpCAM$^+$ SSEA-1^+ and the very rare EpCAM$^+$ N-cadherin$^+$ SSEA-1^+ epithelial cells have been detected and quantified in menstrual fluid (30, 31), indicating that small populations of these cells are shed during menstruation (see section Role of Stem/Progenitor Cells in Menstrual Disorders/Regeneration Disorders).

Atrophic post-menopausal endometrial epithelium also contains N-cadherin$^+$ epithelial cells in the bases of atrophic glands adjacent to the myometrium (26). A similar endometrial epithelial hierarchy has been identified in post-menopausal women taking estrogen replacement therapy in a fully regenerated endometrium with a basalis and functionalis. Atrophic post-menopausal endometrial epithelium also contains nuclear AXIN2$^+$ epithelial cells (23).

New information on endometrial cell lineages using next generation sequencing technologies at the single cell level is rapidly being generated. Since most studies have been undertaken on endometrial biopsies (32–34), gene expression signatures for basalis epithelial cells have not always been available. However, a Visium spatial transcriptomics study of several cadaveric full thickness uterine tissues has captured signatures of luminal, glandular, and basalis epithelium and revealed that *SOX9*-expressing epithelial cells with a cell-cycling profile are widely distributed in proliferative-stage endometrium (34). Although, this Visium spatial analysis was unable to determine if *SOX9* was expressed in the nucleus or cytoplasm, it is possible that nSOX9$^+$SSEA-1^+ epithelial cells may extend further into the functionalis than first observed and behave as transit amplifying cells that contribute to the rapidly expanding glandular epithelium during endometrial regeneration. However, the *SOX9*$^-$ expressing basalis epithelial cells have a non-cycling gene expression profile indicating their quiescence, as shown many years ago in tritiated thymidine incorporation *ex vivo* into endometrial tissue (2). Mouse endometrial epithelial progenitor cells were first identified as quiescent label retaining cells (LRC) by pulse-chase experiments using bromo-deoxyuridine (BrdU), a DNA synthesis label, to detect rarely dividing cells which retain the label (35, 36). Mouse endometrial epithelial LRC were identified in the luminal epithelium and did not express nuclear ERα (35), but were the first cells to proliferate on estrogen replacement of ovariectomised BrdU-labeled LRC mice, thereby driving endometrial luminal and glandular regeneration (37).

More recently, a single cell pulse-chase lineage tracing study using *Cre-loxP-Keratin19* reporter system was used to identify mouse endometrial epithelial stem cells and their niche (38). The epithelial stem cells which generated EpCAM$^+$ FoxA2$^-$ luminal epithelial cells and EpCAM$^+$ FoxA2$^+$ glandular epithelial cells were located in the intersection zone of the luminal and glandular epithelium. They had capacity to repair the luminal epithelium and regenerate the glandular component over numerous estrus cycles and following pregnancy. Other lineage tracing studies have identified *Lgr5*$^+$-expressing cells at the tips of the glands invaginating into the uterine mesenchyme in neonatal mice (39) and *Axin2*-expressing epithelial cells deep in the gland bases of adult mice which regenerated endometrial glands during estrus cycling (24). It appears that there are several stem/progenitor populations in mouse endometrium that are responsible for endometrial repair and regeneration, however the hierarchy of these stem/progenitor cells is yet to be determined.

Endometrial Mesenchymal Stem Cells

Most postnatal tissues, whether highly regenerative or not, contain a population of mesenchymal stem cells (MSC), including human endometrium (eMSC) (40). MSC were first identified in bone marrow aspirates as clonogenic cells with a fibroblastic morphology (CFU-F) with capacity to differentiate into multiple mesodermal lineages (41). MSC were later defined by the International Society for Cell & Gene Therapy (ISCT) as plastic adherent stromal cells that differentiated into adipocytes, chondrocytes and osteocytes *in vitro* and had a characteristic surface marker phenotype distinguishing them from haemopoietic stem cells (42). However, stromal fibroblasts also fulfill the ISCT criteria, which does not distinguish clonogenic MSC with adult stem cell properties (self-renewal, proliferative potential and differentiation *in vivo*) (43). More recently, functional and morphological studies have identified numerous tissues with perivascular MSC with adult stem cell function (44, 45), including human endometrium (46, 47). In this review we will focus on perivascular endometrial MSC (eMSC) rather than endometrial stromal fibroblasts. For a more detailed discussion on the differences between perivascular eMSC and endometrial stromal fibroblasts in endometrial tissue and menstrual fluid see Bozorgmehr et al., (48).

The endometrium has a substantial vascularized stroma which regenerates during the proliferative stage of each menstrual cycle, and is likely mediated by eMSC. eMSC were first identified as clonogenic stromal cells (1.25% of stromal cells) (21, 49), which fulfill the ISCT criteria and also undergo self-renewal and *in vitro* differentiation to multiple mesodermal lineages (22). Their perivascular niche was discovered when specific surface markers were identified that enriched for the clonogenic endometrial stromal cells, first as pericytes co-expressing CD140b (PDGFRβ) and CD146 (46) and as SUSD2$^+$ perivascular cells (47).

SUSD2$^+$ eMSC can also generate endometrial stromal tissue *in vivo* when transplanted underneath the kidney capsule (47). In this model, SUSD2$^+$ eMSC generate vimentin$^+$ fibroblasts and induce the migration of endothelial cells that promote angiogenesis (47). The pro-angiogenic activity of eMSC has also been shown *in vitro* (50). Taken together, these data suggest that eMSC may be responsible for stromal vascular regeneration of the endometrium during the proliferative phase, mediated by both growth and differentiation and also by paracrine effects promoting angiogenesis.

These perivascular eMSC were identified around small and large blood vessels in both the basalis as expected and also in the functionalis, which indicated they would be shed in menstrual fluid (see section Role of Stem/Progenitor Cells in Menstrual Disorders/Regeneration Disorders). Gene expression profiling has demonstrated that CD140b$^+$CD146$^+$ eMSC rapidly lose their gene signature in culture and adopt the CD140b$^+$CD146$^-$ stromal fibroblast signature (51). This suggests eMSC differentiate into stromal fibroblasts, further contributing to the confusion between MSC and stromal fibroblasts (52). However, this differentiation has not been identified *in vivo*. SUSD2$^+$ and SUSD2$^-$ endometrial cells differentiate into decidual cells with similar but distinct gene profiles and both contribute to the formation of the maternal placenta (53–55).

Other markers of perivascular eMSC include NG2, Stro-1, EphA3, W8B2, and CD271 and CD34 which are found in the adventitia of blood vessels rather than a pericyte or medial location [reviewed in (48, 56)]. However, the CD34 population failed to regenerate human endometrium in functional studies (57). The perivascular niche suggests that eMSC likely contribute to vascular and stromal regeneration each menstrual cycle.

Endometrial thickness can be restored in post-menopausal women via oral estrogen therapy, suggesting that endometrial stem/progenitor populations remain quiescent after menopause but when exposed to exogenous estrogen rapidly respond to regenerate both glands and stromal vascular tissue (26). Like N-cadherin$^+$ eEPC, SUDS2$^+$ eMSC can be isolated from post-menopausal (PM) endometrium (58). They have a lower cloning efficiency than in pre-menopausal endometrium, but are detected in similar numbers (58).

Single cell RNA sequencing of human endometrium has identified a small smooth muscle population expressing *SUSD2*, CD146 (*MCAM*), and CD140b (*PDGFRB*) (32). In mouse endometrium, scRNAseq of *Pdgfrb*-BAC-eGFP reporter mice also identified a perivascular population of *Pdgfrb*$^+$ *MCAM*$^+$ cells with a perivascular gene profile and perivascular niche *in vivo*, that was distinct from 3 novel endometrial fibroblast populations also identified (59). These studies confirm our biological findings and provide further insight into the role of perivascular eMSC in endometrial regeneration.

Bone Marrow Derived Stem Cells

Bone marrow derived stem cells (BMDSC) have been suggested as an exogenous source of stem cells in the endometrium (60). In humans, it has been reported that BMDSC contribute up to 48% of the epithelium and 52% of the stroma (60). Most of the supporting data has been generated from mouse models, which have shown BMDSC contribute to epithelial, stromal, and endothelial lineages (61–64). However, a 2018 study disputed their contribution. Using two different transgenic fluorescent tagged mouse lines to repopulate the bone marrow of irradiated recipient mice and sophisticated imaging and microscopy, Ong et al. demonstrated that BMDSC did not contribute to epithelial or stromal lineages (65). Instead they highlighted that intra-epithelial and stromal-derived cells were CD45$^+$ leukocytes and that bone marrow-derived macrophages failed to immunostain with CD45 (65). This highlighted the limitations in the previous body of work relying solely on the identification of CD45$^+$ cells. BMDSC contribution to other body organs has been similarly controversial but the body of evidence suggests their plasticity or ability to transdifferentiate does not occur for similar technical issues (66). For the purpose of this review we will be focusing on endometrial-derived eMSC and eEPC.

Menstrual Fluid

Menstrual fluid, discharged via the vagina following declining circulating progesterone levels, is a complex fluid containing shed endometrial tissue (31, 48), secreted proteins (67, 68), immune cells, peripheral blood components, vaginal epithelial cells, clots, and mucous (69). Shed menstrual fluid cells mainly comprise CD45$^+$ leukocytes (>90%), with the remainder being endometrial cells (31, 69). Menstrual fluid endometrial cells include stromal cells, for example fibroblasts (69), SUSD2$^+$ eMSC (70), and epithelial cells such as N-cadherin$^+$ and SSEA-1$^+$ eEPC (31). A similar proportion of these stem/progenitor cells is identified in menstrual fluid compared to endometrium (31, 70). Both SUSD2$^+$ eMSC and N-cadherin$^+$ eEPC have also been detected and their abundance reported in menstrual blood collected from the uterine cavity during surgery before efflux into the vagina (30). The proportion of clonogenic units in menstrual fluid endometrial cells is comparable for epithelial clones (0.31% for menstrual fluid and 0.22% for eutopic tissue) and somewhat lower for stromal clones (0.22% for menstrual fluid and 1.25% for eutopic tissue) than in endometrial tissue (31).

Menstrual fluid also contains cells described broadly as menstrual stem cells (MenSC) that fulfill the ISCT criteria which are easily isolated based on their adherence to plastic. However, they are likely heterogeneous due to non-specific and non-standardized isolation procedures (48, 71, 72). MenSC express markers for MSC, with the exception of STRO-1 (70, 73). MenSC likely comprise a combination of eMSC and predominantly endometrial stromal fibroblasts. A comparison of MenSC and eMSC has been extensively reviewed elsewhere, which highlights the need for standard isolation and characterization of MenSC (48).

Viable eMSC can be reliably isolated from menstrual fluid, their proportions studied, their clonogenicity assessed (31), and their behavior characterized *in vitro* (70). Together this indicates that menstrual eMSC are a reliable source for research that characterizes the biology of eMSC. Menstrual eMSC do tend to be more apoptotic and necrotic than other sources of MSC, however

they can be prevented from undergoing apoptosis and senescence by A8301 treatment (70), thus indicating menstrual eMSC are also a viable option for cellular therapy (48).

STEM/PROGENITORS IN *IN VITRO* MODELS OF MENSTRUATION, REPAIR AND REGENERATION

3D Endometrial Organoids/Menstrual Fluid Organoids

The study of human primary endometrial epithelial biology has previously been limited to 2D culture such as (1) epithelial monolayers, which either become overgrown by stromal cells or senesce (74, 75), (2) colony forming unit assays, seeded at very low density and generally terminal experiments that do not permit long-term culture or functional assays (21, 49), or (3) serial subcloning which permits up to four passages for large colonies derived from CFU-F (22). However, our recent evidence that the endometrium contains N-cadherin$^+$ and putative SSEA-1$^+$ eEPC (26, 76) capable of forming gland-like structures in 3D culture maintained in ECM, together with organoid technology (77, 78), has enabled generation of human endometrial organoids (EMO) using defined serum-free culture conditions (79, 80) (**Figure 2**, yellow box).

EMO have since been derived from biopsies, hysterectomy tissue, endometrial cancer, endometrial hyperplasia, endometriosis lesions from various locations, and placental decidual tissue (79–81). They can be expanded for >6 months in culture, cryopreserved and generated from cryopreserved gland fragments (82) and show responsiveness to estrogen and progesterone (79, 83). The gene expression profile of EMO has been studied at both the bulk and single cell level (79, 80, 83, 84), however many of these studies are limited by their use of endometrial biopsies instead of hysterectomy tissue, which contain the full hierarchy of eEPC, including those located in the rhizome-like glandular structures of the basalis (85, 86). Changes in EMO cell fate in response to hormones (83) and inhibition of key developmental signaling molecules (NOTCH) (87) enhance our understanding of endometrial epithelial cell fate trajectory and its role in development and disease—an area that has previously been very challenging to study. EMO also show promise for drug screening, demonstrating sensitivity to specific compounds (88).

EMOs are derived from bulk endometrial epithelial fragments and therefore comprise a heterogenous population. They potentially include contamination of surrounding stroma, which recede from culture after multiple passages, but may influence organoid formation and contribute to variation in patient-derived organoid lines. While the ability to generate single cell EMO (scEMO) from existing EMO cultures has been demonstrated (79, 80, 84), the ability of naïve single endometrial cells to form EMO has been underexplored. Generating scEMO from FACS-sorted epithelial subpopulations has potential for investigating the roles of epithelial progenitor cells in endometrial regeneration.

Organoids can be generated from shed endometrial tissue in menstrual fluid (MFO) (89, 90) (**Figure 2**, red box).While MFO are less abundant than EMO, they appear to represent EMO in proliferation rates, responses to hormones, and gene expression profiles (89, 90). They can also be derived from disease states including endometriosis and adenomyosis. They can also be derived from girls and young women without the need for an endometrial biopsy, enabling the study of early disease mechanisms, precision medicine, and diagnosis (91).

While EMO and MFO represent major advancements in studying menstrual biology, these systems largely support epithelial cultures in isolation, and lack the stromal, mesenchymal, vasculature, and immune cells important for a functioning endometrium. Biomaterial engineering has generated synthetic hydrogels (92, 93) that permit co-culture of endometrial epithelial and stromal cells and these are being rapidly applied to EMO (**Figure 2**, orange box) (81, 94, 95). They are also being applied to the cells/tissues required to support the endometrium such as the vasculature (92) and immune cells [reviewed in (96)]. Coculture in defined hydrogel systems has enabled development of organ-on-chip systems for disease modeling and low-cost drug screening for other organs and disease states (97)—their potential for endometrium, endometriosis, and adenomyosis are exciting prospects to be explored (96). Organ-on-chip and micro-physiological systems (**Figure 2**, orange box) have multiple advantages, including infinite tunability (cell input, matrix composition, hormonal, and nutrient delivery), scalability, enabling coupling for modeling the complexity of endometrial regeneration.

In vitro 3D Slice Cultures

Whilst single cell and co-cultured 3D organoids overcome some of the issues of using 2D *in vitro* models to study endometrial dynamics, endometrial repair/regeneration is a multicellular/multizonal, tightly controlled process which has previously been difficult to replicate in a dish. 3D thin tissue slice cultures provide a culture system that maintains endometrial structure (98). These tissue slice approaches have shown that the tissues can respond to estrogen and progesterone over 21 days *in vitro* (**Figure 2**, blue box). Histology of the slices indicates that zone-specific changes *in vitro* mimic *in vivo* hormone responses (98). Whilst stem/progenitor populations were not assessed in this study, the authors did show non-specific transduction of lacZ via adenovirus-mediated gene delivery and therefore the model has promise for studying stem/progenitor populations and interactions in an "*in vivo*-like" system, however 3D endometrial slice cultures are limited to terminal experiments.

Tissue Clearing

Historically, the location and expression profiles of endometrial stem/progenitor cells have been presented in 2D via standard immunohistochemistry/immunofluorescence of thin tissue sections. The introduction of tissue clearing, whereby a tissue sample is rendered optically transparent via solvent- or aqueous-based solutions, has enabled a more detailed

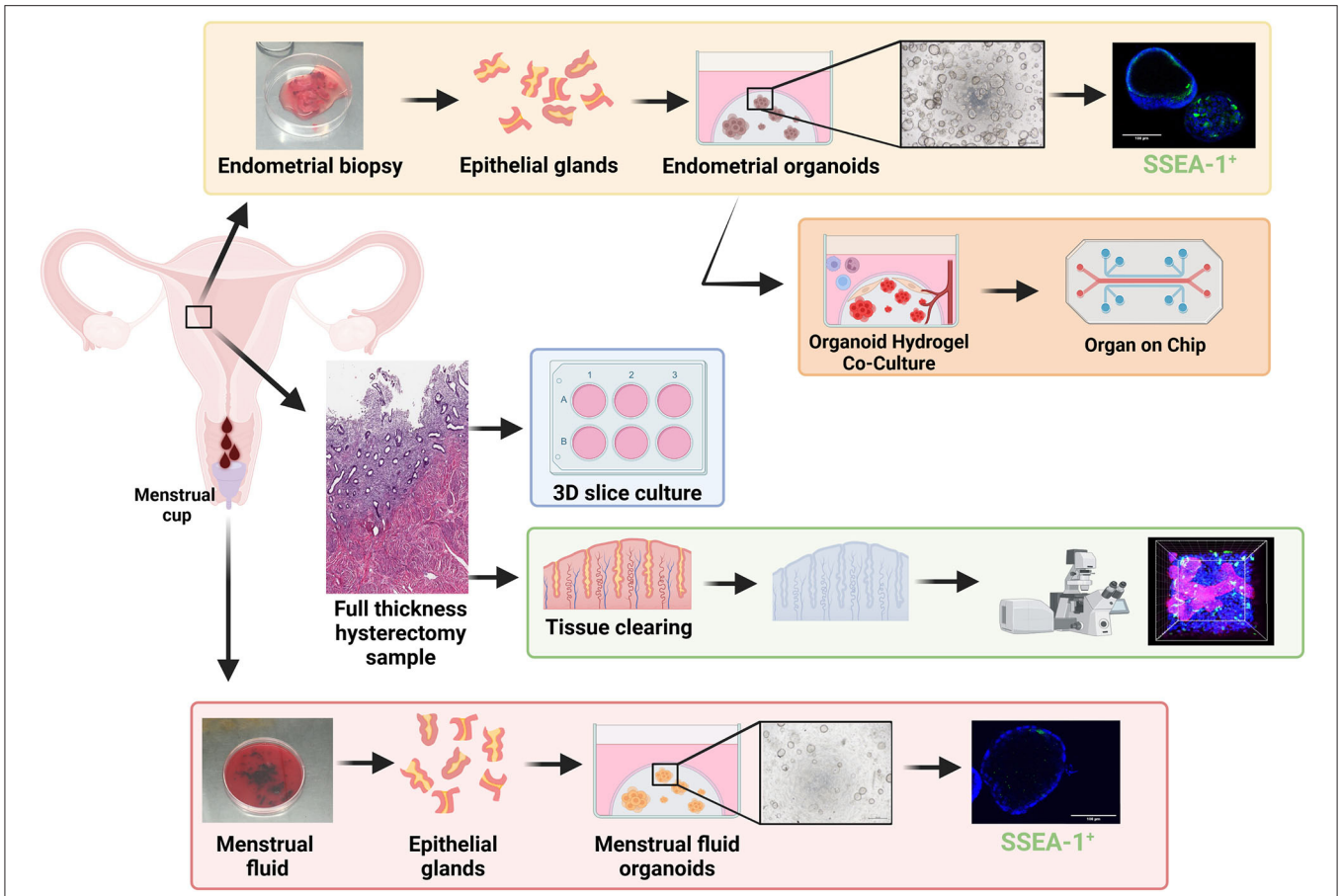

FIGURE 2 | Stem/progenitor cells in *in vitro* models of repair and regeneration. Endometrial biopsies can be used in 3D organoid experiments including single cell organoid culture (yellow box) and hydrogel-based co-culture and organ-on-chip systems (orange box) which show great promise for future endometrial research. Full thickness endometrium collected at hysterectomy has been used for; tissue clearing (green box) to visualize structures in 3D via high powered microscopy and used for 3D slice cultures (blue box). Menstrual fluid can be easily collected in a menstrual cup, from which menstrual fluid organoids (red box) can be generated. Created with BioRender.com.

morphological examination of the endometrium in 3D. Samples are fixed, permeabilized, and then cleared so that light scattering and absorption caused by cellular contents are minimized. Optimal tissue clearing maintains native tissue architecture and preserves fluorescent proteins (99) (**Figure 2**, green box). Two recent studies have reconstructed the 3D morphology of full thickness endometrium and have shown that basalis glands form a rhizozome-like plexus structure horizontally across the endometrial basalis using tissue clearing (85) or genetic lineage tracing (86), challenging the long held view of vertical glands extending from blind-ended glands in the basalis. This plexus structure remains during menstruation, from which branched glands arise and vertically penetrate the functionalis. 2D studies clearly indicate that a hierarchy of epithelial/stem progenitor cell types with specific markers exists extending from the basalis to the luminal epithelium. Now these new technologies are available it will be exciting to see whether this epithelial hierarchy can be reconstructed in 3D, whilst also investigating the relationship between MSCs and the endometrial vasculature across all cycle phases.

