The presence and role of hypoxia in the endometrium

Accepted Manuscript published as REP-20-0268.R1. Accepted for publication: 03-Aug-2020

The presence and role of hypoxia in the endometrium

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Short Title: Hypoxia in the endometrium

Word Count: 7552

ABSTRACT

The endometrium is a multicellular tissue that is exquisitely responsive to the ovarian hormones. The local mechanisms of endometrial regulation to ensure optimal function are less well characterised. Transient physiological hypoxia has been proposed as a critical regulator of endometrial function. Herein, we review the literature on hypoxia in the non-pregnant endometrium. We discuss the pros and cons of animal models, human laboratory studies and novel *in vivo* imaging for the study of endometrial hypoxia. These research tools provide mounting evidence of a transient hypoxic episode in the menstrual endometrium and suggest that endometrial hypoxia may be present at the time of implantation. This local hypoxia may modify the inflammatory environment, influence vascular remodelling and modulate endometrial proliferation to optimise endometrial function. Finally, we review current knowledge of the impact of this hypoxia on endometrial pathologies, with a focus on abnormal uterine bleeding. Throughout the manuscript areas for future research are highlighted with the aim of concentrating research efforts to maximise future benefits for women and society.

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INTRODUCTION

The human endometrium is a heterogeneous and dynamic tissue that undergoes cyclical breakdown and repair/regeneration more than 400 times during the female reproductive lifespan (Short, 1976; Critchley *et al.*, 2020)**.** This occurs each month without scarring or loss of function. However, the regulation and local mechanisms of this endometrial breakdown and repair remain elusive. In particular, our knowledge of the contribution of local endometrial hypoxia to this process is in its infancy. The presence of hypoxia, usually defined as a partial oxygen pressure below 10 mmHg, is not an uncommon phenomenon in human physiology, e.g. bone marrow and intestinal mucosa (Suda, Takubo & Semenza, 2011; Zheng, Kelly & Colgan, 2015). Its presence in the menstrual endometrium has been proposed following progesterone withdrawal and intense vasoconstriction of the specialised spiral arterioles (Markee, 1940). Unravelling the role of hypoxia in the endometrium has the potential to improve our understanding of menstrual and implantation disorders and reveal novel therapeutic strategies for those suffering from these common, devastating conditions.

ENDOMETRIAL HISTOLOGY AND OVARIAN HORMONE REGULATION

Histologically, the endometrium can be divided into the functional and basal layer (Noyes, Hertig & Rock, 1950). The functional layer occupies the upper two thirds of the endometrium and is composed of stroma and glands. This layer undergoes constant remodelling throughout the menstrual cycle and is shed during menstruation. The basal layer, adjacent to the myometrium, comprises the lower third of the endometrium.

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Oestradiol is the dominant hormone in the first half of the menstrual cycle, during the proliferative phase. It acts via the oestrogen receptor (ER), which has two structurally related subtypes, ERα and ERβ (Lessey *et al.*, 1988; Critchley *et al.*, 2002). After ovulation, levels of oestradiol decline and the corpus luteum increases its progesterone production, prompting endometrial differentiation and decidualisation. This process, driven by cAMP signalling, reshapes the stromal compartment in order to keep the endometrium receptive for future implantation (Dunn, Kelly & Critchley, 2003). In contrast with non-menstruating species, where implantation of an embryo is required to trigger decidualisation (Brasted *et al.*, 2003), the human endometrium spontaneously decidualises with endometrial stromal cells in close proximity to spiral arterioles initiating their own transformation (Gellersen & Brosens, 2014). They morphologically transition from fibroblast-like cells to rounded epithelioid-like cells (Dunn, Kelly & Critchley, 2003).