STEM/PROGENITORS IN MOUSE MODELS OF REPAIR AND REGENERATION

Like women, the mouse endometrium also responds to cyclical changes of ovarian-derived steroids. In the mouse this occurs over a much shorter timeframe, ∼4 days. Pro-estrous and estrus mimic the proliferative phase of the human cycle, where increasing concentrations of estrogens result in ovulation, followed by a progesterone dominant metestrus (100). Unlike women, the mouse endometrium does not undergo spontaneous decidualization in the presence of progesterone, but requires an implanted blastocyst. In the absence of implantation, the endometrium is reabsorbed during diestrus, and the cycle begins again. Murine stem/progenitor markers involved in the cyclical turnover of the endometrium during the estrous cycle have been extensively studied, readers interested in this area are referred to our recent reviews (28, 48).

Despite their lack of menstruation, mice are routinely used as an *in vivo* model for menstruation, repair, and regeneration, using several different approaches. The most commonly used

mouse model of menstruation (MMoM) was first described by Finn and Pope, where exogenous hormones were administered to ovariectomised mice to mimic a human cycle. Decidualization was artificially induced by a physical stimulus (sesame oil) to the endometrium, to mimic blastocyst implantation. Exogenous hormones were then withdrawn to stimulate a menses-like event (101). This model has been optimized to use silastic hormone-secreting pellets and transvaginal delivery of oil to reduce variation in the decidual response (14, 102). This model has been used to assess a role for stem/progenitor cells during repair (0–24 h after P4 withdrawal) and regeneration (24–72 h) of the endometrium at menses (14, 103). Pseudopregnancy models of menstruation (PMoM) are also used, where female mice are mated with a vasectomized male to induce decidualization and then ovariectomy or mifepristone to induce menstruation (16, 104). Both models exhibit overt bleeding, breakdown/shedding, re-epithelialization, and regeneration of the endometrium, however breakdown and initial repair occur over a shorter time period in the MMoM (24 h) compared to the PMoM (48–72 h).

An early study used BrdU pulse-chase to assess LRCs in the glandular and luminal epithelial compartments during re-epithelialization in the MMoM. Glandular epithelial cells (GE) retain BrdU for longer periods than luminal epithelial cells (LE) and GE strongly express ERα during initial repair of the endometrium (81.6% ERα positive) (103). Proliferation of LE significantly increases during re-epithelialization (repair phase), in contrast to GE which only commences proliferation once breakdown and repair are complete. These data suggest a stem/progenitor population in the residual basal glands that support the formation of glandular growth during the subsequent regenerative phase (103).

Mouse telomerase reverse transcriptase (mTert), a putative stem/progenitor marker in the regenerative intestine (105), marks rare stromal, epithelial, and leukocyte populations in the cycling mouse endometrium (106). They are positively regulated by estrogen (106) and negatively regulated by progesterone as shown by lack of mTert$^+$ cells in the LE or GE prior to progesterone withdrawal in the MMoM (107). During repair, mTert$^+$EpCAM$^+$ cells are rare (0.08% of total EpCAM population) and localized to the repairing LE, and no mTert$^+$ cells were identified in the GE. In the repairing LE, mTert$^+$Ki67$^-$ cells were localized next to mTert$^-$Ki67$^+$ clusters (107). This suggests that mTert$^+$ cells are progenitor cells that are located in the residual LE and undergo asymmetrical division to form transit amplifying cells which contribute to form the new LE during the steroid-depleted window of epithelial repair. It is likely the GE-derived mTert$^+$ cells are present, but estrogen supplementation has not been studied in this model. Interestingly, mTert does not co-localize with BrdU$^+$ LRC or CD44 suggesting that mTert$^+$ cells may identify different progenitor cell types within the LE (106).

The contribution of the stromal cell compartment to repair via mesenchymal-epithelial transition (MET) has also been studied using cytokeratin as an epithelial marker in combination with different mesenchymal markers. Using the MMoM double immunostaining for cytokeratin and vimentin during early and late repair revealed rare cells undergoing MET close to the repairing epithelium (**Figure 1**) (14). Cytokeratin$^+$vimentin$^+$ cells have also been observed in the stromal cell compartment of repairing endometrium in a PMoM (16), and Amhr2$^+$cytokeratin$^+$ cells were observed in the new luminal epithelium in a post-partum model of endometrial repair (15). A recent study using a number of lineage tracing mouse reporter lines disputed the role of MET in the endometrium, however all of those studies were either in intact estrous cycling mice or mice treated with tamoxifen to induce epithelial expansion and did not investigate endometrial repair or regeneration following a major remodeling event (menses-like or post-partum models) (108). Taken together these data suggest that during normal cyclical turnover each cell compartment supports its own cell type but when the endometrium needs to repair following a major shedding event, such as parturition, the stromal compartment supports re-epithelialization.

A more recent study has identified SM22α-derived CD34$^+$KLF4$^+$ cells as a putative stromal progenitor cell involved in endometrial regeneration (109). SM22α$^+$CD34$^+$ cells were located in the endometrial stroma below the repairing epithelium, where they also co-stained with the epithelial marker E-cadherin. Like other stem/progenitors, proliferation of SM22α$^+$CD34$^+$KLF4$^+$ cells is likely mediated by estrogens. Deletion of SENP1 (SUMOendopeptidase-1) induced SUMOylation, which in turn promoted ERα expression in the repairing endometrium. SM22α$^+$CD34$^+$KLF4$^+$ cell proliferation was significantly increased in SENP1sm22αKO mice in comparison to WT mice and an increase in transdifferentiation of stromal cells into epithelial cells was observed. Repair of the endometrium was completed by 72 h post-progesterone withdrawal in SENP1smKO mice compared to 96 h in WT mice (109). In addition, SENP1 likely mediates stem/progenitor regulation of regeneration, as deletion of SENP1 leads to epithelial hyperplasia (109), highlighting the importance of a tightly controlled repair and regeneration process to prevent endometrial dysfunction.

We acknowledge the limitation that mice do not naturally menstruate and therefore any information gleaned from mouse studies into stem/progenitor dynamics must be carefully related to human studies. At the time of writing, Axin2 appears to be the only marker that has a similar role in glandular epithelial cell turnover in both mice and humans.

ROLE OF STEM/PROGENITOR CELLS IN MENSTRUAL DISORDERS/REGENERATION DISORDERS

Endometriosis

Retrograde Menstruation Theory

Retrograde menstruation, where shedding endometrial fragments flow backwards through the Fallopian tubes and into the peritoneal cavity, is likely the main cause of endometriosis pathogenesis (**Figure 3A**). However, not all women who exhibit retrograde menstruation develop endometriosis. Other contributing factors likely play a part in determining who does and who does not develop endometriosis. The total number

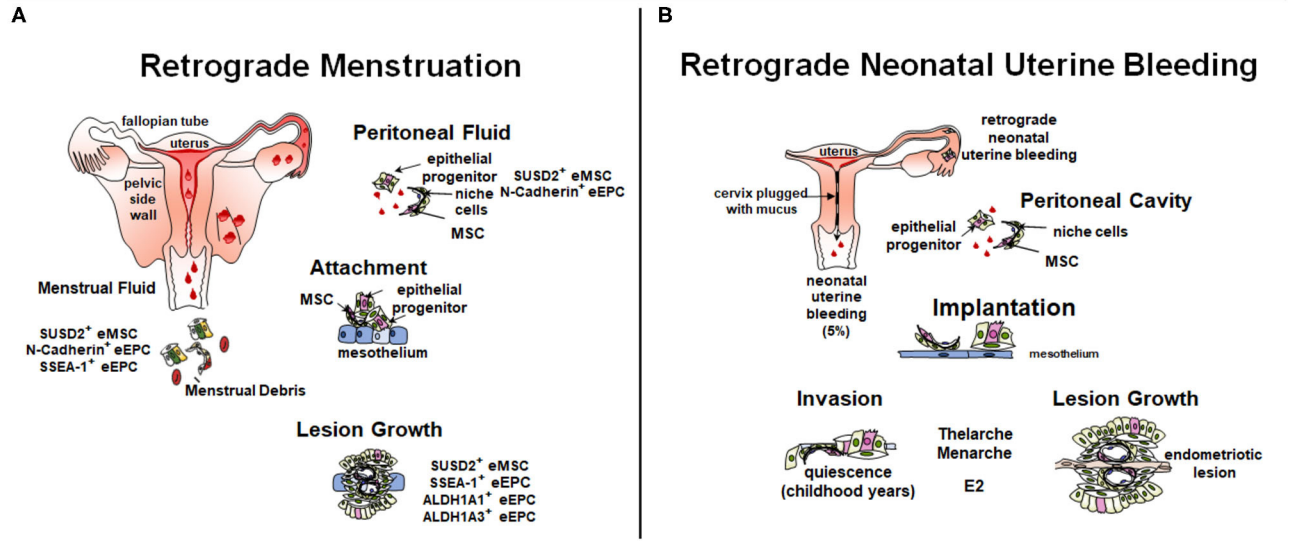

FIGURE 3 | Role of stem/progenitor cells in the pathology of endometriosis. **(A)** In the adult, retrograde menstruation, results in stem/progenitor cells entering the peritoneal cavity. In some circumstances, stem/progenitor cells are able to survive and contribute to lesions establishment and progression. **(B)** Retrograde neonatal bleeding is observed in 5% of female born babies. Epithelial progenitors and eMSC enter the peritoneal cavity and remain quiescent until puberty, where increasing concentrations of estradiol (E2) stimulate lesion growth. Created with BioRender.com, adapted from (110).

of endometrial cells is unlikely to play a role, given their similar prevalence in the peritoneal cavity of both women with and without endometriosis (30, 111). Rather, the type of cells deposited in the cavity may play a role in pathogenesis. Leyendecker et al. have suggested that endometriosis is caused by the shedding of basalis endometrium, as women with endometriosis have a higher prevalence of basalis fragments in their menstrual blood compared to controls (112). Furthermore, we have shown increased proportions of SSEA-1$^+$ basalis cells in the functionalis layer normally shed with menstruation (76).

The initiation of lesions in the peritoneal cavity likely depends on the ability of stem/progenitor cells to adhere to, and for deep infiltrating endometriosis (DIE) invade, ectopic sites and subsequently give rise to epithelial and stromal progeny. Different theories exist on how they contribute to lesions (91, 110, 113). Factors likely contributing include the type of cells shed at menstruation, the influence of genetics such as, endometriosis risk variants and somatic mutations (114–117), site of attachment in the peritoneal cavity and the surrounding micro-environment. Stromal and epithelial stem/progenitor cells have been identified in menstrual blood, peritoneal fluid, and ectopic lesions (**Figure 3A**) (30, 31, 70, 118, 119).

Menstrual and Peritoneal Fluid Stem/Progenitor Cell Populations in Endometriosis

Study of endometrial stem/progenitor cells in menstrual and peritoneal fluid is still in its infancy, partly due to recent discovery of appropriate markers (26, 47, 76) and challenges in acquiring, processing, and analyzing complex fluids. Whilst easily acquired tissue fragments and stromal fractions have been studied by some, far fewer have attempted to identify, isolate, and characterize the endometrial stem/progenitor cells from menstrual fluid (48, 111). We identified clonogenic endometrial cells, SUSD2$^+$ eMSC and N-cadherin$^+$ eEPC concurrently in menstrual fluid and peritoneal fluid of women with and without endometriosis (31) and also clonogenic endometrial cells and SSEA-1$^+$ eEPC in menstrual fluid of normal women (30, 31).

In menstrual fluid the proportions of SUSD2$^+$ eMSC and SSEA-1$^+$ eEPC endometrial stem/progenitor cells show minimal variation from one menstrual cycle to the next in both groups (31). On the other hand, the proportion of N-cadherin$^+$ eEPC showed a poor agreement from one menstrual cycle to the next, indicating variability in the numbers of N-cadherin$^+$ eEPC shed, likely due to their deep basalis location on the rhizomal-like gland structures. The concentration of SUSD2$^+$ eMSC and N-cadherin$^+$ eEPC in uterine menstrual blood appears similar between women with and without endometriosis (30).

Recently we have described the first ever stem/progenitor cell evidence of Sampson's 100 year-old theory of retrograde menstruation (30, 120). While we hypothesized that the concentrations of endometrial stem/progenitor cells retrogradely shed into the pelvic cavity would be higher in women with endometriosis, surprisingly our study did not find a significant difference in the concentrations of eMSC, eEPC, or clonogenic cells in peritoneal fluid during the menstrual phase of the cycle. This unexpected finding may be limited by sample size and a control group confounded by pelvic pain—women undergoing laparoscopy are not "normal" and thus a true control for peritoneal fluid is a rare occurrence (e.g., tubal ligations).

The clonogenic cells persisted in peritoneal fluid beyond the menstrual phase in women with endometriosis, whereas in controls they declined during the non-menstrual phase

(30). This may indicate enhanced survival or persistent shedding of clonogenic cells in women with endometriosis. Other studies have shown an increased pro-invasive cytokine profile of peritoneal fluid from women with endometriosis (121) and this may aid the survival of stem/progenitor cells beyond the menstrual phase. Alternatively, the shed cells may exhibit different behavior due to underlying genetic or other regulatory programs (91). Finally, there was noticeable variation in the concentration of eMSC and N-cadherin$^+$ eEPC in women with endometriosis, indicating the possibility of sub-groups with different pathophysiology that is worthy of further investigation.

Stem/Progenitors in Ectopic Lesions

Sequencing of epithelial and stromal cells in superficial and deep infiltrating lesions reveals characteristic somatic mutation profiles for each cell type derived from different clones suggesting each cell type is supported by their own stem/progenitor population (91, 115, 122). In support of this, both mesenchymal and epithelial stem/progenitor markers have been identified in ectopic lesions (25, 29, 29, 76, 123–125) (**Figure 3**).

The clonogenicity of eutopic and ectopic stromal and epithelial cells was lower for both epithelial and stromal cells from endometriomas when compared to matched eutopic endometrium (118). Lower cloning efficiency was also observed when control endometrium was compared to endometriomas. However, no significant difference was found in either cell population when eutopic endometrium from women with and without endometriosis was compared (118). That study did not compare the clonogenicity of either DIE or superficial endometriosis lesions, however a lower clonogenicity of endometrioma cells is in keeping with a lower organoid yield from ectopic rather than eutopic tissue (80). Furthermore, eMSC in ectopic lesions have a higher proliferative potential (119). Cultured endometrial stromal cells, fulfilling the ISCT criteria, show increased migration capacity, enhanced angiogenic potential, and exhibit altered expression of adhesion molecules in comparison to eutopic MSC (126). This suggests that the peritoneal cavity provides a micro-environment which promotes or selects for stem/progenitor cell activity. Perivascular SUSD2$^+$ NTPDase2$^+$ MSC have been identified in ovarian endometriomas via immunofluorescence (123).

Basalis epithelial stem/progenitor markers SSEA-1 and SOX9 are increased in eutopic secretory phase functionalis of endometriosis women in comparison to healthy controls (76). *In vitro*, these cells can form 3D gland-like structures highlighting their potential at supporting lesion development *in vivo*. SSEA-1$^+$ cells are present in endometriosis lesions (25) as are the deep basalis epithelial markers ALDH1A1 and ALDH1A3 (29) and N cadherin (29, 124, 125), supporting Leyendecker's basalis theory of endometriosis pathogenesis.

Neonatal Uterine Bleeding

A new theory to explain the pathogenesis of pre-menarchial early onset endometriosis involves neonatal uterine bleeding (**Figure 3B**), a forgotten phenomenon occurring in ∼5% of neonatal girls (127). Its incidence is highest in post-term babies. This neonatal vaginal bleed observed in the first week of life results from maternal progesterone withdrawal from the neonatal circulation upon birth. Unlike the mouse, the fetal uterus is fully formed *in utero* and autopsy studies have shown that the endometrium can undergo decidualization and there is evidence of endometrial shedding. The neonatal uterus is predominantly cervix which is functionally blocked with mucous thereby allowing any shedding endometrium to flow back into the pelvic cavity undetected, and only allowing minimal blood and cell numbers to permeate the cervical mucous. The neonatal endometrial stem/progenitor cells present in such shed endometrial tissue could invade the mesothelium and remain dormant in a similar manner to the dormancy of endometrial stem/progenitor cells in estrogen-depleted post-menopausal endometrium (128). As estrogen levels rise with thelarche and menarche, these potent stem/progenitor cells would commence proliferation and generation of clonal endometriotic tissue in the pelvic cavity and on the ovary. It is suggested that the overt bleeding observed in 5% of neonatal girls is indicative of a substantial "menses" with a greater degree of retrograde shedding and therefore greater risk of developing early onset endometriosis.

Asherman's Syndrome

Asherman's syndrome (AS) is characterized by intrauterine adhesions/scarring and loss of a functional endometrium. Adhesions can be caused via surgical scraping/cleaning of the uterus or via uterine infection in a setting of low circulating estrogen e.g., post-partum and pregnancy termination. This trauma, to the endometrial basalis, causes loss of the germinal compartment for regenerating the endometrium. It has been proposed that trauma damages stem/progenitor populations and their surrounding stem cell niche in the basalis, preventing regeneration of the functionalis (40, 129, 130).

The use of endometrium-derived stem cells for treatment of Asherman's is still very much in its infancy. At the time of writing, few studies have investigated using menstrual fluid as a potential therapeutic option. Menstrual blood derived eMSC form spheroids that, when injected into the uteri of rats with induced AS, can improve fertility rates (131). In a small study of 7 human patients, autologous transfer of cultured MenSC resulted in an increase in endometrial thickness in 5 patients, four of which were able to undergo embryo transfer and two patients conceived successfully (132). Given menstrual fluid is a plentiful, easily available resource, research into autologous MenSC transfer would be worthy of further investigation.

CONCLUSIONS

The endometrial stem cell field has advanced considerably since the first description of clonogenic cells in 2004. The recent identification of epithelial stem/progenitor markers has revealed a glandular epithelial hierarchy which likely supports re-epithelialization at menstruation as well as the growth of the functionalis during the proliferative phases. The remarkable regenerative capacity of endometrial stem cells shows promise for use in regenerative medicine, for endometrial disorders such as

Asherman's but also for the treatment of infertility or miscarriage. The localization of eMSC and eEPC in menstrual and peritoneal fluid and ectopic lesions supports Leyendecker's theory that stem cells are involved in the pathogenesis of endometriosis, highlighting a potential target for future therapeutics. More importantly, the presence of both eMSC and eEPC in menstrual fluid has the potential to provide a new diagnostic tool for endometrial disorders. Advances in single cell sequencing will likely advance our understanding of the epithelial hierarchy and contributions of both eMSC and eEPC to the basalis and the functionalis.

AUTHOR CONTRIBUTIONS

FC was involved in the conception and design, acquisition of data, analysis and interpretation of the data, writing of the manuscript, editing and formatting the manuscript, and the drawing of the figures. CF provided acquisition, analysis and interpretation of data, writing of the manuscript, critical review of content, and drawing of figures. CG was involved in data acquisition, analysis and interpretation of data, writing of the manuscript, and critical review of content. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by an Australian National Health and Medical Research Council Investigator Fellowship to CG (1173882), an EndoFound Grant to CF and CG, and Victorian Government's Operational Infrastructure Support Program and the United States Department of Defense through the Congressionally Directed Medical Research Program under Award No. E01 W81XWH1910364 to CG.

ACKNOWLEDGMENTS

The authors acknowledge the support of Wentong Xu in figure preparation.

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frontiers in Reproductive Health

REVIEW
published: 31 January 2022
doi: 10.3389/frph.2021.794352

The Menstrual Endometrium: From Physiology to Future Treatments

Marianne Watters[1†], *Rocío Martínez-Aguilar*[2†] *and Jacqueline A. Maybin*[2*]

[1] Simpson Centre for Reproductive Health, Royal Infirmary of Edinburgh, NHS Lothian, Edinburgh, United Kingdom, [2] MRC Centre for Reproductive Health, University of Edinburgh, Edinburgh, United Kingdom

Abnormal uterine bleeding (AUB) is experienced by up to a third of women of reproductive age. It can cause anaemia and often results in decreased quality of life. A range of medical and surgical treatments are available but are associated with side effects and variable effectiveness. To improve the lives of those suffering from menstrual disorders, delineation of endometrial physiology is required. This allows an increased understanding of how this physiology may be disturbed, leading to uterine pathologies. In this way, more specific preventative and therapeutic strategies may be developed to personalise management of this common symptom. In this review, the impact of AUB globally is outlined, alongside the urgent clinical need for improved medical treatments. Current knowledge of endometrial physiology at menstruation is discussed, focusing on endocrine regulation of menstruation and local endometrial inflammation, tissue breakdown, hypoxia and endometrial repair. The contribution of the specialised endometrial vasculature and coagulation system during menstruation is highlighted. What is known regarding aberrations in endometrial physiology that result in AUB is discussed, with a focus on endometrial disorders (AUB-E) and adenomyosis (AUB-A). Gaps in existing knowledge and areas for future research are signposted throughout, with a focus on potential translational benefits for those experiencing abnormal uterine bleeding. Personalisation of treatment strategies for menstrual disorders is then examined, considering genetic, environmental and demographic characteristics of individuals to optimise their clinical management. Finally, an ideal model of future management of AUB is proposed. This would involve targeted diagnosis of specific endometrial aberrations in individuals, in the context of holistic medicine and with due consideration of personal circumstances and preferences.

Keywords: menstruation, endometrial, adenomyosis, abnormal uterine bleeding, inflammation, coagulation, vascular, hypoxia

OPEN ACCESS

Edited by:
Fiona L. Cousins, Hudson Institute of Medical Research, Australia

Reviewed by:
Vinay Shukla, University of Kansas Medical Center, United States
Juan S. Gnecco, Massachusetts Institute of Technology, United States

***Correspondence:**
Jacqueline A. Maybin
jackie.maybin@ed.ac.uk

[†] These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Gynecology, a section of the journal Frontiers in Reproductive Health

Received: *13 October 2021*
Accepted: *20 December 2021*
Published: *31 January 2022*

Citation:
Watters M, Martínez-Aguilar R and Maybin JA (2022) The Menstrual Endometrium: From Physiology to Future Treatments. Front. Reprod. Health 3:794352. doi: 10.3389/frph.2021.794352

INTRODUCTION

The symptom of abnormal uterine bleeding (AUB) is defined by the International Federation of Gynecology and Obstetrics (FIGO) as bleeding from the uterine corpus that is outside the normal parameters defined by AUB System 1 (**Table 1**) for duration, volume, frequency and/or regularity (1, 2). It encompasses heavy menstrual bleeding (HMB) and intermenstrual bleeding. AUB becomes chronic when symptoms are present for the majority of the preceding 6 months.

AUB is a symptom and not a diagnosis. The FIGO AUB System 2 PALM-COEIN provides a system for diagnosing the underlying pathology resulting in the symptom of AUB

TABLE 1 | International Federation of Gynaecology and Obstetrics (FIGO) Abnormal Uterine Bleeding System 1 definitions for normal menstrual bleeding [(2). doi: 10.1002/ijgo.12666].

Parameter	Normal
Frequency	24–38 days
Duration	Up to 8 days
Regularity	Regular variation (shortest to longest ≤ 9 days)
Flow volume	Normal
Intermenstrual bleeding (bleeding between the cyclically regular onset of menses)	None
Unscheduled bleeding on progestins ± oestrogen	Not applicable if not on hormonal medication or none

(1, 2). PALM encompasses structural disorders such as polyps (AUB-P), adenomyosis (AUB-A), leiomyoma (AUB-L) and malignancies (AUB-M). COEIN refers to non-structural causes including coagulopathies (AUB-C), ovulatory dysfunction (AUB-O), endometrial disorders (AUB-E), iatrogenic causes (AUB-I) and not otherwise classified (AUB-N).

Approximately one third of women of reproductive age experience AUB at some point in their reproductive lives (3). This translates to approximately 600 million women worldwide with debilitating symptoms that negatively impact their quality of life (4). In the UK, over 800,000 women seek medical help for AUB annually and it is the fourth most common referral to UK gynaecological services (5). The impact amongst those who do not present for medical review is unquantifiable. It is thought that many women endure years of AUB that may result in anaemia, affect mental health and result in financial hardship (4).

There are a range of current medical treatments for AUB, but these are often limited by lack of effectiveness and intolerable side effects. Hormonal medications are the mainstay of medical management for AUB. These preparations act to override physiological ovarian hormone production and do not specifically target the underlying cause of AUB in many cases. Hormonal medications have a range of contraindications and side effects and are particularly unsuitable for women who wish to conceive. Most importantly, women with AUB often find these treatments ineffective meaning that up to 60% of these women resort to fertility-ending surgical procedures with associated surgical risk (5). To improve medical strategies for AUB it is important to understand endometrial physiology and accurately diagnose the aberrations that result in AUB.

This review examines our current knowledge of menstrual physiology and the key processes involved. Aberrations which lead to AUB will be discussed, with a focus on AUB-E as an example of a non-structural cause and AUB-A as a structural diagnosis. Reference is also made to AUB-O, as most of the current treatments for AUB act at the ovarian level. However, detailed review of the processes and subsequent management of AUB –P/L/M/C/O/I/N is not included and we signpost readers to existing comprehensive reviews covering AUB-L (6), AUB -M (7) and AUB-C (8). Current management of AUB-A and AUB-E and its limitations will be reviewed, followed by discussion of recent advances and potential new therapeutic targets. Finally, a model of future management of AUB will be proposed with the aim of providing more effective, personalised treatments for those who are suffering with this debilitating symptom.

PHYSIOLOGY OF MENSTRUATION

The endometrium forms the inner lining of the uterus and requires an ability to change across the menstrual cycle to regenerate, decidualise, shed and to support implantation and pregnancy when necessary. In the absence of implantation, shedding of the luminal two-thirds of the endometrium occurs in a process known as menstruation. This occurs under the strict control of endocrine, immune, vascular and coagulation systems.

Physiology: Endocrine Regulation

The endometrium is a complex and dynamic multicellular tissue that responds to the ovarian hormones. Oestradiol is most abundant in the first half of the menstrual cycle, coincident with high rates of endometrial cell proliferation (9). Following ovulation, the endometrial secretory phase commences, where high levels of progesterone produced by the *corpus luteum* lead to altered endometrial morphology to prepare for implantation. If implantation does not occur, the *corpus luteum* regresses. Subsequent withdrawal of progesterone and oestradiol triggers a series of molecular and cellular events that resemble a classical inflammatory episode (pain, heat, redness and swelling) (10) and culminates with menstruation.

Physiology: Endometrial Breakdown and Inflammation

The progesterone withdrawal that occurs during the late secretory phase releases the transcription factor nuclear factor kappa B (NFκB) from its association with inhibitory proteins, such as IκB (11, 12). Once free, NFκB translocates to the nucleus, where it enhances the expression of inflammatory mediators (13, 14) including cytokines [tumour necrosis factor (TNF), interleukin-6 (IL6)] and chemokines [C-C motif chemokine ligand 2 (CCL2), interleukin-8 (CXCL8)] (13, 14). These mediators promote specific leukocyte trafficking and recruitment of myeloid cells (15). Activation of the NFκB pathway has been immunohistochemically detected in the endothelial, glandular and stromal compartments of the secretory endometrium (11). However, a thorough delineation of the particular cells types responsible for the initiation of the inflammatory cascade at menstruation is still lacking.

More recently, the effects of the inflammasome on the release of inflammatory cytokines during menses has been described, *in vitro* and *ex vivo* (16). The inflammasome is a multiprotein assembly that is classically associated with inflammatory signalling amplification (17). Both the NFκB and inflammasome systems may act simultaneously at menses to recruit immune cells to the endometrium. During menses, the most prevalent myeloid cells present in endometrial tissue are neutrophils and macrophages (18). Both myeloid cells activate and release matrix metalloproteinases (MMPs) in the endometrial milieu.

MMPs are widely accepted as being responsible for the shedding of the upper layers of the endometrium during menses (18), although the contribution of reactive oxygen species has also been suggested (19).

Once endometrial shedding has been accomplished, the inflammatory events which led to tissue destruction must be controlled in order to allow repair processes to begin. Several anti-inflammatory mediators emerge as candidates responsible for limiting the inflammatory response, including the glucocorticoid cortisol and lipid mediators.

Exposure of epithelial ovarian cells to the pro-inflammatory cytokine interleukin-1α (IL1A) has been found to increase hydroxysteroid 11-β dehydrogenase 1 mRNA (*HSD11B1*) (20). *HSD11B1* catalyses the final step of cortisol synthesis, regulating the availability of this anti-inflammatory steroid. Similar regulation may be present in the menstrual endometrium, where the pro-inflammatory environment may activate anti-inflammatory pathways to resolve inflammation. Indeed, *HSD11B1* mRNA was found to be increased in the endometrium at the time of menses (21), consistent with cortisol having a role in menstrual inflammatory resolution. A local increase in endometrial cortisol levels at menstruation may also result in a pro-repair environment. *In vitro* treatment of human endometrial stromal cells with cortisol was found to increase active transforming growth factor β (TGFB) in cell culture supernatants (22), an ambivalent soluble mediator with context-dependent pro-inflammatory or restorative properties (23). Cortisol has also been shown to affect the macrophage secretome, with supernatant from cortisol-treated peripheral blood monocyte-derived macrophages resulting in altered endometrial endothelial cell expression of angiogenic genes C-X-C motif chemokine ligand 2 (*CXCL2*), *CXCL8*, connective tissue growth factor (*CCN2*), and vascular endothelial growth factor C (*VEGFC)* with a putative role in vascular repair (24). Cortisol has also been involved in the regulation of the platelet factor 4 (CXCL4/PF4) released by endometrial cells *in vitro*. This factor may be involved in endometrial repair by promoting the recruitment of reparative macrophages (25). Therefore, cortisol may limit the inflammatory response and create a pro-repair endometrial environment.

The presence of lipid mediators has also been associated with the resolution of inflammation. Specifically, lipoxins are lipid mediators with anti-inflammatory and pro-resolution properties that are present systemically (26). During menses, the lipoxin A4 receptor was increased in the endometrium at the mRNA level (27). Furthermore, *in vitro* studies showed that addition of lipoxin A4 to endometrial explants primed with an inflammatory stimulus mitigates the subsequent pro-inflammatory response (27). Hence, lipoxin A4 and other lipid mediators may play a role in limiting the inflammatory response within the menstrual endometrium and merit further study.

Physiology: Limiting Blood Loss

In addition to the resolution of menstrual inflammation, further mechanisms exist to limit menstrual blood loss. NFκB activation promotes the expression of prostaglandins and enzymes involved in their synthesis, such as cyclooxygenase-2 (COX2/PTGS2). While COX2 plays a role in endometrial breakdown (28, 29), prostaglandin F2α (PGF2α) (30), along with other vasoconstrictors like endothelin-1 (EDN1) (31), may curtail menstrual blood loss by constricting endometrial arterioles. Haemostatic mechanisms are also required to limit menstrual blood loss (8). During primary haemostasis, platelets adhere to the injured vascular endothelium and interact with the surrounding matrix, creating a platelet plug. The resulting platelet aggregation triggers activation of the coagulation system which, through complex interactions, converts soluble fibrinogen into an insoluble fibrin clot (32). In the endometrium, pre-clinical studies predict that platelet aggregation events are less crucial than vasoconstriction and fibrin clot formation (33). However, both the dysregulation of platelet aggregation and/or fibrin clot formation may have a negative impact upon menstrual blood loss (8).

Physiology: Endometrial Repair and Regeneration

After endometrial shedding, the denuded surface needs to be restored to minimise blood loss and recover its functionality for the next cycle. Endometrial hypoxia has been proposed as an important regulator of endometrial repair. Intensive vasoconstriction of the spiral arterioles during menstruation was directly observed in endometrial explants transplanted into the anterior chamber of the eye of rhesus monkeys by Markee in 1940 (34). More recently, markers of endometrial hypoxia have been detected in both in pre-clinical models (35–37) and the human endometrium (38, 39) during menses.

Although hypoxia does not appear to be required for endometrial breakdown (40), it may be important for triggering menstrual endometrial repair (37). Hypoxia inducible factor (HIF), is composed of an oxygen regulated alpha subunit (HIF1A) and a constitutively expressed beta subunit to form a transcription factor responsible for the cellular adaptative response to hypoxia (41). It is proposed that HIF1A is required for normal endometrial repair during menstruation, due to its exclusive presence in the perimenstrual phase, alongside evidence of delayed endometrial repair during menstruation with genetic or pharmacological reduction of HIF1A in mouse studies (37). HIF1A enhances the endometrial transcription of several genes involved in endometrial repair and blood vessel formation such as adrenomedullin, *CCN2, CXCL8* and *VEGF* (42–44). Interestingly, some of these mediators can also be synergistically upregulated via prostaglandin action (44), which may represent dual regulation to ensure timely repair and cessation of menstrual bleeding.

PATHOLOGY OF MENSTRUATION

As described above, menstruation relies on meticulously coordinated endocrine, immune, vascular and haemostatic responses to limit blood loss and ensure optimal repair. Thus, repression or overactivation of the biochemical pathways involved in this process may result in pathological

manifestations. The role of (i) endocrine regulation, (ii) tissue breakdown and inflammation, (iii) vascular function and coagulation and (iv) endometrial repair in AUB is discussed below, with a focus on AUB-E and AUB-A.

AUB-E is a non-structural cause of AUB and is diagnosed when other causes of AUB have been excluded clinically. AUB-E represents an under-researched area where precise mechanisms resulting in this particular subtype of AUB remain undefined. AUB-A is an example of a structural cause of AUB. Adenomyosis develops a result of endometrium or endometrial-like tissue being present within the myometrial layer of the uterus. Adenomyosis may be asymptomatic or may cause symptoms such as dysmenorrhoea, subfertility or AUB (AUB-A) (45). Traditionally, adenomyosis has been diagnosed retrospectively following hysterectomy. There now is evidence to support ultrasound diagnosis of adenomyosis (46) but there remains a pressing clinical need to improve our understanding of the mechanisms causing AUB-A to improve diagnosis and management.

Pathology: Endocrine Regulation

Aberrant endocrine regulation of the endometrium is not known to be present in AUB-E or AUB-A but does occur due to ovulatory dysfunction (AUB-O). Menstrual disturbance in AUB-O occurs due to persistence of oestradiol signalling and lack of *corpus luteum* formation (47). The resulting lack of progesterone and subsequent progesterone withdrawal may result in heavy, infrequent and/or irregular menstrual bleeding. AUB-O occurs frequently at menarche and during peri-menopause or in those with polycystic ovary syndrome. Many current treatments for AUB act to override physiological ovarian hormone production and are often helpful in AUB-O. However, as endocrine dysregulation is rarely the primary cause of AUB-E and AUB-A, treatment failures occur and are discussed in further detail below.

Pathology: Endometrial Breakdown and Inflammation

Patients with AUB-E have been shown to have higher levels of TNF protein in their menstrual effluent when compared to those with normal menstrual blood loss (NMB) (48). TNF is a downstream inflammatory target in NFκB signalling, which can also act as an NFκB inducer (49). COX is another NFκB downstream inflammatory effector that is dysregulated in AUB. This biosynthetic enzyme possesses two isoforms (COX1/PTGS1 and COX2/PTGS2) and the mRNA of both were found to be increased in endometrium from those with AUB-E (50). This supports the hypothesis that excessive endometrial inflammation may be one mechanism causing AUB-E.

In the early stages of endometrial breakdown, local inflammation is tightly controlled. It may be hypothesised that those with AUB-E have disproportionate endometrial recruitment of neutrophils and macrophages that generate an inflammatory positive loop, where more mediators are released and further myeloid cells are recruited. In turn, these cells may over activate the secretion and release of MMPs leading to excessive or prolonged endometrial breakdown. Mechanistic preclinical studies focusing on the impact that upregulation of TNF, COX and other NFκB-induced inflammatory mediators have on endometrial leukocyte recruitment and MMPs activation are needed to confirm or refute this hypothesis.

An altered inflammatory response may also contribute to the symptom of AUB-A. In the presence of adenomyosis, NFκB binding activity is constitutively overactivated both in the eutopic endometrium (51) and adenomyotic lesions (51, 52). The increase in inflammatory cytokines released by leukocytes isolated from both the eutopic endometrium and adenomyotic lesions of women with adenomyosis compared to healthy controls (53) also suggests inflammatory dysregulation. Moreover, NFκB activity has been positively associated with the symptom of AUB-A (54). Interestingly, in those with AUB-A, *COX2/PTGS2* mRNA levels both in the adenomyotic lesions and the eutopic endometrium are increased compared to adenomyotic patients with NMB. This *COX2/PTGS2* increase correlated with higher expression of the pro-inflammatory mediators *IL6* and *CXCL8* (55), consistent with a pro-inflammatory endometrial environment increasing menstrual blood loss in those with adenomyosis.

As previously discussed, endometrial cortisol may play a role the resolution of the menstrual inflammatory response. Patients with AUB-E have been shown to have an increased endometrial expression of the cortisol-inactivating enzyme hydroxysteroid 11-β dehydrogenase 2 (*HSD11B2*) (56) as well as a decrease in the downstream cortisol target CXCL4/PF4 during menses (25). Hence, cortisol deficiency may play a key role in AUB-E. At present, there are no studies exploring a potential correlation between cortisol levels and AUB-A.

Pathology: Limiting Blood Loss

As described above, arteriole vasoconstriction is a key process to limit menstrual blood loss. Defective vasoconstriction may have its origin in alterations in vasoconstrictive mediators. In AUB-E, a decrease in the vasoconstrictor EDN1 at the protein level has been described, as well as an increase in the enzyme responsible for its inactivation, neutral endopeptidase (57). There is also evidence of higher endometrial levels of the vasodilating prostaglandin PGE_2 in those with HMB compared to NMB (50). These effects in conjunction with decreased mRNA levels of the PGF2α receptor (*PTGFR*) (58), cause a reduction in the ratio PGF2α/PGE_2 which may result in a defective ability to limit menstrual blood loss (50, 58).

In patients with AUB-A, there are no reports of endometrial differences in EDN1 or prostaglandins. Studies are required to examine the eutopic endometrium in those with AUB-A and control groups of those with adenomyosis who do not experience AUB and those free from disease and symptoms. However, within adenomyotic lesions, the vasoconstrictor/vasodilator balance appears to be disrupted. Preliminary data from *in vitro* studies (59) show that cells from adenomyotic lesions have higher mRNA concentrations of prostaglandin E synthase 2 (*PTGES2*)- the enzyme responsible of PGE_2 synthesis- compared to eutopic endometrial cells from those without adenomyosis (59). In a pre-clinical mouse model, prostaglandin D2 genetic deficiency increased endometrial COX2/PTGS2 and PGE_2 levels and increased adenomyosis lesion development (60). Therefore, an

imbalance of vasoconstrictor and vasodilator molecules may be involved in the development of adenomyotic lesions but its impact on the development of AUB remains to be determined.

AUB may additionally or alternatively result from aberrant endometrial haemostatic processes, such as fibrin clot formation/degradation ratios. Patients with AUB-E were found to have increased activity of the tissue plasminogen activator (PLAT), compared to those with NMB (61). This mediator activates plasminogen, which is the enzyme responsible for the degradation of fibrin clots. In contrast, the eutopic endometrium and lesions of those with adenomyosis were found to have higher levels of the plasminogen activator inhibitor 1 (SERPINE1) -a PLAT inhibitor-when compared to healthy controls (62). Whether such aberrations result in less fibrinolysis or represent compensatory mechanisms remains to be determined.

Defects in primary haemostasis might also be involved in AUB-A. Tissue factor (F3), a protein involved in the initiation of the coagulation cascade, was found to be increased in the eutopic endometrium as well as in the lesions of adenomyotic patients when compared to healthy controls (63). Interestingly, F3 endometrial immunohistochemical staining of glandular epithelial cells was significantly higher in women with AUB-A than those adenomyosis patients with normal menses (63). To determine the role of haemostasis dysregulation in AUB-A, combining a pre-clinical mouse model of adenomyosis (64) with simulated menstruation (36, 37) would allow genetic and/or pharmacological alteration of the platelet cascade to examine the impact on menstrual blood loss/endometrial repair.

Pathology: Endometrial Repair and Regeneration

Defective vasoconstriction may also affect endometrial hypoxia and menstrual endometrial repair. Patients with AUB-E have been shown to have decreased menstrual endometrial HIF1A protein when compared to those with NMB, as well as a reduction in HIF1 downstream targets (37). In a mouse model of simulated menses, *HIF1A* genetic deficiency or HIF1 pharmacological inhibition, delayed endometrial repair (37). However, specific cell types driving these HIF1A mediated effects in the menstrual endometrium remain undefined. Those with AUB-E have also been shown to have menstrual deficiencies in other putative endometrial repair factors, with lower protein levels of TGFB perimenstrually when compared to those with NMB (22).

The decreased bioavailability of HIF1A and TGFB in patients with AUB-E may affect vascular repair and/or angiogenesis after endometrial shedding (65). Defects in spiral arteriole maturation have been described in AUB. Patients with AUB-E display greater focal discontinuities in endometrial blood vessel walls than those experiencing NMB (66). A lower proliferation rate of vascular smooth muscle cells (VSMCs) in spiral arterioles of those with AUB-E has also been demonstrated and these cells appear critical for vessel integrity and blood flow (67). In addition, VSMCs in the spiral arterioles of AUB-E patients exhibit a lower expression of maturation markers [alpha smooth muscle actin (ACTA2), myosin heavy chain (MYH)] (68). Moreover, some components of the endometrial endothelial extracellular matrix [laminin (LAM), osteopontin (SPP1), fibronectin (FN1), collagen IV (COL4)] are dysregulated in AUB-E, which may contribute to reduced endothelial vascular integrity (69).