ENDOMETRIAL BREAKDOWN AND REGENERATION

In the absence of implantation, the corpus luteum regresses causing significant progesterone withdrawal (Corker *et al.*, 1976; Maybin, Hirani, *et al.*, 2011). This decrease in progesterone levels triggers a cascade of local physiological inflammatory events that initiate menstruation. Progesterone withdrawal leads to the induction of the transcription factor NFκB, which up-regulates the expression of pro-inflammatory cytokines (IL-6, TNF) and chemokines (CCL2, CXCL8)(King, Critchley & Kelly, 2001). In addition, this fall in progesterone levels increases endometrial cyclooxygenase 2 (COX-2), responsible for the synthesis of prostaglandins (PG)(Critchley *et al.*, 1999). Increased levels of these inflammatory mediators drive the recruitment of myeloid leukocytes, activation of matrix metalloproteinases (MMPs) and the shedding of the upper endometrial layers (Critchley *et al.*, 2001; Kelly, King & Critchley, 2001). Hypoxia has been identified in the endometrium

following progesterone withdrawal (Fan *et al.*, 2008; Cousins, Murray, *et al.*, 2016; Maybin *et al.*, 2018) and may be due to vasoconstriction of the endometrial vessels. $PGF_{2\alpha}$ and endothelin-1 (ET-1) are two endometrial factors with known vasoconstrictive properties that are present following progesterone withdrawal (Baird *et al.*, 1996; Marsh *et al.*, 1997). Vasoconstriction of specialised endometrial spiral arterioles may limit blood loss during menstruation. The subsequent tissue hypoxia does not appear to be necessary for endometrial breakdown but may have an important role in endometrial repair/regeneration (Maybin *et al.*, 2018; Chen *et al.*, 2020).

Shedding of the functional endometrial layer necessitates repair of the denuded endometrial surface and regeneration of endometrial tissue. This takes place when oestradiol and progesterone levels are low but local glucocorticoid action may be increased (McDonald *et al.*, 2006; Kaitu'u-Lino, Morison & Salamonsen, 2007a; Rae *et al.*, 2009). Evidence from mouse models and human tissue studies suggest that hypoxia is required for physiological endometrial repair (Fan *et al.*, 2008; Maybin *et al.*, 2018). The processes involved are likely to be similar to those of wound healing, involving haemostasis, inflammation, proliferation and remodelling (Velnar, Bailey & Smrkolj, 2009; Mutsaers *et al.*, 2015).

DETECTION OF HYPOXIA THROUGHOUT THE MENSTRUAL CYCLE

The first suggestion that hypoxia was present at menses derived from findings in a primate model in 1940 (Markee, 1940). Transplantation of endometrial explants to the Rhesus macaque eye allowed direct observation of intense vasoconstriction of spiral arterioles and focal bleeding following

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136 progesterone withdrawal. Since then, the use and refinement of animal models for the study of
137 menstrual physiology and endometrial hypoxia has become more common.
138

139 *In vivo* animal models

140 Menstruation is restricted to humans and few other species. These include higher order primates
141 (baboons, Rhesus macaques), the elephant shrew (Van der Horst & Gillman, 1940), certain bats
142 (Hamlett, 1934; Rasweiler & de Bonilla, 1992; Zhang *et al.*, 2007) and the spiny mouse (Bellofiore *et*
143 *al.*, 2017). The majority of menstrual studies have been carried out in rodents and non-human
144 primates, including the Rhesus macaque (Brenner & Slayden, 2012).
145

146 Rodent models

147 Despite physiological differences between mice and humans (e.g. a shorter length of cycle and lack
148 of spontaneous decidualisation) mouse models replicate the events of human menstruation and
149 decidualisation well (Wang *et al.*, 2013; Cousins, Kirkwood, *et al.*, 2016; Armstrong *et al.*, 2017)**.** The
150 feasible management of large experimental groups, short breeding times and availability of
151 laboratory antibodies/reagents provide advantages over macaque models. Mouse models also offer
152 the possibility of genetic, environmental and pharmacological manipulation of hypoxia (see Role of
153 hypoxia throughout the menstrual cycle below). Technically, euthanasia by carbon dioxide (CO_2)
154 inhalation can impact tissue hypoxia and may distort results. Hence, cervical dislocation is the
155 recommended euthanasia method for these studies. Great care must be taken to handle, process
156 and fix tissue rapidly to capture the physiological events of menstruation.
157