Patients with adenomyosis have also been shown to have evidence of abnormal endometrial vascularisation. The microvascular density of both the eutopic endometrium and adenomyotic lesions was higher than in the endometrium of healthy controls (70, 71). Furthermore, VEGF protein levels show a similar trend, being higher in eutopic endometrium (71, 72) and lesions of adenomyotic patients (52, 70, 73) when compared to healthy controls. None of these studies quantified menstrual blood loss or considered the presence of the symptom of AUB in their analysis. This is necessary to determine if abnormal endometrial vascularisation is causing the symptom of AUB and/or is involved in the development of adenomyotic lesions.

CURRENT TREATMENT

There are a range of medical treatments currently available for the treatment of AUB. This section discusses these options in the context of the menstrual physiological processes on which they exert their actions.

Current Treatment: Endocrine Manipulation

Most current medical treatments for AUB act to override physiological ovarian hormone production. As discussed, however, there are multiple pathways involved in the pathogenesis of AUB-E and AUB-A and endocrine manipulation fails to directly target these processes. Hormonal medication may be more suitable for those with AUB-O where aberrant hormone secretion and regulation leads to AUB symptoms. Despite this, medical therapy in the form of the combined oral contraceptive pill (COCP), oral progestins and levonorgestrel-releasing intrauterine system (LNG-IUS) are commonly offered to patients who present with AUB as first line treatment (74). The COCP has been shown to be effective in reducing HMB and regulating unscheduled bleeding (75) but it is not suitable for those with a history of migraine with aura (sensory disturbance accompanying migraine symptoms), a personal or strong family history of venous thromboembolism, a body mass index (BMI) > 35, smokers over the age of 35 or those wishing to conceive (76). Side effects such as mood changes, skin changes and fluctuation in weight are reported. Oral progestins are available to a larger group of patients, with fewer contraindications, but again these medications come with similar reported side effects and a negative impact on conception which may render them unsuitable for certain populations.

Interestingly, of the hormonal treatments outlined above, only the LNG-IUS has been shown to improve quality of life (77) and there is evidence that it improves HMB (78). A randomised control trial comparing the LNG-IUS and COCPs for symptomatic treatment of adenomyosis, showed the LNG-IUS to be more effective in reducing pain and bleeding (79). However, some women may find IUS insertion painful and the risks of uterine perforation or infection may be unacceptable. It may also cause unpredictable bleeding patterns and hormonal side effects of acne, breast tenderness and mood changes (80).

Gonadotropin releasing hormone (GnRH)-agonists may also be used for AUB management (81, 82). The sustained activation of GnRH receptors leads to their desensitisation, ultimately inhibiting luteinizing hormone (LH) and follicle-stimulating hormone (FSH) synthesis by the pituitary gland. Abrogation of these hormones suppresses ovulation and consequently oestrogen (oestradiol) production. This ovarian hormonal suppression also usually results in the absence of menstruation. However, one of the main downsides of this treatment is the negative side effects caused by oestrogen deficiency. In addition to hot flushes and/or loss of libido, patients may experience a reduction in bone mineral density, increasing the risk of osteoporosis (83). Therefore, its use is limited in younger women. If used in young women, hormone replacement therapy (HRT) is recommended to reduce menopausal symptoms and risk of loss of bone density.

Current Treatment: Breakdown and Inflammation

More specific correction of the aberrations present in those experiencing menstrual disorders is currently possible. Non-hormonal medical treatment includes the use of non-steroidal anti-inflammatories (NSAIDs). NSAIDs target the excessive endometrial inflammation observed in AUB-E (48) by inhibiting the COX enzymes that are responsible for the synthesis of prostaglandins (28, 29). The ability of NSAIDs to reduce menstrual blood loss is highlighted in a meta-analysis of 18 randomised controlled trials which demonstrates that NSAIDs are more effective in reducing menstrual blood loss when compared with placebo (84). Mefenamic acid and naproxen have both been shown to result in a reduction in levels of menstrual blood loss, with no significant difference noted between the two (85, 86). NSAIDs use may be limited in patients with a history of gastrointestinal bleeding, inflammatory bowel disease, severe asthma, renal disease, congestive heart failure and cerebrovascular disease. NSAIDs can also affect platelet function and when used in individuals with underlying coagulopathies NSAIDs may be ineffective and may lead to increased bleeding (87).

Current Treatment: Limiting Blood Loss

As discussed, a key process involved in limiting menstrual blood loss is primary haemostasis and the creation of a platelet plug. This triggers a series of interactions resulting in conversion of soluble fibrinogen to an insoluble fibrin clot. Tranexamic acid (TXA) is a medication which may be used to reduce the breakdown of the fibrin clot. TXA competitively blocks plasminogen binding sites and reduces the production of plasma and the breakdown of fibrin. In a meta-analysis, which predominantly incorporated results from studies in patients with AUB-E, tranexamic acid was demonstrated to be superior to placebo in reducing menstrual blood loss (88). It has fewer contraindications when compared with hormonal therapies but its use is limited in patients with a history of thromboembolic disease (76).

EMERGING TREATMENTS

Current treatment options for AUB remain suboptimal, as highlighted by the results of the Royal College of Obstetricians and Gynaecologists (RCOG) UK HMB audit (89). In this audit of patients attending hospital gynaecology clinics ($n = 8183$), 37% of women remained "unhappy" or "very unhappy" with their ongoing HMB symptoms (89). There is a clear need for new and improved medical treatment strategies for AUB (**Figure 1**).

Emerging Treatments: Endocrine Manipulation

Ulipristal acetate (UPA) is a selective progesterone receptor modulator with prevailing inhibitory effects on the progesterone receptor that may be used as a treatment for AUB-L (90). The safety and effectiveness of UPA has been assessed in three clinical studies under the PEARL (PGL4001 Efficacy Assessment in Reduction of Symptoms Due to Uterine Leiomyomata) programme, where UPA administration resulted in effective control of AUB (90). Interestingly, UPA reduces AUB as effectively as the GnRH agonist Leuprorelin but without hypoestrogenic side effects (90). Some adverse effects associated with UPA treatment have been noted, such as weight gain and fatigue and recently potential concerns regarding negative liver effects have been highlighted (91).

The effectiveness of UPA in AUB-L management raises the possibility of trialling this therapeutic strategy in those with AUB-E and AUB-A (**Figure 1B**). In an observational study in women with adenomyosis, treatment with UPA for 12 weeks reduced symptoms of AUB (92). A phase II, randomised, double-blind controlled trial with UPA 10 mg/day for 3 months in patients with adenomyosis has been registered (NCT02587000). UPA has been shown to impact the endometrium at a cellular and molecular level. In particular, it has been shown to reduce to expression of endometrial steroid metabolising enzymes. A reduction in *HSD11B1*, known to metabolise cortisol, was observed (93). As previously discussed, cortisol is thought to play a role in regulating the menstrual inflammatory response. These findings support the potential use of UPA in patients with AUB-E.

Aromatase (CYP19A1*)* has been detected in the endometrium of women with endometriosis, adenomyosis and leiomyomas but not in normal endometrium and has therefore been proposed as a potential therapeutic target (94). A small prospective randomised controlled study compared the oral aromatase inhibitor letrozole and the subcutaneous GnRH agonist Goserelin in the treatment of adenomyosis. In both groups, a similar reduction in uterine volume and adenomyoma volume was observed and two patients in the letrozole group became pregnant during treatment (95).

GnRH-antagonists have also emerged as a new strategy for improving AUB (**Figure 1B**). As opposed to conventional GnRH agonists, GnRH antagonists directly inhibit LH and FSH synthesis. This mechanism of action skips the initial surge of the pituitary hormones aforementioned that occurs with GnRH agonist treatment. Moreover, the dose of GnRH-antagonist used may be titrated to allow only partial suppression of oestrogen. This reduces the potential consequences of a hypoestrogenic state and additional add-back HRT may not be required.

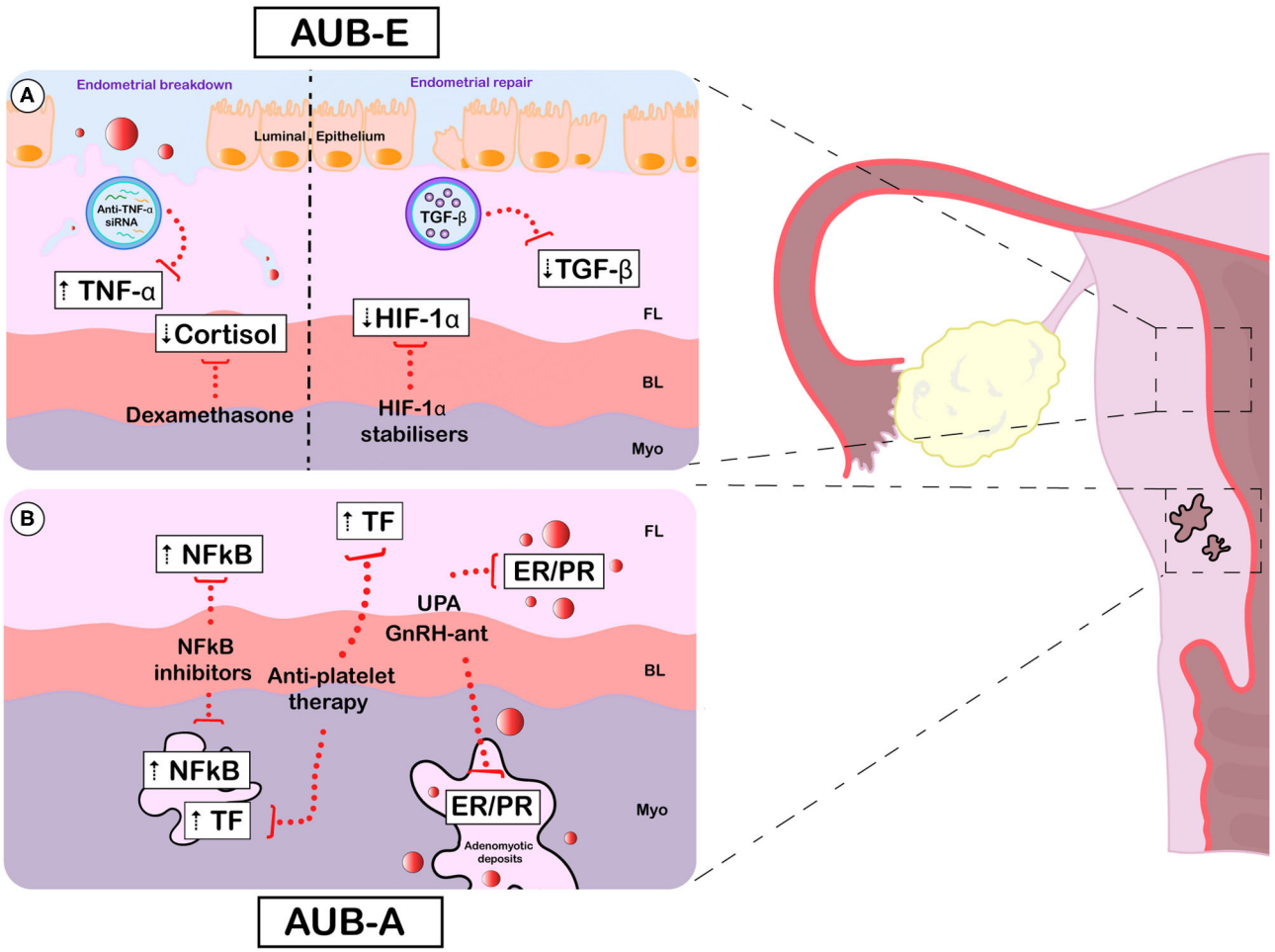

FIGURE 1 | Emerging and potential future treatments for AUB-E and AUB-A. Specific aberrations (white boxes) and emerging therapeutic targets (red dotted lines) for AUB-E **(A)** and AUB-A **(B)**. [**(A)**, left] During active endometrial breakdown, the TNF upregulation observed in AUB-E could be targeted using nanoparticles loaded with small interfering RNA against TNF. Cortisol deficiency at menses may be restored by administration of the glucocorticoid dexamethasone, which effectively reduces HMB. [**(A)**, right] During endometrial repair, pharmacological stabilisation of HIF1A may prevent the delayed repair thought to occur in those with AUB-E. Correcting TGFB deficiency with nanoparticles carrying soluble TGFB may be another strategy to improve the symptom of AUB-E. **(B)** In adenomyosis, AUB-A may be improved via endocrine manipulation. The selective progesterone modulator UPA and GnRH antagonists have shown promising results in clinical trials by targeting PR and/or ER in adenomyotic deposits and the eutopic endometrium. Therapeutic strategies focused on compensating the overactivation of NFκB (using NFκB inhibitors) and TF (using anti-platelet therapy) observed both in the eutopic endometrium and adenomyotic lesions may also be beneficial in AUB-A. AUB, abnormal uterine bleeding; AUB-E, AUB of endometrial origin; AUB-A, AUB due to adenomyosis; FL, functional layer; BL, basal layer; Myo, myometrium; TNF-α, tumour necrosis factor; siRNA, small interfering RNA; TGF-β, transforming growth factor beta; HIF-1α, hypoxia inducible factor 1 alpha; NFκB, nuclear factor kappa B; TF, Tissue factor; UPA, ulipristal acetate; GnRH-ant, gonadotropin releasing hormone antagonists; ER, oestrogen receptor; PR, progesterone receptor.

As an example, the GnRH-antagonist Elagolix significantly reduced HMB in patients with uterine fibroids and coexisting adenomyosis (96).

Emerging Treatments: Breakdown and Inflammation

As discussed above, inflammation and NFκB signalling pathways may play a crucial role in the initiation of endometrial breakdown, with overactivation resulting in AUB (**Figure 1B**). Andrographolide is an active ingredient from the plant *Andrographis paniculata* and has been used for many years in traditional Chinese medicine for the treatment of inflammatory disorders. It has been shown to suppress NFκB activation (97). In a pre-clinical model of adenomyosis, the intragastric administration of andrographolide reduced myometrial leukocyte infiltration as well as adenomyosis-derived pain (98). The impact of andrographolide treatment on menstrual blood loss was not reported in this study and merits further examination.

A more refined strategy may be to target downstream mediators of NFκB that have been shown to be dysregulated in AUB, such as TNF (48). At present, there is a wide range of anti-TNF antibodies which effectively block the biological function of

TNF (99). However, systemic administration of these drugs is not without side effects, given the key role of TNF against pathogen invasion (99). Therefore, local endometrial anti-TNF delivery may be beneficial. As an example, polymer-coated nanoparticles containing small interfering (si)RNA targeting TNF have been proven to be successful in a mouse model of rheumatoid arthritis, reducing TNF levels in serum and arthritic joints (100). This technology has the potential to be adapted for endometrial disorders once the endometrial source of TNF is confirmed (**Figure 1A**).

Cortisol deficiency may also play a key role in the development of AUB-E via dysregulation of the endometrial repair processes. The role of low dose dexamethasone as a treatment to reduce HMB has been demonstrated in a recent response-adaptive randomised placebo-controlled dose-finding parallel group trial (DexFEM) (101). This trial showed that dexamethasone 1.8 mg once daily over 5 days in the mid-secretory phase reduced menstrual blood loss when compared with placebo. However, 75% of the participants in the dexamethasone group reported adverse events, compared with 58% of those taking placebo. Nonetheless, this study demonstrated that dexamethasone may provide an effective treatment option for AUB in women who wish to avoid ovarian hormone based treatments (**Figure 1A**).

Emerging Treatments: Limiting Blood Loss

Treatments that correct aberrations identified in the endometrial coagulation system of those with AUB have potential as novel therapeutics or preventative strategies for AUB-A (**Figure 1B**). In a mouse model of adenomyosis, treatment with Ozagrel, a platelet aggregation inhibitor, supressed myometrial infiltration and improved adenomyosis related pain (102). As highlighted by the authors of this proof-of-concept study, concerns remain about the possible risk of haemorrhage associated with such an anti-platelet therapy. While this study adds to our understanding of the role of activated platelets in the pathogenesis of adenomyosis, further examination of the safety profile of such medications is required before translation to clinical trials.

Emerging Treatments: Endometrial Repair and Regeneration

Markers of hypoxia have been detected in the human endometrium at menstruation (38) and physiological hypoxia has been shown to be important in endometrial repair during simulated menses in the mouse (37). Pharmacological stabilisation of HIF1 in a mouse model of delayed endometrial repair rescued the phenotype and improved repair, indicating that targeting the hypoxia pathway may be a valid approach in the treatment of AUB (37) (**Figure 1A**). HIF1A stabilisation is not an unfamiliar therapeutic strategy in the treatment of non-gynaecological disorders. Pharmacological stabilisation of HIF1A has proven to be effective and safe in the treatment of anaemia in chronic kidney disease (103, 104). In addition, a small HIF1A stabiliser has showed promise in accelerating diabetic wound healing in different pre-clinical models (105). These compounds may have therapeutic benefits in the endometrium, which is amenable to local and intermittent treatment.

Correcting the partial deficiency of TGFB during menstruation that has been detected in the endometrium of those with AUB-E may present another valid therapeutic strategy for AUB (**Figure 1A**). Due to the numerous pleiotropic effects of TGFB as a cytokine, the systemic administration of a soluble version is far from ideal (106). However, recent literature offers different strategies for local delivery of soluble mediators. Using inert biodegradable nanoparticles loaded with TGFB, McHugh et al. achieved T cell-specific delivery both *in vitro* and in animal models (107). This technique has the potential to be applied in the endometrial environment, perhaps via transvaginal administration, targeting endometrial, immune and/or vascular endothelial cells. Liposomes are another alternative nanoparticle with good pre-clinical results (108) that could be of use in TGFB delivery if designed for local endometrial action.

A FUTURE MODEL FOR MANAGEMENT OF AUB

Despite advances in understanding of the mechanisms and pathological processes that lead to AUB, many treatments remain broad spectrum and generic. Often the focus is on AUB symptom control rather than specific diagnosis and targeted treatment.

Achieving an accurate diagnosis starts with focused history taking and clinical examination and should involve reference to FIGO AUB System 1 (nomenclature) and System 2 (classification; PALM-COEIN) (1). This directs further relevant investigations and personalises management. For example, identifying and understanding a patient's wish for fertility, previous experience with treatments and assessment of the size, position, regularity, mobility, and tenderness of the uterus will help aid clinicians to tailor management options specifically suited to that patient and their diagnosis. Further research to determine how patient demographics such as age, BMI and physical activity influence menstrual blood loss and response to treatment may also help to select more effective treatments for individuals (109).

To assist clinical diagnosis, bedside tests that identify the presence of structural uterine disorders and/or identify the specific cause(s) of endometrial dysfunction would be highly valuable. For example, the ability to identify if a women with AUB-E had aberrations in endometrial hypoxia, inflammation and/or coagulation at the time of endometrial sampling would facilitate personalised medicine and correction of the specific underlying defect causing AUB. These tests may also assist in the selection of appropriate investigations such as ultrasound or hysteroscopy.

The gold standard for diagnosis of adenomyosis is histopathological confirmation of eutopic endometrium within the myometrium. However, the use of transvaginal ultrasound (TVUS) in achieving accurate diagnosis of adenomyosis is highlighted in a systematic review (46). Of the 8 studies included, TVUS 2D and TVUS 3D were shown to be effective methods for diagnosis of adenomyosis with pooled sensitivity of 84 and 89%, and pooled specificity of 64 and 56%, respectively (46). These findings support the use of imaging in obtaining an accurate

diagnosis but focused research to improve the specificity of such methods would have significant clinical and scientific benefits.

Several biomarkers for adenomyosis have also been proposed. For example, proteomic analysis has shown that moesin, a cytoskeletal adaptor protein, is higher in the endometrium of those with adenomyosis vs. controls (110). This finding may also help our understanding of the development of adenomyosis as moesin expression is correlated with the extent of invasiveness seen in some tumours, such as gastric adenocarcinoma (110). Full discussion of all emerging biomarkers for AUB is not possible in this review but such findings highlight the potential for improving the non-invasive diagnosis of this debilitating symptom.

Improved diagnosis of structural and non-structural causes of AUB will not only direct treatment but will facilitate research into the pathogenesis of specific diagnoses (e.g., AUB-E/A) and reveal new therapeutic targets and preventative strategies to improve the lives of those who suffer from AUB.

CONCLUSIONS

Furthering our understanding of menstrual physiology confirms that there is a complex interplay of endocrine, immune, haemostatic and vascular regulatory functions. This complexity is mirrored in the pathological aberrations that may occur in each of these pathways, resulting in AUB. We have demonstrated that current medical management of AUB is suboptimal by highlighting poor satisfaction rates and the lack of specific, targeted treatments. Emerging treatments offer the promise of more specific targeting of the underlying pathology causing AUB-E and AUB-A. Accurate diagnosis of the underlying cause of AUB should be a clinical and research priority. Future research should also focus on developing therapies which have direct actions against the pathological processes which have been demonstrated to result in AUB-E and AUB-A. Focused clinical assessment and imaging techniques may help toward this goal but the development of non-invasive biomarkers would be a significant step toward improving management. These efforts would facilitate the development of personalised, effective, acceptable treatments to improve the lives of those who experience AUB.

AUTHOR CONTRIBUTIONS

MW and RM-A wrote the manuscript. JM, MW, and RM-A planned and edited the manuscript. All authors have read and approved the final version of this manuscript.

FUNDING

Some aspects of studies described herein were undertaken in the MRC Centre for Reproductive Health which was funded by MRC Centre Grants G1002033 and MR/N022556/1. This work was also in part funded by Wellcome Trust Grant 209589/Z/17/Z and Tenovus Scotland.

ACKNOWLEDGMENTS

We would like to acknowledge the participants and clinical research team who have helped with our studies to date.

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frontiers in Reproductive Health

REVIEW
published: 28 February 2022
doi: 10.3389/frph.2022.820029

Historical Perspectives and Evolution of Menstrual Terminology

Rohan R. Chodankar[1], *Malcolm G. Munro*[2] *and Hilary O. D. Critchley*[3]*

[1] Department of Obstetrics and Gynaecology, NHS Lothian, Edinburgh, United Kingdom, [2] Department of Obstetrics and Gynecology, David Geffen School of Medicine at UCLA, Los Angeles, CA, United States, [3] Medical Research Council Centre for Reproductive Health, The Queen's Medical Research Institute, The University of Edinburgh, Edinburgh, United Kingdom

Abnormal uterine bleeding (AUB) in the reproductive years in non-pregnant women comprises a group of symptoms that include abnormal frequency and the irregular onset of flow as well as prolonged and heavy menstrual bleeding. It is a common, chronic, and debilitating condition affecting women worldwide with an adverse impact on their quality of life. Until the last decade, the "menstrual" terminology used to describe both normal and abnormal uterine bleeding and its underlying causes was inconsistent, creating considerable confusion. Using standardized terminology may potentially improve clinical management as well as help designing and interpreting basic, translational, epidemiological, and clinical research in women with menstrual problems. In this article, we explore the history and evolution of menstrual terminology and discuss the two International Federation of Gynecology and Obstetrics (FIGO) systems on i.e., (A) menstrual terminology and definitions (B) and the causes of AUB, achieved through international consensus of relevant stakeholders through a long multistage journey.

Keywords: menstruation, menstrual terminology, abnormal uterine bleeding, PALM-COEIN, menstrual disorders

OPEN ACCESS

Edited by:
Fiona L. Cousins, Hudson Institute of Medical Research, Australia

Reviewed by:
Moamar Al-Jefout, United Arab Emirates University, United Arab Emirates
Antonio Simone Laganà, University of Insubria, Italy

***Correspondence:**
Hilary O. D. Critchley
hilary.critchley@ed.ac.uk

Specialty section:
This article was submitted to Gynecology, a section of the journal Frontiers in Reproductive Health

Received: *22 November 2021*
Accepted: *24 January 2022*
Published: *28 February 2022*

Citation:
Chodankar RR, Munro MG and Critchley HOD (2022) Historical Perspectives and Evolution of Menstrual Terminology. Front. Reprod. Health 4:820029. doi: 10.3389/frph.2022.820029

INTRODUCTION

Abnormal uterine bleeding (AUB) in the reproductive years in non-pregnant women comprises a group of symptoms that include abnormal frequency and the irregular onset of flow as well as prolonged and heavy menstrual bleeding; the latter referred to as HMB. Individually or collectively, the symptoms frequently have an adverse effect on the quality of life (QoL) and can be debilitating. The precise prevalence of AUB is not well-understood since many women normalize their symptoms, do not present for care, or are deemed "normal" by healthcare providers, but it has been estimated that at least 1 in 4 women of reproductive age are affected, however the prevalence may be as high as 53% (1–3). It is important to remember that AUB is a collection of symptoms and that, in each instance, there exists one or more underlying causes that are almost always benign, but occasionally, and especially in the later reproductive years, may be premalignant or malignant. Heavy menstrual bleeding especially is typically chronic, and in addition to the cyclical adverse impact on QoL, the chronic blood loss frequently leads to iron deficiency with all the attending adverse effects on cognitive and physical function (4).

Until the last decade, the "menstrual" terminology used to describe both normal and abnormal uterine bleeding and its underlying causes was inconsistent, leading to the widespread use of a variety of poorly defined terms. In the past, this circumstance hampered both teaching and clinical management and made challenging the process of designing and interpreting basic, translational, epidemiological, and clinical research in women with menstrual disorders. A well-known example

includes two contemporaneous clinical trials, in the USA and in Europe, established to answer the same clinical question (5) due to lack of clarity on menstrual disorder terminology.

In this article, we explore the history of menstrual terminology, the potential causes of AUB symptoms, the continuing evolution to the current versions of the two systems developed by the International Federation of Gynecology and Obstetrics (FIGO) as per the FIGO Committee on Menstrual Disorders, known as the MDC.

HISTORICAL PERSPECTIVES

Although it is beyond the scope of this chapter to explore all the historical perspectives associated with abnormal menstruation, we discuss below the presumed origin of three of the terms commonly used in the medical literature to describe menstrual disorders, i.e., menorrhagia, metrorrhagia, and dysfunctional uterine bleeding. It is difficult to ascribe these above-mentioned terms to the exact descriptions in the historical texts as discussed below. Much of this history of menstrual terminology is addressed in depth in the publication by Woolcock et al. (6).

In the early literature (430BC until the 1800s), what is currently defined as HMB was described variously as "excessive evacuations of the menses, inordinate flowing, the immoderate flux, an overflowing of the courses, excessive flooding's, uterine hemorrhage, and so on." Hippocrates (born around 460 BC) in his Aphorisms, translated from Greek and Latin to English in 1822 (potentially addresses HMB in the following descriptions: "To stop excessive evacuations of the menses, a large cupping glass may be applied to the breast," and "Menstruation if too abundant produces disease" (7).

The popular Greek philosopher Aristotle (Third century BC) also addressed excessive menstrual bleeding, as referenced in the English translations of his work Aristotle's Masterpieces, although it is believed that he relied heavily upon the works of Hippocrates for medical reference. For example: "In quantity, bleeding is excessive, saith Hippocrates, when they flow about eighteen ounces;" "In time when they flow about 3 days;" and "but it is inordinate flowing when the faculties of the body are thereby weakened." These menstrual volumes fit with those of women in clinical trials of drugs and devices designed to treat causes of HMB, and the "weakened faculties" could be perceived to be the result of iron deficiency!

Other historical references include the Bible (New Testament, Gospel of St. Mark, King James I translation from the original Greek, 1611) where excessive bleeding is described as "And a woman, which had an issue of blood 12 years, and had suffered many things of many physicians and straightway the fountain of her blood was dried up."

Avicenna, the Persian philosopher, via his book Canon of Medicine describes a scenario where "menstruation is profuse and is arrested with difficulty." In 1666, Thomas Sydenham, the English physician, when addressing "immoderate menstrual flow" described how "the natural flow of the menses would fill a vessel the size of a goose's egg," perhaps reflecting a desire to communicate the quantity of blood lost at menstruation. Furthermore, the same author describes that "when inordinate, there is difficulty, weakness, anorexia, cachexia, cadaverous complexion, and swelling of the feet." The latter content may be capturing the symptoms of (gross) anemia associated with heavy menstrual loss. None of the historical publications concerning menstruation used the term "heavy menstrual bleeding" or "menorrhagia," but clearly addressed the symptom through other descriptors (6).

The term "menorrhagia" is believed to have been first used by Professor William Cullen, Professor of the Practice of Physic at the University of Edinburgh, in the 1700s. Its usage appears in his textbook of lectures to medical students (8). One of the earliest written uses of the term was in a discourse in Latin written by one of his student's and attributed to Cullen. The word "menorrhagia" is derived from the Greek noun "mene" meaning moon, and the verb "regnumi" meaning to burst forth, to let loose or break asunder, the implication being sudden severe blood loss. Cullen also used the term "maetrorrhagia" in his lectures. The origin is from the Greek noun, "metra," meaning uterus, and the verb "regnumi" again, perhaps suggesting bleeding bursting forth from the uterus at any time, that is, much less regular than implied by "menorrhagia." The English physician Fleetwood Churchill (one of the first true specialist obstetrician/gynecologists clearly summarizes early nineteen century use of the term "menorrhagia" in his textbook on "Principal Diseases of Females. There in, "metrorrhagia" appears to have been a less popular term than "menorrhagia," and Churchill omits use the term (6).

The causes of menstrual disorders receive considerably less attention in historical literature before the 1800s, predominantly attributed to the lack of knowledge. During the late nineteenth century and early twentieth century that the causes of AUB were starting to be recognized. With the advent of anesthetic safety, histological assessments and radiology, the causes of AUB were becoming more apparent. The confusing term "dysfunctional uterine bleeding," or DUB, did not appear until the 1930s. it is then that possible causes for AUB in a group of women who did not have recognizable local pelvic pathology began to be considered.

THE PROBLEM WITH TRADITIONAL MENSTRUAL TERMINOLOGY

There is considerable confusion in the existing medical literature when describing normal menstrual bleeding and AUB symptoms and distinguishing those symptoms from their underlying etiology. In the past, and too often in the present, terms such as HMB and AUB, and previously, menorrhagia and DUB, have been often used to indicate either or both a symptom and a diagnosis. Such a circumstance can adversely impact the design and interpretation of clinical and basic research, and, thereby, undermine clinical care. Historically, the two most common descriptors used are the terms menorrhagia and DUB, and we will use these as examples to highlight the problem with menstrual terminology that ultimately led to the design of the two FIGO systems.

TABLE 1 | Analysis of the use of the term menorrhagia.

Category	Usage
1(a) Defined	56
1(b) Undefined	44
	$n = 100$
2(a) Used as symptom of heavy uterine bleeding, irregular or regular, with or without pathology	34
2(b) Used as symptom of heavy uterine bleeding, regular, with or without pathology	28
2(c) Used as a symptom of heavy uterine bleeding, regular with no detectable pathology	16
	$n = 78$
3(a) Primarily reflecting patient complaint	59
3(b) Primarily reflecting the doctor's definition	19
	$n = 78$
4(a) Used as a diagnosis	5
4(b) Used as a diagnosis when combined with another term (e.g., "idiopathic")	17
	$n = 22$

Adapted from Woolcock et al. (6).

The term "menorrhagia" appears to have been universally employed as a description of some aspect of excessive, heavy, or prolonged menstrual blood loss; however, no clear definition existed. Woolcock et al. (6) reviewed 100 articles (in English) appearing on Medline (Ovid Technologies, Inc, New York, USA) between 2000 and 2006 where the term "menorrhagia" appeared in the article title. The articles were classified based on the usage of the term menorrhagia in 4 major categories:

- If the term menorrhagia was defined or not,
- If the term menorrhagia was used as a symptom of heavy uterine bleeding, with or without pathology, with irregular or regular bleeding,
- If the term menorrhagia was recognized as a patient complaint or a doctors' definition,
- If the term menorrhagia was used as a diagnosis by itself or in combination with other adjectives (see **Table 1**).

The analysis of these 100 articles suggested that nearly 1 in 5 authors used the term menorrhagia to describe a diagnosis rather than a symptom and nearly 75% of these authors used a qualifying adjective preceding the term menorrhagia e.g., idiopathic, essential, and so on. Overall, the authors concluded that the use of the term was sometimes so uncertain that approximations had to be made as to which of the 4 categories was suitable with an overlap in several instances.

Similarly the term DUB in the UK referred to regular, (i.e., cyclic and predictable) HMB following the exclusion of other pathologies i.e., likely describing ovulatory bleeding. In the USA the term DUB usually referred to irregular uterine bleeding related to anovulation (9). The term DUB was first used by Graves in the 1930s to ascribe the "impairment of endocrine factors that normally control menstruation." Whereas, the confusion in terminology is apparent, this lack of clarity may also impact the interpretation and implementation of clinical trial data. An example includes a UK-based randomized controlled trial RCT ($n = 204$) which randomized women with a clinical diagnosis of DUB to a hysterectomy or hysteroscopic surgery (endometrial resection or endometrial laser ablation). The final histology however, revealed the presence of fibroids, adenomyosis and endometrial cancer, a circumstance that reflects the diagnostic heterogeneity of the enrolled subjects (10). The inclusion of such intervention based RCTs in systematic reviews, and, if performed, meta-analysis, can produce misleading results, as the primary inclusion criteria could be considered flawed since they were based on a symptom such as DUB rather than the underlying cause of the symptom.

EVOLUTION OF MENSTRUAL TERMINOLOGY

Achieving an international consensus on menstrual terminology has been a multistage journey. The process was designed to include a wide spectrum of stakeholders representing national and subspecialty gynecological societies worldwide, relevant medical journals, the FDA, and a variety of recognized experts from six continents. The initial result was a consensus-based system that defined both normal and abnormal menstrual bleeding with simple terms translatable into multiple languages. Ultimately, the process evolved to include a second system classifying the potential causes or contributors to AUB symptoms and called the PALM-COEIN system. In 2011 the two systems were initially presented together in a seminal paper that was then updated in 2018 following an additional rigorous process of clarification and revision (11, 12). The entire process was initially conducted under the aegis of a FIGO Menstrual Disorders Working Group, that subsequently became the Committee on Menstrual Disorders (usually called the Menstrual Disorders Committee or MDC).

- Terminology and Definitions (FIGO-AUB System 1)
- Classification of Causes of AUB in the Reproductive Years, the PALM-COEIN system (FIGO-AUB System 2).

The evolution of this process is shown in **Figure 1**.

The Paris Meeting

The first step in the development of a standardized system was to deal with AUB associated with systemic disorders of hemostasis. The core group began by assembling an international group of clinician-investigators from the gynecological and hematological communities with expertise in the field of AUB and/or inherited haemostatic disorders. The goals developed for the group were:

1. Collaborative review of the evidence base concerning the prevalence and clinical impact of disorders of haemostasis in reproductive-aged females with AUB.
2. Development of a consensus on an appropriate screening methodology and tests of coagulation function suitable for use in the evaluation of females with AUB.

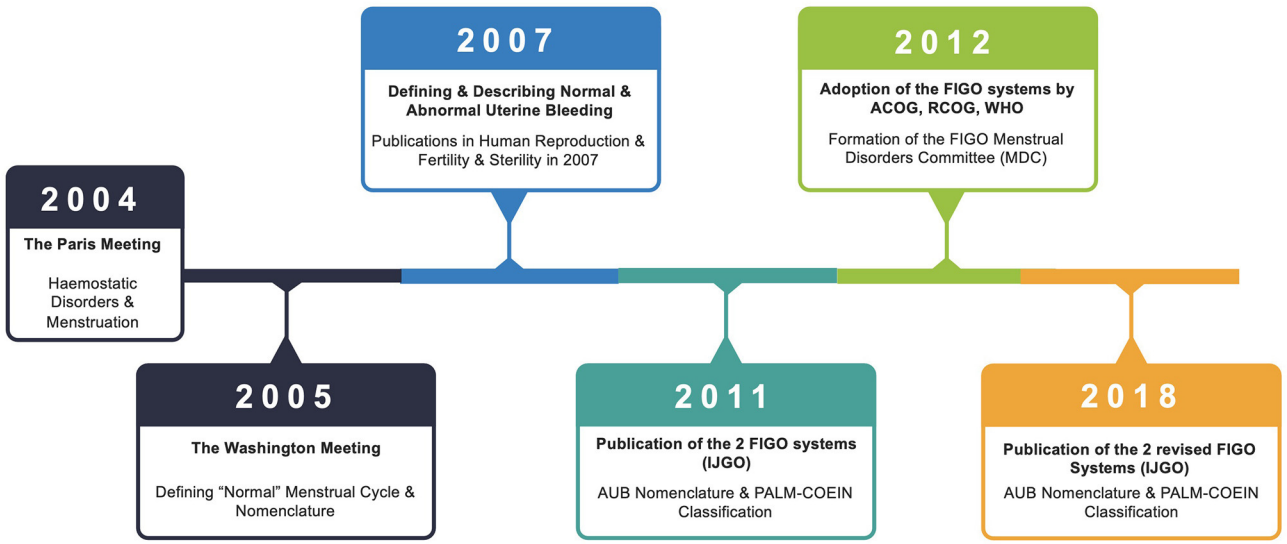

FIGURE 1 | Evolution of the two FIGO Systems. Relevant publications include 2005 (13–15), 2007 (16, 17), 2008 (6), 2011 (11), and 2018 (12). FIGO, International Federation of Gynecology and Obstetrics; IJGO, International Journal of Gynecology & Obstetrics; ACOG, American College of Obstetricians & Gynecologists; RCOG, Royal College of Obstetricians & Gynecologists; WHO, World Health Organization; AUB, Abnormal Uterine Bleeding; MDC, Menstrual Disorders Committee.

3. Evidence-based evaluation of AUB therapeutic approaches in females with known disorders of haemostasis.
4. Identification and prioritization of targets for clinical and basic research in the future.

Following development of draft documents, the members of the interdisciplinary consensus group assembled in Paris, France in May 2004. It was a less formal process that started with presentations and was followed by group discussion. Recommendations required the consensus of members and areas of disagreement were recorded. Following the meeting, manuscripts were drafted and circulated to subgroup members for required revisions. Each manuscript was distributed to each member of the consensus group for approval. This then culminated into the development of several important publications (13–15).

The Washington Meeting

In 2004 the core organizers of the Paris meeting started to develop a process where the aim was to recommend clear, simple terminologies and definitions that would have the potential for wide acceptance. The process was called "Terminologies, Definitions and Classifications of Abnormal Uterine Bleeding (AUB)" and the aim was to determine consensus to support clinical care, trainee education, and the future design and interpretation of basic, translational, clinical and epidemiologic research related to non-gestational abnormal uterine bleeding in the reproductive years (16, 18).

The process began by performing a detailed literature review for terms commonly used to describe menstrual disorders (i.e., menorrhagia, dysfunctional uterine bleeding, and abnormal uterine bleeding) with the search including a variety of publications such as clinical trials, review articles, and well-read popular gynaecologic textbooks. This review confirmed that there was significant inconsistency and resulting confusion regarding the terminology used to describe normal and abnormal menstruation. With this material, the organizers sought and received support from FIGO, the American Society for Reproductive Medicine (ASRM) and the European Society of Human Reproduction and Embryology (ESHRE) and received unconditional grants from several donors. With this support, the organizers established contact with relevant international and national organizations, journal editors, representatives of the US Food and Drug Administration (FDA), and experts including reproductive endocrinologists, gynecologists, and investigators to develop an expert panel. Ultimately this panel comprised 35 representatives that included a broad spectrum of stakeholders including those from both developed and developing countries.

The Washington process included experts in the use of the RAND corporation's Delphi process (M. Broder and the Partnership for Health Analytic Research, Beverly Hills, CA). The Delphi method is a validated nominal group process designed to determine consensus on a clearly defined issue using a series of anonymous polls with individualized feedback designed to provide context in a non-confrontational fashion (19). For the Washington meeting a modification of the model was used that initially comprised a series of e-mail-based surveys of the panel members designed to determine their understanding of the use of terminology to describe normal and abnormal menstrual

bleeding, as well as the causes of AUB in the reproductive years. The polls were designed so that most items were rated on a 4-point scale, and agreement was defined as at least 80% of respondents rating the item either 1 and 2, or 3 and 4. For example, if the rating scale was 1 = strongly disagree, 2 = disagree, 3 = agree, and 4 = strongly agree, at least 80% of respondents were required to provide either a "disagree" answer (1 or 2) or an "agree" answer (3 or 4) for there to be agreement on that item. Results were reported as the mean of the responses.

The aggregate ratings were shared when the expert group met in person for 3 days in February 2005 in Washington, D.C. (USA). Delphi rounds performed at the Washington meeting were conducted using an anonymous electronic survey system (Audience Response System) allowing for instantaneous polling of the participants and display of the results (11). The aggregate survey responses were considered in a plenary session of all meeting participants and also in smaller groups dedicated to aspects of classification and terminology.

Following extensive discussions, the smaller groups identified areas of agreement and disagreement, which were used to create new survey questions. These modified surveys were subsequently administered to all participants during a plenary session using electronic voting. During this In second round of ratings, two levels of agreement were identified. Panelists were considered to have "agreed" on an item if ratings met the original criteria (0.80% of answers were either 1 and 2 or 3 and 4). Panelists were considered to have "unanimously agreed" if all rated an item either 1 and 2 or 3 and 4 (e.g., 100% of respondents selected either 4, "strongly agree," or 3, "agree").

The "Washington" meeting and its Delphi process led to the following major outcomes:

- There was no consensus definition for terms such as menorrhagia, metrorrhagia, hypermenorrhea, and dysfunctional uterine bleeding.
- These terms and similar ones such as oligomenorrhea, polymenorrhea, hypermenorrhea, and others should be abandoned.
- Simple, descriptive terms with clear definitions should be used which should be understood by health professionals and patients alike, and importantly, any terminology adopted should be suitable for translation into most languages.
- These simple terms should describe the parameters of menstrual frequency, regularity, duration, and volume, with norms defined by the 5th to 95% centiles as determined by analyses of large menstrual databases (20, 21).
- There exists a need for a separate system designed to categorize the causes, not the symptoms, of non-gestational AUB in the reproductive years. General concepts and categories were discussed and debated and there was substantial support for a system that recognized structural causes as well as those that are secondary to non-structural disorders.

Following the Washington meeting the FIGO Menstrual Disorders Working Group (MDWG) was established in early 2006 and the results of the Delphi process published simultaneously in two journals, Fertility and Sterility and Human Reproduction, in 2007 (16, 17).

TABLE 2 | Terms used to describe menstrual disorders that should no longer be used.

- Anomalous uterine hemorrhage
- Anovulatory menorrhagia
- Dysfunctional uterine bleeding;
- Excessively heavy menstrual loss
- Epimenorrhea
- Epimenorrhagia
- Essential menorrhagia
- Functional uterine hemorrhage
- Functional menorrhagia
- Genuine menorrhagia
- Hypermenorrhea
- Idiopathic menorrhagia
- Idiopathic uterine hemorrhage
- Menorrhagia
- Meno-metrorrhagia
- Metropathia hemorrhagica
- Ovulatory menorrhagia
- Polymenorrhea
- Polymenorrhagia
- Primary menorrhagia
- Persistent menorrhagia
- Symptomatic menorrhagia
- Unexplained menorrhagia
- Uncomplicated menorrhagia

Based on the consensus developed in the Washington meeting, the MDWG recommended that the following terms (see **Table 2**) that have been used over the last 100 years or so should no longer be used (6, 11, 12, 16, 17).

The MDWG had several activities relating to work surrounding menstrual terminology including presentations, publications, workshops, and meetings. However, and most importantly, it paved the way for planning a focused working group meeting during the 2009 FIGO World Congress in Cape Town and an AUB symposium was held within the main scientific program of the 2009 FIGO World Congress.

The Cape Town Meeting

FIGO's 19th triennial World Congress of Gynecology and Obstetrics, held in October 2009 in Cape Town, South Africa, provided a further forum for a pre-congress menstrual disorders workshop. In preparation for the meeting the MDWG recruited additional participants and initiated development of a draft system for classification of potential causes supported by telephonic and person to person discussion. At the workshop, members of the MDWG discussed and refined the elements of the system for classification of causes of AUB in the reproductive years. Following that, in the main AUB symposium "Let us Talk about How We Can Improve Clinical Management through Clear Language and Disease Classification," there was a unique opportunity to ascertain opinions concerning the

proposed systems (definitions of symptoms, and classification of causes) from over 800 participants with diverse national and socioeconomic backgrounds, and aided by the use of an audience response system (ARS). This process was also designed to gauge the ability of participants from a spectrum of countries, including those defined as low and middle income countries (LMIC), to have the resources needed to evaluate patients using imaging and laboratory tests. The main outcomes from this meeting were as follows:

- 215/237 (90.7%) respondents agreed that "AUB" was a suitable overarching term for abnormal menstrual symptoms.
- 96/141 (68.1%) and 171/223 (76.7%), respectively, supported proposals that terms such as "menorrhagia" and "DUB" be discarded.
- 198/237 (83.5%) agreed that the term "heavy menstrual bleeding (HMB)" should replace the term "menorrhagia" for the symptom of excess menstrual bleeding.
- agreement on the principles, structure, and content of a "discussion" document for "Classification of causes of abnormal uterine bleeding."
- Format and content of a proposed "Structured menstrual history" with widespread applicability.

THE TWO FIGO AUB SYSTEMS

In 2011, recognizing the international unmet need created by the impact of AUB, the FIGO MDWG published two systems (FIGO Systems 1 and 2) and a set of clinical recommendations in order to inform and aid clinicians and investigators in the design and interpretation of investigations into AUB in the reproductive years, as well as the provision of evidence-based clinical care (11). In 2012 FIGO endorsed the systems and, at the same time, "promoted" the MDWG to a standing committee called the "Committee on Menstrual Disorders," or the "Menstrual Disorders Committee" known as the MDC. FIGO's Systems 1 and 2 are living entities designed to adapt to the evolving nature of menstrual norms and the classification process in light of ongoing debate and the assimilation of new knowledge from appropriately designed research. The most recent update was published in 2018 where the contributions from the FIGO MDC, as well as epidemiologists, gynecologists, and other experts from around the world between 2012 and 2017 were utilized. Where major change was considered, anonymous voting, in some instances using a modified RAND Delphi technique (described previously), was utilized (12).

Terminology and Definitions (FIGO-AUB System 1)

So, what specifically is FIGO AUB System 1? To start with, System 1 describes non-gestational abnormal uterine bleeding (AUB) in the reproductive years as an overarching term for disturbances in one or more aspects of menstruation including the frequency, regularity, duration, and volume of menses including the presence of bleeding between periods and unanticipated bleeding associated with the use of medications such as gonadal steroids for contraception. The objective measurement of the volume of menstrual blood loss correlates poorly with presenting symptomatology and health seeking behavior. Consequently, FIGO has adopted the National Institute for Care Excellence definition of heavy menstrual bleeding (HMB) which, for clinical purposes, defines it as "excessive menstrual blood loss which interferes with the woman's physical, emotional, social and material quality of life, and which can occur alone, or in combination with other symptoms" (22).

In the original system published in 2007, FIGO introduced the concept of acute non-gestational AUB in the reproductive years, distinguishing it from chronic AUB. These definitions remain unchanged for 2018. Chronic non-gestational AUB in the reproductive years is defined as "bleeding from the uterine corpus that is abnormal in duration, volume, frequency, and/or regularity, and has been present for the majority of the preceding 6 months." Acute AUB, on the other hand, is defined as "an episode of heavy bleeding that, in the opinion of the clinician, is of sufficient quantity to require immediate intervention to minimize or prevent further blood loss."

When AUB occurs between well-defined cyclical episodes of menstrual bleeding, the symptom described as intermenstrual bleeding and may be further sub divided as:

- *Cyclic Midcycle IMB*—Small quantity of frank vaginal bleeding or discharge around midcycle. This may be physiological due to the nadir in circulating oestradiol levels that follow the oestradiol surge that initiates ovulation.
- *Cyclic Pre or Postmenstrual IMB*—Cyclical IMB that predictably occurs either early in the cycle (follicular phase) or late (luteal phase), and typically presents as very light vaginal bleeding for one or more days.
- *Acyclic IMB*—When the IMB is not cyclical or predictable.

The summary of the terminology recommended by the FIGO MDC is shown in **Figure 2** (12).

Classification of Causes of AUB in the Reproductive Years, the PALM-COEIN System (FIGO-AUB System 2)

System 2 describes the known potential causes or contributors to the symptoms categorized in System 1. There are nine main categories, arranged according to the acronym PALM-COEIN (pronounced "palm-koin"): Polyp; Adenomyosis; Leiomyoma; Malignancy and hyperplasia; Coagulopathy; Ovulatory dysfunction; Endometrial disorders; Iatrogenic; and Not otherwise classified. Since the original publication in 2011, category N has undergone a change from "not yet classified" to "not otherwise classified" recognizing that some entities may never have a specific classification category. The components of the PALM group are generally discrete (structural) entities that can be evaluated or measured visually using some combination of imaging techniques and histopathology; the COEI group comprises entities

Parameter	Normal	Abnormal	☑
Frequency	Absent (no bleeding) = amenorrhea		☐
	Infrequent (>38 days)		☐
	Normal (≥24 to ≤38 days)		☐
	Frequent (<24 days)		☐
Duration	Normal (≤8 days)		☐
	Prolonged (>8 days)		☐
Regularity	Normal or "Regular" (shortest to longest cycle variation: ≤7-9 days)*		☐
	Irregular (shortest to longest cycle variation: ≥8-10 days)*		☐
Flow Volume (patient determined)	Light		☐
	Normal		☐
	Heavy		☐

Intermenstrual Bleeding (IMB) Bleeding between cyclically regular onset of menses			
	None		☐
	Random		☐
	Cyclic (Predictable)	Early Cycle	☐
		Mid Cycle	☐
		Late Cycle	☐

Unscheduled Bleeding on Progestin ± Estrogen Gonadal Steroids (birth control pills, rings, patches or injections)		
	Not Applicable (not on gonadal steroid medication)	☐
	None (on gonadal steroid medication)	☐
	Present	☐

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FIGURE 2 | FIGO AUB System 1 Nomenclature and Definitions of AUB symptoms—The normal menstrual cycle is based on 4 parameters i.e., frequency, duration, regularity, and volume (subjectively determined by patient). The table shows normal values in unshaded areas and abnormalities in each of the parameters in shaded areas. The middle and lower panels are new (vs. 2011 paper); the middle panel is used to describe the presence or absence of IMB, whereas the lower panel is for the description of unscheduled bleeding while using gonadal steroid medication, most often progestogen or estrogen and progestogen-containing preparations.

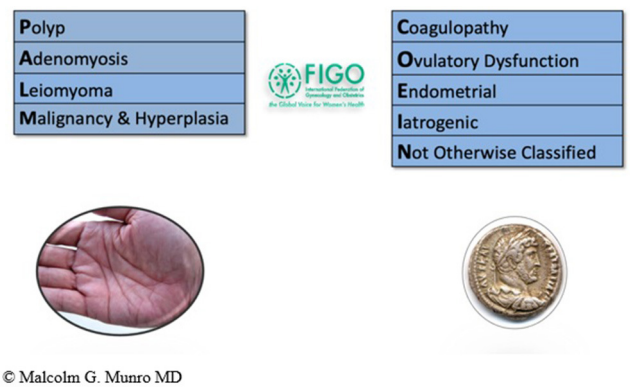


FIGURE 3 | FIGO AUB System 2. PALM-COEIN system for classification of causes of AUB in the reproductive years. Adapted from Munro et al. (12).

that are not defined by imaging or histopathology (non-structural). By its nature, the "Not otherwise classified" category includes a spectrum of potential entities that may or may not be measured or defined by histopathology or imaging techniques, but are not considered qualified for their own category or inclusion in an existing category (12) (see **Figure 3**).

The FIGO MDC is currently working on an international consensus for an imaging-based adenomyosis classification system designed to phenotype the disorder in a standardized fashion. However, for diagnosis the use of the transvaginal ultrasonography-based MUSA criteria have been defined (23).

Polyps (AUB-P)

Endometrial polyps are epithelial proliferations arising from endometrial stroma and glands (24). The reported prevalence of endometrial polyps ranges from 7.8 to 34.9%, depending on the definition of a polyp, the diagnostic method used, and the population studied (25–28). Exocervical polyps may be diagnosed by clinical examination, but those within the uterine cavity by one or a combination of ultrasonography, sonohysterography (US with simultaneous infusion of contrast into the endometrial cavity), hysteroscopy and histopathology. Blind endometrial sampling may identify polyps, however, have a low accuracy as compared to hysteroscopic directed biopsies (29, 30). Hysterosalpingography has a high sensitivity (98%), yet low specificity (35%) compared with hysteroscopic diagnosis (31). The gold standard for diagnosis of intrauterine polyps is hysteroscopy with a guided biopsy. Diagnostic hysteroscopy alone only has a reported sensitivity of 58–99%, specificity of 87–100%, positive predictive value (PPV) of 21–100%, and NPV of

66–99% when compared with hysteroscopy with guided biopsy as a diagnostic tool (32, 33).

Adenomyosis (AUB-A)

Adenomyosis is present when endometrial-like glands and stroma are identified in the myometrium, and is associated with hypertrophy and hyperplasia of the myometrium surrounding the ectopic endometrial tissue. The genesis of adenomyosis remains unclear, along with its association with AUB and infertility. Consequently, the appropriate diagnosis and management of adenomyosis remains poorly understood. Given the many uncertainties surrounding this condition, it has been recently described as an enigma (34).

Traditionally, the diagnosis of adenomyosis was made in retrospect, following histopathological assessment after hysterectomy for AUB. Defined sonographic criteria and magnetic resonance imaging (MRI) criteria for diagnosis of adenomyosis are described (23, 35). Despite this, the prevalence however remains unclear with a reported a 5–70% occurrence in histological diagnosis in hysterectomy specimens (36). Recent metanalyses have compared the accuracy of various imaging modalities in the non-invasive diagnosis of adenomyosis. Tellum et al. observed that pooled MRI, 2D-TVUS, and 3D-TVUS had a sensitivity of 78, 74, and 84% and a specificity of 88, 76, and 84% for diagnosing adenomyosis, respectively. 3D-TVUS could detect changes in the JZ, which was one of the more important diagnostic determinants (37). There was no statistically significant difference between the diagnostic quality of MRI and TVUS (35, 37).

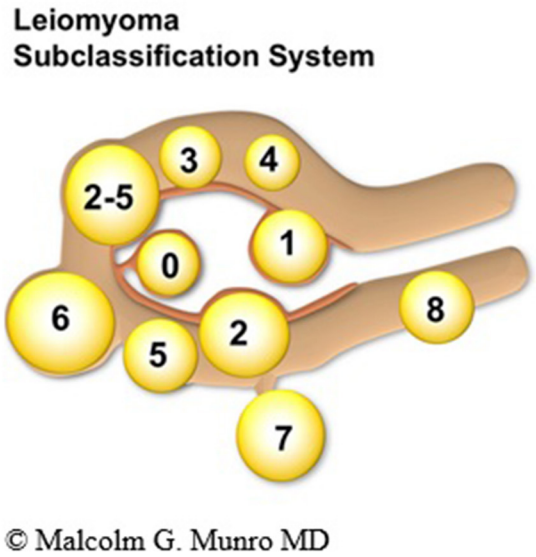
Recent evidence illustrates that adenomyosis may also be present in a nearly a third of young (<30 years) nulliparous women with symptoms of HMB and /or dysmenorrhoea (38). Adenomyosis may also co-exist in up to 60% of women with severe forms of endometriosis when evaluated using MRI (39). Studies using ultrasound have also found a similar relationship between ovarian endometriosis and adenomyosis in young women (<30 years) (40). This emerging evidence dispels the previously held belief that adenomyosis is largely a disease of parous women in the 4 or 5th decade of their life, Newer modalities such as elastography (ultrasound mode) have made progress in reaching a diagnosis (41). There remains limited evidence to guide the management of women with adenomyosis, either medically or surgically.

Leiomyomas (AUB-L)

Leiomyomas (fibroid, myoma), are very common with the estimated cumulative incidence by age 50 is >80% for black women and nearly 70% for those who are white (42). Fibroids may be asymptomatic (incidentally diagnosed) or commonly contribute to AUB when submucous (43–45).

Uterine fibroids may be diagnosed by clinical examination, which may reveal an enlarged uterine or pelvic mass. The most common modality used in the diagnosis of uterine fibroids is ultrasound (US), which may be transabdominal (TA) or transvaginal (TV). Its low cost and accessibility often make it primary choice as a diagnostic modality. TV US is considered more sensitive than TA US for detection of small fibroids, for submucous fibroids and in obese patients (46, 47). The reproducibility, sensitivity, and specificity of US lacks consistency between different studies. Sensitivity and specificity ranged from 24–96 to 29–93%, respectively, in published literature (47). A recent meta-analyses observed that saline infusion sonography (SIS) has a pooled sensitivity in the detection of all intrauterine abnormalities (polyps, sub-mucous fibroids, adhesions) of 0.88 [95% confidence interval (CI): 0.85–0.90] and a pooled specificity of 0.94 (95% CI 0.93–0.96) and is comparable to hysteroscopy in this context (48). The sensitivity and specificity of SIS have been reported to be as high as 85–91 and 83–100%, respectively (47). The current NICE guidance recommends hysteroscopy as a first line investigation for AUB in women with suspected submucous fibroids vs. a TV US and thereby remains a gold standard in the diagnosis of suspected intrauterine pathology or where an endometrial biopsy is indicated (22). Magnetic resonance imaging (MRI) has the highest sensitivity and specificity (88–93, 66–91%), respectively, in the diagnosis of fibroids and differentiating fibroids from focal adenomyosis when compared to other discussed modalities. It has excellent reproducibility vs. US, SIS and hysteroscopy and can identify unusual fibroids e.g., parasitic fibroids, and the extent of fibroid degeneration. Despite these advantages, the routine use is precluded by cost and accessibility (47).

The FIGO PALM-COEIN leiomyoma system is extensive and to date, the only sub-classification to be ratified by the FIGO. The system includes primary, secondary, and tertiary classification of leiomyomas with the first level being presence or absence, the second submucous or "other" and the third a categorization that includes the submucous group according to the original Wamsteker et al. system (49). The FIGO system adds additional categorisations for submucous, intramural, subserosal, and transmural lesions. Intracavitary lesions are attached to the endometrium by a narrow stalk (≤10% or the mean of three diameters of the leiomyoma) and are classified as Type 0, whereas Types 1 and 2 require a portion of the lesion to be intramural—with Type 1 being <50% of the mean diameter and Type 2 at least 50%. Type 3 lesions are intramural but also abut the endometrium. Although they can be diagnosed with imaging techniques such as sonohysterography and MRI, Type 3 lesions are formally distinguished from Type 2 with hysteroscopy using the lowest possible intrauterine pressure necessary to allow visualization. Type 4 lesions are intramural leiomyomas that are entirely within the myometrium, without extension to the endometrium or to the serosa. Subserous (Types 5, 6, and 7) leiomyomas represent the mirror image of the submucous leiomyomas—with Type 5 being at least 50% intramural, Type 6 being <50% intramural, and Type 7 being attached to the serosa by a stalk that is also ≤10% or the mean of three diameters of the leiomyoma. Classification of lesions that are transmural are categorized by their relationship to both the endometrial and the serosal surfaces. The endometrial relationship is noted first, with the serosal relationship second (e.g., Type 2–5). An additional category, Type 8, is reserved for leiomyomas that do not relate to the myometrium at all, and would include cervical lesions (demonstrated), those that exist in the round or broad ligaments without direct attachment



SM - Submucous	0	Pedunculated intracavitary
	1	<50% intramural
	2	≥50% intramural
	3	Contacts endometrium; 100% intramural
O - Other	4	Intramural
	5	Subserous ≥50% intramural
	6	Subserous <50% intramural
	7	Subserous pedunculated
	8	Other (specify e.g. cervical, parasitic)

Hybrid (contact both the endometrium and the serosal layer)	Two numbers are listed separated by a hyphen. By convention, the first refers to the relationship with the endometrium while the second refers to the relationship to the serosa. One example is below	
	2-5	Submucous and subserous, each with less than half the diameter in the endometrial and peritoneal cavities, respectively.

FIGURE 4 | FIGO leiomyoma sub-classification system. Adapted from Munro et al. (12).

to the uterus, and other so-called "parasitic" lesions (12) (see **Figure 4**).

Location seems to be a more important factor than size in determining bleeding symptoms. Submucous myomas, those in or partially intruding into the endometrial cavity, are most likely to cause heavy menstrual bleeding. The reason why these tumors cause disproportionate bleeding is not clear (44).

Malignancy and Hyperplasia (AUB-M)

FIGO System 2, the PALM-COEIN system, aims to complement pre-existing classification systems by the World Health Organization (WHO) and FIGO for atypical endometrial hyperplasia (also known as endometrial intraepithelial neoplasia, or EIN) and gynecological malignancies, in particular endometrial cancer (50, 51). There are several risk factors for EIN and endometrial cancer in premenopausal women that include obesity, a family history, and chronic anovulation from a spectrum of causes that typically manifest with irregular menstrual bleeding (AUB-O). These have been defined by the RCOG (52) and reflect the increasing incidence relating to the increased prevalence of obesity in many populations (53). WHO first proposed a classification system for endometrial hyperplasia in 1994 (54), which was subsequently revised in 2004 (55).

The current NICE guidance recommends that women presenting with AUB, where an endometrial biopsy is deemed necessary, this should be done in the context of outpatient hysteroscopy, rather than blind sampling (22). The high accuracy, sensitivity, and specificity of hysteroscopy in assessing intrauterine pathology are well-studied (56, 57).

Cervical cancer may present as persistent IMB or post-coital bleeding.

Leiomyosarcoma (LMS) is an aggressive uterine tumor (sarcoma) and may present with AUB often associated with a rapid increase in fibroid size. The incidence of uterine sarcoma is a topic of current interest and good quality data are required, specifically given the high utilization of power morcellation of fibroids during minimal access surgery. These tumors are aggressive, have a poor prognosis and a high recurrence rate following treatment, with intraperitoneal dissemination having potentially disastrous consequences due to seeding of malignant cells. Age and peri-menopausal status are important considerations. Recent data highlights an increased incidence of expected uterine sarcomas for women undergoing hysterectomy for benign indications, including fibroids (58–60). This risk increases with age and the risk is higher in women >45 years (58, 60). As the evidence base concerning risk of LMS in women with uterine fibroids builds important information will be available to clinicians to inform management discussions. This is an important finding as it provides important insights in clinical practice in guiding management in women with fibroids i.e., an informed discussion of the potential risks of a conservative approach (fibroid surveillance) in older women. Symptomatic postmenopausal women with uterine fibroids represent a particularly high-risk group (61) and may need more definitive treatment.

At the present time, there remains no laboratory test, for example, a tumor maker or an imaging study (ultrasound, MRI, CT scan) that can reliably diagnose uterine LMS preoperatively (62).

Recent evidence emphasizes the importance of performing endometrial sampling in women with AUB with suspected benign disease. Although the likelihood of diagnosing uterine sarcomas is low, a liberal approach to endometrial sampling may reduce the risk of unexpected non-benign histology in women undergoing hysterectomy (60). Furthermore, younger women who are obese are also at a risk of endometrial cancer and as such should be considered for endometrial sampling (63).

TABLE 3 | Screening for hematological abnormalities in women with AUB.

Structured history—positive screen if
a. Excessive menstrual bleeding since menarche, or
b. History of one of the following—postpartum hemorrhage, surgery-related bleeding, or bleeding associated with dental work, or
c. History of two or more of the following—bruising >5 cm once or twice/month, epistaxis once or twice/month, frequent gum bleeding, family history of bleeding symptoms

Adapted from Kouides et al. (14).

TABLE 4 | Screening for hematological abnormalities in women with AUB.

1	a. PT and APTT (if APTT prolonged, do mixing assay for inhibitor or factor deficiency)
	b. VWF antigen
	c. ristocetin cofactor d. Factor VIII
	e. ABO type
	f. Ivy bleeding time and/or PFA-100 closure time
	Nonhematologic testing: Consider TSH, especially if VWF levels reduced, and baseline iron profile if anemic prior to intervention
2	If #1 is normal, then consider platelet aggregation and release studies
	If #2 is normal, then consider specific factor levels (e.g., FXI, FXIII), and euglobuin clot lysis and other measures of fibrinolysis (α_2-antiplasmin level, plasminogen activator inhibitor level)
	For females without positive screen as noted in structured history above, but who are considering major surgical intervention, consider secondary evaluation. This because up to 8% of women without a positive screen (**Table 3**) will have underlying VWD (65)

Adapted from Kouides et al. (14).

Coagulopathy (AUB-C)

Underlying bleeding disorders are reported to affect 12–14% of the women presenting with the symptom of HMB, most commonly von Willebrand disease (64). While it is generally perceived that these diagnoses are made in adolescence, around menarche, when subtle abnormalities exist the first presentation of AUB-C may occur in adult life. A simple set of screening questions may allow identification of women at high risk, such that an appropriate laboratory testing can be performed, with or without onward referral to a hematologist. The system presented is 90% sensitive for the presence of a coagulopathy (14, 65) (see **Table 3**).

Based on the screening results a secondary evaluation may need to be undertaken in consultation with a hematologist as summarized in **Table 4**. A primary full blood count should be undertaken in all women presenting with AUB/HMB and a normal platelet count should be established prior to the secondary evaluation below. The evaluation of thrombocytopenia's is beyond the scope of this chapter.

Ovulatory Disorders (AUB-O)

Ovulatory disorders comprise a spectrum of disturbance in normal ovulatory function ranging from irregular or infrequent ovulation to anovulation. By its nature anovulation results in exposure of the endometrium to various levels of unopposed estrogen, which, absent progesterone, typically result in a persistent proliferative state and a consequent increase in the incidence of endometrial hyperplasia. Women with ovulatory disorders may be amenorrheic (a term retained by FIGO) or can manifest with infrequent and/or prolonged cycles and bleeding of a variety of durations and volumes, either related to spontaneous endometrial sloughing or to periodic ovulation and progesterone withdrawal. AUB-O is common in the early years following menarche and again during the perimenopausal transition due to changes in the hypothalamic-pituitary-ovarian axis, typically evolving to cyclical bleeding in the adolescent and, with menopause, the onset of amenorrhea. Ovulatory disorders may also be associated with or caused by other conditions such as hypothyroidism, hyperprolactinemia, extremes of weight (including sudden changes in weight), mental stress, and excessive exercise. The diagnosis of ovulatory disorders is largely based on a detailed menstrual history that is descripted in FIGO System 1. The use of serum progesterone measured in the presumed luteal phase, or the results of endometrial sampling may have occasional utility but can also be misleading since they reflect only a single cycle.

The recent FIGO classification systems update (2018) recommends that therapies interfering with the H-P-O axis and associated with AUB, now be placed in the "AUB-I" category.

Endometrial (AUB-E)

AUB that occurs with regular menstrual cycles in the absence of a bleeding disorder and unrelated to structural abnormalities is likely to represent a primary endometrial disorder. The exact etiology remains poorly understood, although defective local haemostasis may contribute (66–68). It is important to understand that structural anomalies such as uterine leiomyomas NOT in contact with the endometrium are unlikely to contribute to AUB, and in such instances, AUB-E or AUB-O should be considered depending on the characteristics of the menstrual cycle. There are no validated tests currently available for clinical use to diagnose AUB-E, which is a primary disorder of endometrial haemostasis. It is a diagnosis when no other explanation is found following clinical assessment (history, physical examination) conduct of appropriate blood tests and uterine imaging.

Iatrogenic (AUB-I)

AUB-I occurs secondary to the use of several medications or to the use of intrauterine systems, typically designed for contraception, but also those used primarily for the treatment of selected causes of AUB. They may be allocated to one of 5 major categories.

- Exogenous gonadal steroids, including levonorgestrel-releasing intrauterine systems (LNG-IUS), long-acting progestin preparations e.g., etonogestrel implants, gonadotrophin releasing hormone modulators including agonists and antagonists. These drugs alter the prevailing endocrine environment and often contribute to unscheduled or breakthrough bleeding (69). Up to 1 in 5 women using

progestin only contraception may develop AUB-I (70). Hormonal polytherapy may also be contributory.
- Pharmaceutical agents that alter drug bioavailability by modifying hepatic enzyme metabolism. Examples include anti-epileptic or anti-tuberculous drugs, which may alter the circulating level of gonadal steroids.
- Anticoagulants such as warfarin, unfractionated heparin, low molecular weight heparin with impaired formation of an adequate "plug" or clot within the vascular lumen.
- Agents that impact dopamine physiology. These include tricyclic antidepressants (e.g., amitriptyline and nortriptyline) and phenothiazines that can result in hyperprolactinemia with subsequent ovulatory dysfunction.
- Inert intrauterine systems or that contain copper or alloys that are designed for contraception.

Not Otherwise Classified (AUB-N)

On occasion AUB may be associated with rare or uncommon conditions or those for which there is an unclear association with symptoms. Worldwide, the incidence of cesarean delivery (CD) is rising substantially, and it has been recognized that there exists in many a defect at the incision site on the uterus that has been called variously a niche, an isthmocele or simply a cesarean scar defect (CSD). These defects at the site of CD may contribute to AUB and FIGO is currently undertaking a systematic review to study this relationship as a prelude to considerations of how and if this putative mechanism should be included in FIGO System 2 (71, 72). Uterine arteriovenous malformations may also be responsible for acute uterine bleeding but are not known to contribute to chronic AUB in the reproductive years (73, 74).

CONCLUDING THOUGHTS

The two FIGO classification systems are designed to define the nomenclature used to describe menstrual symptoms (System 1) and with System 2, to categorize the potential underlying causes or contributors to the spectrum of symptoms described in System 1. Indeed, it is important to understand that FIGO System 1 should be considered to be a mandatory gateway to the application of System 2, since many diagnoses require clear description of the menstrual symptoms experienced by the woman. This approach is clinically important in instituting the most appropriate approach to investigation and to the identification of a menu of treatment options that can be tailored to the individual patient considering her current clinical situation, future desires regarding fertility, and to her cultural and religious norms. In addition to helping the practicing healthcare professional manage patients with AUB, including the coordination of care, the two systems are also excellent tools for teaching and training due to the simplified expression of the concept of AUB. Since their introduction, the FIGO systems have received worldwide acceptance; at the time of writing this article there are approximately 2,444 citations in the literature, 1,480 of the two IJGO papers (11, 12). The systems are designed to be flexible with further classifications and subclassifications proposed in the future, thus allowing clinicians to refine and provide optimum care to patients, and bench, epidemiological and clinical investigators a structure within which to design and interpret AUB-related research.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

HC receives support from Medical Research Council Centre for Reproductive Health (MRC CRH) Grant MR/N022556/1.

ACKNOWLEDGMENTS

Authors wish to thank Tatiana White, MRC Center for Reproductive Health, University of Edinburgh for assistance with finalization of manuscript preparation and submission.

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Conflict of Interest: HC has received clinical research support for laboratory consumables and staff from Bayer AG (paid to Institution) and provides consultancy advice (no personal remuneration) for Bayer AG, PregLem SA, Gedeon Richter, Vifor Pharma UK Ltd, AbbVie Inc, and Myovant Sciences GmbH. HC receives royalties from UpToDate for article on abnormal uterine bleeding. RC has been supported as a clinical research fellow by Bayer AG. MM has received research support from AbbVie Inc and Pharmacosmos, and for consulting services from AbbVie Inc, Daiichi-Sankyo, Hologic Inc, Myovant Sciences, Pharmacosmos, and Vifor Pharma.

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frontiers in Reproductive Health

REVIEW
published: 04 March 2022
doi: 10.3389/frph.2022.818243

Uterine Fibroids (Leiomyomata) and Heavy Menstrual Bleeding

Outi Uimari[1,2], *Kavita S. Subramaniam*[3,4], *Beverley Vollenhoven*[5,6] *and Thomas T. Tapmeier*[4,6]*

[1] Department of Obstetrics and Gynecology, Oulu University, Oulu, Finland, [2] Research Unit for Pediatrics, Pediatric Neurology, Pediatric Surgery, Child Psychiatry, Dermatology, Clinical Genetics, Obstetrics and Gynecology, Otorhinolaryngology and Ophthalmology (PEDEGO) Research Unit and Medical Research Center Oulu, Oulu University Hospital, Oulu, Finland, [3] St John's Institute of Dermatology, King's College London, Guy's Hospital, London, United Kingdom, [4] Endometriosis CaRe Centre, Nuffield Department of Women's and Reproductive Health, University of Oxford, Oxford, United Kingdom, [5] Women's and Newborn Program, Monash Health, Clayton, VIC, Australia, [6] Department of Obstetrics and Gynaecology, Monash University, Clayton, VIC, Australia

Uterine Fibroids, or leiomyomata, affect millions of women world-wide, with a high incidence of 75% within women of reproductive age. In ∼30% of patients, uterine fibroids cause menorrhagia, or heavy menstrual bleeding, and more than half of the patients experience symptoms such as heavy menstrual bleeding, pelvic pain, or infertility. Treatment is symptomatic with limited options including hysterectomy as the most radical solution. The genetic foundations of uterine fibroid growth have been traced to somatic driver mutations (*MED12*, *HMGA2*, *FH*$^{-/-}$, and *COL4A5-A6*). These also lead to downstream expression of angiogenic factors including IGF-1 and IGF-2, as opposed to the VEGF-driven mechanism found in the angiogenesis of hypoxic tumors. The resulting vasculature supplying the fibroid with nutrients and oxygen is highly irregular. Of particular interest is the formation of a pseudocapsule around intramural fibroids, a unique structure within tumor angiogenesis. These aberrations in vascular architecture and network could explain the heavy menstrual bleeding observed. However, other theories have been proposed such as venous trunks, or venous lakes caused by the blocking of normal blood flow by uterine fibroids, or the increased local action of vasoactive growth factors. Here, we review and discuss the evidence for the various hypotheses proposed.

Keywords: uterine fibroid, leiomyoma, heavy menstrual bleeding (HMB), somatic mutation, vascular architecture

OPEN ACCESS

Edited by:
Philippa Saunders, University of Edinburgh, United Kingdom

Reviewed by:
Moamar Al-Jefout, United Arab Emirates University, United Arab Emirates
Essam R. Othman, Assiut University, Egypt

***Correspondence:**
Thomas T. Tapmeier
thomas.tapmeier@monash.edu

Specialty section:
This article was submitted to Gynecology, a section of the journal Frontiers in Reproductive Health

Received: *19 November 2021*
Accepted: *09 February 2022*
Published: *04 March 2022*

Citation:
Uimari O, Subramaniam KS, Vollenhoven B and Tapmeier TT (2022) Uterine Fibroids (Leiomyomata) and Heavy Menstrual Bleeding. Front. Reprod. Health 4:818243. doi: 10.3389/frph.2022.818243

HEAVY MENSTRUAL BLEEDING AND UTERINE FIBROIDS

As many as 1 in 20 women aged between 30 and 49 years consult their GP each year because of heavy menstrual bleeding (HMB) or menstrual problems, with menstrual disorders the reason for 12% of all referrals to gynecology services in the UK (1). While HMB was historically given as a blood loss of more than 80 mL per day (2)—a definition not considered useful any longer given the large variation in women's physique and the fact that most women who seek treatment for HMB do not actually meet this criterion (3)—HMB is now defined as "excessive menstrual blood loss which interferes with a woman's physical, social, emotional and/or material quality of life" (1). It can occur on its own or in combination with other symptoms such as acute and chronic pelvic pain, or infertility (4). The severity can be estimated by self-reporting in questionnaires (5). The potential causes for HMB are many, such as ovulatory disorders, adenomyosis, endometriosis, endometrial polyps, and endometrial hyperplasia (6); however, the most common condition underlying HMB are uterine fibroids.

Uterine fibroids, or leiomyomata, are benign tumors of the myometrium arising within the uterus. Despite the name, fibroids largely comprise of myocytes rather than fibroblasts and are characterized by the excessive deposition of extracellular matrix substances, mainly collagen, within the tumor (7). The bulk growth of this extremely dense tissue leads an enlarged and deformed uterus and to some of the key symptoms associated with uterine fibroids in addition to HMB such as pressure symptoms, abdominal pain, and infertility (4, 6). In the United States, fibroids are cited to be the cause for over 50% of hysterectomies (8), and direct costs for their treatment is estimated between 4 and 9 billion USD (9).

Data on the incidence of uterine fibroids varies; while an Italian study of 341 non-care seeking women of reproductive age reported an incidence of 21.4% (10), a US study of 1,346 randomly selected women between 35 and 49 years screened by self-report, medical record and sonography, found an incidence of uterine fibroids by age 35 of 60% among African-American women, increasing to >80% by age 50, whereas Caucasian women in this study showed an incidence of 40% by age 35, and almost 70% by age 50 (11). An online survey of 21,479 women from Brazil, Canada, France, Germany, Italy, South Korea, the UK and the US on the other hand found a self-reported incidence of 4.5–17.8% in women of reproductive age (12), indicating the importance of sample population, age bracket and genetic background to reported susceptibility. In up to 40% of patients, uterine fibroids cause HMB (13), and more than half of the patients experience combinations of symptoms such as HMB, pelvic pain, or infertility (14, 15).

Uterine fibroids are classified according to their location relative to the uterine anatomy in the FIGO system (16), but while intermenstrual bleeding as a symptom of uterine fibroids has been shown to correlate with the position and number of fibroids (13), the causal link to HMB is unknown. The classification of both HMB and the FIGO system are not without problems, as consistency between surgeons is lacking [**Figure 1**, (17)].

CLINICAL CONSIDERATIONS AND TREATMENT OPTIONS

With a comprehensive treatment plan lacking (6), current treatment of uterine fibroids largely provides symptomatic control. Treatment options are dictated by patient compliance, age, fertility preservation, and other common (co)morbidities such as endometriosis, adenomyosis, endometrial polyps, and endometrial hyperplasia that cause overlapping symptoms [pain and abnormal uterine bleeding (4)]. Follow-up evaluations of the tumor growth rate are recommended for asymptomatic fibroids by most evidence-based guidelines (18). Treatment options can be loosely classified by degree of invasiveness and the risk of reintervention (**Table 1**).

MEDICAL MANAGEMENT

Non-steroidal anti-inflammatory drugs (NSAID) reduce heavy menstrual bleeding (20). Although trials included in Cochrane meta-analyses commonly excluded UF and despite controversial evidence (21, 22), NSAIDs are recommended as an alternative

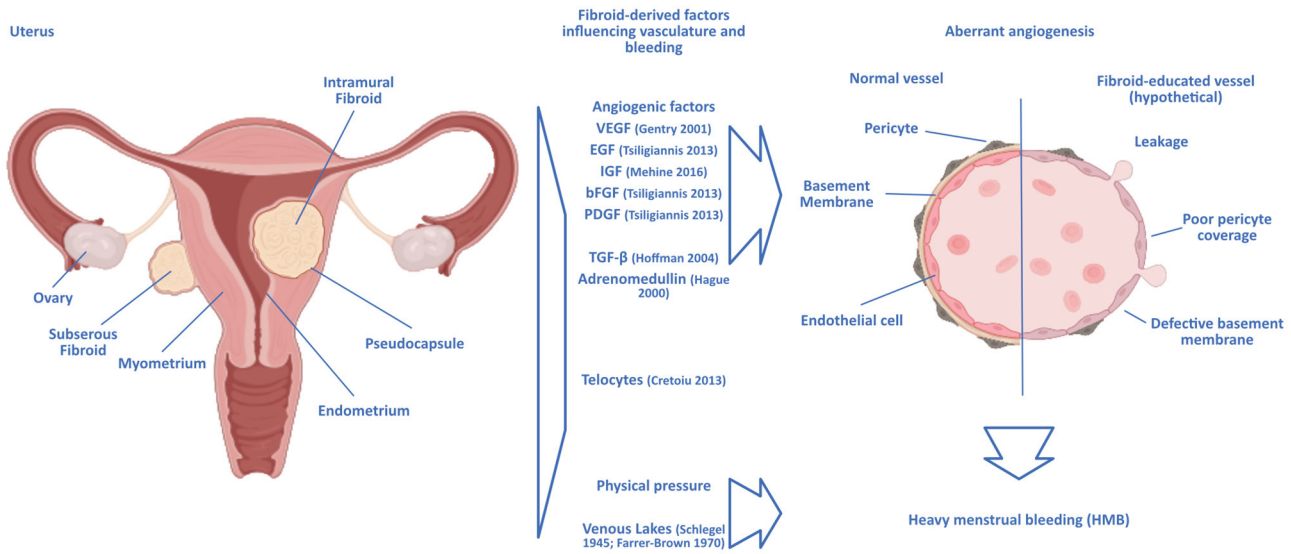

FIGURE 1 | Hypothetical scenarios of a link between uterine fibroids and heavy menstrual bleeding. Intramural uterine fibroids are surrounded by a pseudocapsule of vasculature supplying the growing tumor. The growth and presence of fibroids can lead to aberrant angiogenesis similar to that observed in malignant solid tumors, with the chaotic formation of blood vessels in the vicinity of the fibroids. The vasculature surrounding fibroids would then be structurally deficient, rendering it prone to breaking and leakage. Angiogenic factors identified as expressed by fibroids are displayed. Other hypotheses focus on the connection with the formation of enlarged venous lakes within the endometrium, presumably induced by the mere physical pressure exerted by the fibroids. Ultrastructural analysis of fibroid-educated vasculature would help unravel the actual mechanisms at play.

TABLE 1 | Comparison of treatment options for uterine fibroid-related heavy menstrual bleeding.

Treatment	FIGO type (16)	Preserves fertility	Reversible	Additional outcome	Treatment course	Reintervention rate within 5 years (19)
NSAID	0–8	Yes	Yes	Pain relief	Long-term	N/A
TXA	0–8	Yes	Yes		Long-term	N/A
LNG-IUD	2–8	Yes	Yes	Pain relief	Long-term	N/A
CHC	2–8	Yes	Yes	Pain relief	Long-term	N/A
Progestin-only contraceptive	2–8	Yes	Yes	Pain relief	Long-term	N/A
UPA	1–8	Yes	Yes	Reduction of UF volume	Short-term	N/A
GnRH agonist	1–8	Yes	Yes	Reduction of UF volume	Short-term	N/A
GnRH antagonist	1–8	Yes	Yes	Reduction of UF volume	Short-term	N/A
UAE	1–6	Yes?	No	Reduction of UF volume	One-time intervention with long-term effects	14.4%
HIFU/MRgFUS	2–8	Yes	No	Reduction of UF volume	One-time intervention with long-term effects	53.9%
Hysteroscopic myomectomy	0–1/(2)	Yes	No	Removal of UF, definitive treatment	One-time intervention with long-term effects	7%
Laparoscopic/laparotomy myomectomy	3–8	Yes	No	Removal of UF, definitive treatment	One-time intervention with long-term effects	12.2%
Hysterectomy	2–8	No	No	Removal of uterus, definitive treatment	One-time intervention with long-term effects	0%

NSAID, non-steroidal anti-inflammatory drugs; LNG-IUD, levonorgestrel-releasing intrauterine devices; CHC, combined hormonal contraceptives; UPA, ulipristal acetate; GnRH, gonadotropin-releasing hormone; UAE, uterine artery embolization; HIFU/MRgFUS, high intensity focused ultrasound/magnetic resonance imaging-guided focused ultrasound; TXA, tranexamic acid (most evident contraindications are listed in the manuscript text).

option for levonorgestrel-releasing intrauterine devices (LNG-IUD) to treat HMB in women with fibroids smaller than 3 cm in size (1). The antifibrinolytic agent, tranexamic acid preserves the fibrin matrix structure and is widely used to prevent and treat blood loss, and it has been shown to be effective in fibroid-associated HMB (23).

Intrauterine devices such as the 52 mg LNG-IUD reduces menstrual bleeding in women with fibroids by inducing endometrial atrophy (24–26). The reduction in blood loss is significant, with evidence of successful treatment of anemia and increase in ferritin and hematocrit levels (27). However, the risk of expulsion for the device in a uterus with fibroids is increased compared to a non-fibroid uterus [11 vs. 0–3% (27)], with higher risks again for uteri with large and multiple fibroids. An additional limitation of studies published thus far is that they do not further explore those fibroid cases that fail in their aim to reduce HMB. There is no evidence yet on what type of fibroids LNG-IUD act on long-term, thus avoiding surgery, and for which subtypes other treatment options should be primarily considered. Many studies show a significant drop-out rate of participants with no response to LNG-IUD, who require definitive treatment, i.e., a hysterectomy (28, 29). No data on the effectiveness of lower doses of levonorgestrel are yet available.

Combined hormonal and oral progestin-only contraceptives can be considered in the treatment of fibroid-associated HMB, although evidence is limited (30) and partly based on expert opinion (23). Again, these products can be an option for fibroids smaller than 3 cm in size (1).

Selective progesterone receptor modulators (SPRM) moderate progesterone activity (31). Ulipristal acetate (UPA) binds to the intracellular progesterone receptor and blocks the effects of progesterone; it is thus effective in reducing total fibroid and uterine volume but results in amenorrhea during treatment in most women (32). Several randomized clinical trials have been evaluated in a Cochrane Review, and according to moderate-quality evidence, UPA improves the quality of life and reduces menstrual blood flow more than placebo and leuprolide (31). Similar results on quality of life, pain and bulk symptoms, efficacy and tolerability of UPA have been reported from RCTs in recent years (33–35). An exception are submucosal fibroids, which are less likely to respond with an improved bleeding pattern. Additionally, UPA improves the women's quality of life, and fibroid-related bulk and pain symptoms (36). It can be used preoperatively or as short-term management (usually as 3-month intermittent courses). Due to cases of serious liver injury that an UPA product (Esmya) was suspected of causing, the European Medicines Agency (EMA) has recommended periodic liver monitoring before, during, and after treatment with UPA in all prospective patients, to minimize any risk of developing liver failure (37). More data on UPA vis-à-vis surgical treatment for HMB and fertility outcomes in uterine fibroids are expected in the near future as several new trial protocols have been published recently (clinicaltrials.gov).

The anti-progestin mifepristone has been mainly studied and used for fibroid-associated HMB outside of Western countries. While its anti-glucocorticoid activity may limit its use, like UPA it decreases the size of fibroids, reduces heavy bleeding and improves pelvic pain symptoms and the quality of life, with spotting, elevations in liver enzymes, and endometrial hyperplasia reported as side effects (38, 39).

The estrogen receptor ligands raloxifene and tamoxifen act as selective estrogen receptor modulators (SERM). Their effect on fibroid size and associated symptoms have been investigated, but without significant evidence of alleviation of HMB (23).

Gonadotropin-releasing hormone (GnRH) agonists initially stimulate the pituitary gland and ovaries, then cause downregulation of GnRH receptors with full suppression of estradiol, causing a hypoestrogenic state. *Via* this mechanism they induce amenorrhea and reduce UF and uterine volume significantly but cause menopausal symptoms and bone loss (6). Due to these side effects, GnRH agonists are primarily used as short-course treatment (2–6 months) preoperatively to improve the effects of more conservative and less invasive surgical techniques (40).

GnRH antagonists competitively inhibit GnRH receptors in the pituitary gland and reduce circulating gonadotropins and ovarian sex hormones, including estradiol. Two oral GnRH antagonists with hormonal add-back therapies (indicated to offset hypoestrogenic effects including hot flushes, adverse lipid metabolism, and bone loss), elagolix and relugolix can be considered for the treatment of fibroid-associated HMB (41–43). Both have proven effective in reducing fibroid-associated HMB, and relugolix additionally seems to improve pain and bulk symptoms (43). In 2020, the U.S. Food and Drug Administration (FDA) approved the combination of elagolix (300 mg twice daily) with add-back therapy (1 mg estradiol and 0.5 mg norethindrone acetate once daily) to be used for up to 24 months (44). Relugolix (40 mg once daily) with add-back therapy (1 mg estradiol and 0.5 mg norethisterone acetate) was approved by the European Commission in July 2021 (45), and in October 2021 the agent was granted a license by the UK's Medicines and Healthcare Products Regulatory Agency (MHRA) (46).

RADIOLOGICAL MANAGEMENT

Uterine artery embolization (UAE) can be recommended as a minimally invasive treatment for fibroid-associated bleeding and bulk symptoms in women who desire to preserve their uterus (47). An embolic agent is delivered through catherization of both uterine arteries to cause devascularization and involution of uterine fibroids. After UAE, significant reductions in fibroid and uterine volumes have been observed, which were maintained for up to 5 years (23). The bleeding pattern is usually improved following embolization, and the quality of life 2–5 years after treatment is similar among patients undergoing UAE, hysterectomy, or myomectomy (48). Although the surgical reintervention rate for UAE is higher than that for myomectomy [14.4 vs. 12.2% at 60 months (19)], rates for major post-procedural complications are lower in comparison to any type of surgery for uterine fibroids (19, 49).

Focused ultrasound procedures guided by diagnostic ultrasound (high intensity focused ultrasound, HIFU) or magnetic resonance imaging (Magnetic Resonance-guided Focused Ultrasound, MRgFUS), are non-invasive treatments using multiple high-intensity ultrasound waves to cause coagulative necrosis within fibroids (50). According to the limited published data, HIFU and MRgFUS reduce both fibroid and uterine volume (23) and improve the quality of life—but the evidence is of low quality (51). The rate of reintervention after HIFU is estimated to be as high as 53.9% at 60 months (19).

Radiofrequency ablation (RFA) of fibroids is a minimally invasive procedure that uses heat generated through radiofrequency waves with ultrasound guidance to induce coagulative necrosis in targeted fibroids to reduce their size. RFA can be delivered *via* a laparoscopic, transvaginal, or transcervical approach depending on fibroid location. UF volume reductions have ranged from 32 to 66% at 12 months of follow-up, and at 77% at later time points. RFA improves UF associated symptoms and quality of life. Surgical reintervention rate at 3 years is 11.5% (50). While RFA thus seems to be a good management option for symptomatic fibroids, access to this procedure is currently very limited. Both of these methods rely on adequate imaging to guide treatment.

SURGICAL MANAGEMENT

Endometrial ablation is a procedure performed *via* hysteroscopy that surgically destroys a layer of endometrium to reduce menstrual bleeding. Current evidence is insufficient to assess the effectiveness of this management option to improve fibroid symptoms (23).

Myomectomy describes the surgical removal of fibroid tissue either *via* hysteroscopy, laparoscopy, laparotomy, minilaparotomy, or laparoscopically-assisted minilaparotomy. Hysteroscopic myomectomy is the primary management option for HMB from submucosal (FIGO type 0) and partly submucosal fibroids (FIGO type 1, $\geq$50% of the fibroid situated within the uterine cavity). Myomectomy can also be considered for FIGO type 2 fibroids (partly submucosal fibroid with $\geq$50% in an intramural location), albeit with a higher risk for repeated surgery or further need of medical management. Hysteroscopic myomectomy significantly improves fibroid-associated symptoms and quality of life, and the reintervention rate is as low as 7% at 5 years (19). Myomectomy *via* laparoscopy or laparotomy are a second-line treatment for FIGO type 3–8 fibroids if medication has failed (6, 50). Improvements in the quality of life are similar regardless of surgery type, but laparosopic myomectomy is associated with faster recovery time (50). The surgical reintervention rate for laparoscopic and laparotomy myomectomy is 12.2% (19).

The definitive surgical management for the treatment of fibroid-associated HMB, pain and pressure symptoms is hysterectomy. It is suitable for women who do not desire future pregnancies or do not wish to retain their uterus. Hysterectomy substantially improves hemoglobin levels and anemia, bulk symptoms (23, 52, 53) and the

women's quality of life (54). However, blood transfusions following intraoperative hemorrhage, thromboembolism, and intraoperative bowel/bladder/ureter injury are apparent risks (23). The size and shape of the uterus (deformed by fibroids) directs the chosen hysterectomy route (vaginal, laparoscopic, and laparotomy).

The analysis of data from uterine fibroid registries such as COMPARE-UF in the United States could provide comparative effectiveness data regarding treatment options in the future (55).

ANGIOGENESIS IN UTERINE FIBROIDS

Hardly any tissue in the human body undergoes angiogenesis to the degree and frequency as the endometrium, where angiogenesis is vital during the growth of the endometrium in the proliferative phase of the menstrual cycle, the build-up and elongation of spiral arteries in the secretory phase, and the repairs during and after menstruation (56). In the neighboring myometrium, angiogenesis has been shown to be influenced by the presence of uterine fibroids through an increased proliferative response to estrogen and progesterone in smooth muscle cells in the presence of fibroids compared to normal myometrium [**Figure 1**, (57)].

An array of angiogenic factors involved in the vascularization and growth of uterine fibroids has been identified, including epidermal growth factor (EGF), heparin-binding-EGF, vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), transforming growth factor-β (TGF-β), and adrenomedullin (58). Driver mutations within uterine fibroids have been elucidated in the past two decades as a mutated Mediator Complex subunit 12 (MED12), mutations in the gene encoding the DNA-binding high mobility group AT-hook 2 (HMGA2), fumarate hydratase (FH) deficiency, and mutations in the genes encoding the collagen type IV α5 chain/collagen type IV α6 chains (Col4A5/A6) (59). Interestingly, 65% of tumors show mutations in the *MED12* gene (60), and another 25% show aberrations in *HMGA2*-driven gene expression (15, 61). Uterine fibroids arising from these two mutations show different characteristics; with fibroids driven by *MED12* mutations more numerous but smaller in size (62) compared to fibroids driven by *HMGA2* (63). MED12 has been shown to alter WNT/β-catenin pathway expression (64). In a mouse model of uterine fibroids, this resulted in a breakdown of cytoplasmic and an increase in nuclear levels of β-catenin, associated with an increased fibroid burden (65). Knockdown of the *MED12* gene on the other hand resulted in decreased proliferation of fibroid cells as induced by the WNT/ β-catenin pathway, and thus decreased fibroid growth (66). Apart from growth-promoting effects, these mutations increase the expression of downstream targets including angiogenic factors, most notably the insulin-like growth factor (IGF) system (59). The factors of the IGF family and their binding proteins (IGFBPs) have been shown to regulate tube formation and cell migration in endothelial cells (67), and could explain the formation of blood vessels around the uterine fibroids in the absence of HIF-1α/VEGF signaling.

The role of vascular endothelial growth factor (VEGF) has been studied in detail following its discovery in 1989 (68), and it has become one of the most important targets in anti-angiogenic tumor therapy (69). In uterine fibroids, VEGF levels have been reported as either similar (70) or increased (71) in fibroid tissues vs. adjacent myometrium, with an observation of declining VEGF levels after hysterectomy (72). Compared to myometrium, the expression of EGF was also reported to be higher in fibroid tissue (73), as did FGF (73), PDGF (73), TGF-β (74), IGF-1 (75), and adrenomedullin (76). An indication that angiogenesis in uterine fibroids could follow a different trajectory from the normal hypoxia response—with HIF-1α stabilization and the expression of VEGF as a result, which in turn induces sprouting and outgrowth of endothelial cells nearby to establish a connection to vascular supply (77)—was the finding that despite their hypoxic state, uterine fibroids surprisingly show a down-regulation of key players of the normal hypoxic response, HIF-1α for example has been shown lacking in uterine fibroids, when it was readily shown in leiomyosarcomata (78, 79). Aberrant vasculature as seen in tumors shows a chaotic structure and is prone to leaking; thus, alternative angiogenesis mechanisms, e.g., primarily through IGF signaling, might explain the HMB seen in women with uterine fibroids (80).

PSEUDOCAPSULE

While the fibroid mass itself is poorly vascularised, it can be surrounded by a highly vascularised pseudocapsule in intramural fibroids, a specialized layer of tissue between the tumor and the surrounding myometrium that contains the blood vessels needed to sustain the fibroid (81), which develops in response to the fibroid growth (82). This structure can be seen as a "ring of fire" in ultrasound Doppler imaging, and it is separated from the myometrium by a clear cleft, as observed in histological images. The bursting of the vessels contained within the pseudocapsule could explain the HMB observed in women with uterine fibroids, in which case the symptom should correlate with the position of the fibroids, as the pseudocapsule only develops around intramural fibroids; this seems to be the case (13). The pseudocapsule is made up of the same cell types and shows the same biologic structure as the neighboring myometrium (83); however, the vasculature of the pseudocapsule might harbor structural defects that rend it susceptible to breaking, leading to HMB.

Responsible could be "telocytes," a comparably recent discovery in the interstitial myometrium ($CD34^+$c-$kit^+PDGFR\alpha^+$). Telocytes have elongated telopodes, podomers, and regional podoms that stretch into the surrounding tissue; the cells thus stay in physical contact with many other cell types (84). In the uterus, telocytes are in especially close contact with smooth muscle cells (85) and are speculated to coordinate myometric contractions. Telocytes in the uterus express estrogen and progesterone receptors and are thought capable of regulating smooth muscle cell proliferation (84). Intriguingly, telocytes are not present within uterine fibroids but are found within

surrounding myometrium (86). They express VEGF and could thus contribute to angiogenesis in the pseudocapsule.

VENOUS LAKES

An older, classic theory of how HMB is connected to uterine fibroids suggests that "venous lakes" are responsible for the increased bleeding during menstruation: These large sinusoidal structures have long been known to form physiologically within the uterine vasculature from arteriovenous anastomoses (87). The blood flow into the venous lakes was thought to lead to a loss of pressure in the capillary system supporting the build-up of endometrium until the increase in venous lakes and the limited potential for increasing the supply pressure meant that the endometrium would die off, thus starting menstruation. Because the venous lakes lack any closing mechanism, they would bleed until completely sloughed off, and the menstrual loss of endometrium would only stop once the basal layer, supported by capillaries but without any arteriovenous anastomoses, had been reached. Microradiographic studies in the 1970's supported the notion that HMB resulted not from the fibroid vasculature itself but rather from venous lakes dilated and enlarged by virtue of increased interstitial pressure from the growing tumors (88). Once corrosion cast microscopy methods became available, studies into vascular changes within the myometrium in the presence of uterine fibroids indeed found venous lakes enlarged in uteri bearing fibroids in comparison to normal uteri (89), and further support to the theory was lent by immunohistochemistry studies of cyclic changes in uterine vasculature showing dilated and disintegrating venous vessels in the upper functionalis layer of the endometrium (90), showing that menstrual blood was largely venous in origin. However, the notion that physical pressure was the main factor leading to enlarged venous lakes was challenged once molecular biological analyses of angiogenic factors were able to show that indeed growth factors were largely responsible for the enlargement of veins and venous lakes (91), and that the heavy bleeding resulted from a failure of the fibrin/platelet plugs formed in the coagulation cascade in closing these blood vessels of increased diameter successfully.

VASCULAR ARCHITECTURE, ABERRATIONS

The decisive factor could be the aberrant architecture of the fibroid-educated vasculature. Vasculature growing rapidly around malignant tumors in response to hypoxia is highly irregular, with high tortuosity, shunting of vessels and non-patent ends. Instead of ordered layers of pericytes and α-smooth muscle actin, tumor vasculature lacks stabilization through pericytes, and the α-smooth muscle actin is merely sporadically wrapped around the vessels (92). This renders tumor vasculature leaky and prone to breaking, enables the intravasation of metastatic tumor cells and hinders the successful delivery of drugs to the tumor (93). The vasculature developing around uterine fibroids could similarly show structural defects; however, elucidating these requires microscopy-driven avenues of investigation rather than omics approaches, as the latter would not necessarily flag any difference in vascular architecture as long as the quantities of the building blocks are not altered significantly. If proven to be similarly defective, the architecture of fibroid-educated vasculature could be targeted in analogy to the vascular normalization angle in tumor therapies (94).

PHYSICAL MECHANISMS

Fibroids may cause HMB merely physically, i.e., through an increase in endometrial surface due to underlying fibroid growth; through an influence on normal myometrial contractility patterns; through an ulcerated or degenerating fibroid, or through uterine venous ectasia due to compression from the fibroids (95). Fibroids were shown to secrete increased levels of transforming factor-beta 3 (TGF-β3) in response to steroids, as the estrogen and progesterone native to the uterus (96). TGF-β is associated with fibrotic disease, such as renal (97) or pulmonary fibrosis (98), where it induces the emergence of collagen-producing myofibroblasts *via* an epithelial-mesenchymal transition (EMT) (97). Although distinct from fibrotic disease, a role of TGF-β in uterine fibroids may enhance our understanding of the pathomechanism leading to HMB. TGF-β3 secreted by fibroids has been shown to induce BMP-2 resistance in endometrium by down-regulation of BMPR-2, likely causing defective endometrial decidualization (99). TGF-β3 also reduces expression of plasminogen activator inhibitor-1 (PAI-1), Antithrombin III (ATIII), and thrombomodulin in endometrium, likely contributing to bleeding (99). Interestingly, seasonally different patterns of circulating levels of interleukins (IL)-10, IL-13, and IL-17—all associated with fibrotic disease—have been identified in women with fibroids, similarly pointing toward an involvement of the wound healing immune response in fibroid growth and possibly HMB (100).

CONCLUSION

Uterine fibroids are one of the main indications for heavy menstrual bleeding, a symptom that causes considerable impairment to patients' quality of life. Apart from hysterectomy, none of the currently available treatment options addresses this problem satisfactorily, a conundrum underlined by the fact that many women require definitive surgical management after exhausting their medical management options. Treatment options that would go beyond the merely symptomatic will depend on further knowledge of the influence of uterine fibroids on blood vessel growth and structure. The somatic mutations giving rise to uterine fibroids hold some clues as to their angiogenic potential, but the causal link between uterine fibroids and heavy menstrual bleeding is not yet known. While several hypotheses have been proposed during the last decades, structural studies of the vascular architecture of the blood vessels supplying uterine fibroids and the specific angiogenesis mechanisms that lead to their growth are needed to unravel the causal link between uterine fibroids and heavy menstrual bleeding, and thus enable the search for better therapies.

AUTHOR CONTRIBUTIONS

TT and OU conceived of the idea. OU, KS, BV, and TT wrote and discussed the manuscript. All authors agreed to the final version.

ACKNOWLEDGMENTS

The figure was created with help from Biorender (www.biorender.org).

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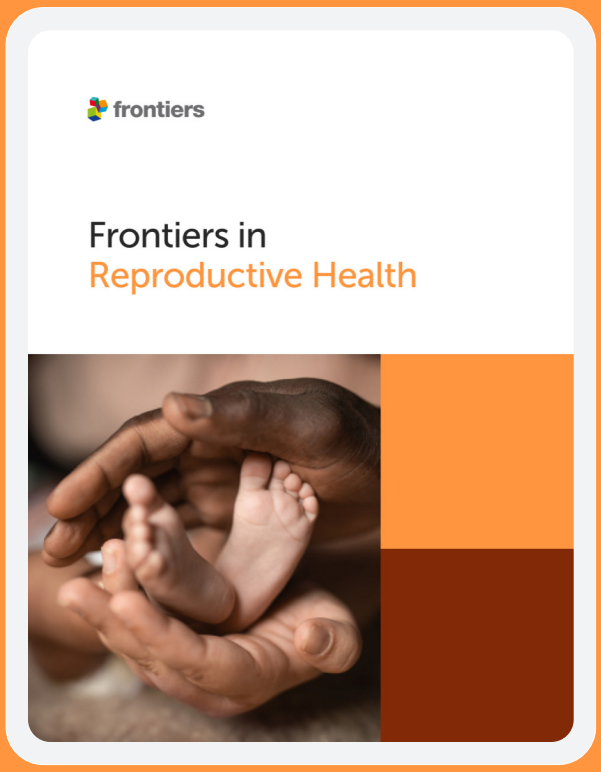

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