158 *The mouse model of simulated menstruation*

159 The *menses-like* model was first described in 1984 (Finn & Pope, 1984) and further optimised in the
160 2000's (Brasted *et al.*, 2003). Since then, it has been the most popular model to investigate the
161 dynamics of endometrial repair (Fan *et al.*, 2008; Evans, Kaitu'u-Lino & Salamonsen, 2011; Cousins *et*
162 *al.*, 2014; Maybin *et al.*, 2018; Chen *et al.*, 2020) (**Fig. 1**)**.** Mice are ovariectomised and supplemented
163 with exogenous oestradiol and progesterone to mimic the human hormonal endometrial
164 environment. They require artificial induction of decidualisation, via a transcervical or surgical
165 intrauterine injection of oil. Once decidualisation has taken place, progesterone withdrawal leads to
166 active bleeding in the mouse uterus and subsequent repair (**Fig. 1a**). Alternatively, simulation of
167 menses can be achieved by inducing pseudopregnancy (**Fig. 1b**). In this model, female mice are
168 mated with vasectomized males to mimic fertilisation events. Progesterone withdrawal occurs
169 naturally or is induced by ovariectomy or administration of a progesterone antagonist (Rudolph *et*
170 *al.*, 2012)**.**
171
172 The first work to describe the presence of hypoxia during endometrial breakdown and repair in the
173 mouse utilised the 'pseudopregnancy' model variant (Fan *et al.*, 2008). Pimonidazole is a hypoxic
174 marker that, when oxygen partial pressures are below 10 mmHg, forms protein adducts which can
175 be visualized using specific monoclonal antibodies. Due to its chemical stability, pimonidazole is
176 considered one of the most reliable means of tissue oxygen level detection, even when it is
177 temporally and spatially transient. Fan *et al.* found the endometrial area undergoing regeneration to
178 be hypoxic and that this hypoxia decreased and eventually disappeared with endometrial
179 reepithelialisation (Fan *et al.*, 2008). Subsequent confirmation of the presence of menstrual hypoxia
180 was found in the 'exogenous hormone' model of simulated menses (Cousins, Murray, *et al.*, 2016;
181 Maybin *et al.*, 2018; Chen *et al.*, 2020). Using pimonidazole, hypoxia was detected during bleeding
182 and later confined to areas undergoing active repair. Hypoxia may also be present in the
183 endometrium at the time of implantation. As the uterine epithelium contains no blood vessels

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during initial embryo contact, it has been suggested that the onset of implantation occurs in a hypoxic environment (Daikoku *et al.*, 2003)**.** The detection of pimonidazole adducts in the area of implantation in mice reinforces this hypothesis (Pringle *et al.*, 2007).

Another method to determine tissue hypoxia is detection of the oxygen-sensing transcription factor hypoxia inducible factor (HIF). HIFs have a key role in the cellular response to oxygen and are heterodimers composed of two subunits: a constitutively expressed beta subunit (HIF-1β) and an O_2-sensitive alpha subunit (Semenza, 2000). There are three known α subunits: HIF-1α, HIF-2α, and HIF-3α. HIF-1α and HIF-2α are the most common alpha isoforms and present overlapping but distinct target gene specificities (Mole *et al.*, 2009). HIF-3α is structurally different from the other isoforms and is the least characterized (Pasanen *et al.*, 2010). Along with promoting genes related to nitrogen metabolism and immune response, HIF-3α has the ability to inhibit HIF-1α/2α action (Zhang *et al.*, 2014).

The regulation of HIF takes place predominantly at the protein level. In normoxia*,* prolyl hydroxylase domain enzymes (PHDs) hydroxylate specific residues within the alpha subunit, leading to its ubiquitination and subsequent degradation via the proteasome (Salceda & Caro, 1997). In hypoxia these PHDs are inhibited, resulting in HIF-α stabilization. HIF-α translocates to the nucleus, dimerizes with HIF-1β and binds to hypoxia-response elements (HREs) to enhance transcription of a plethora of genes involved in energy metabolism, angiogenesis, tissue remodelling and inflammatory responses (Semenza, 2012)**.**

The presence of nuclear HIF-1α protein is therefore indicative of active HIF-1 and consistent with tissue hypoxia. Using this approach, HIF-1α has been detected during menstruation in both the exogenous hormone (Maybin *et al.*, 2018; Chen *et al.*, 2020) and pseudopregnancy menstruation models (Chen *et al.*, 2015), decreasing during endometrial regeneration. Examination of HIF-1α and

HIF-2α in the mouse uterus during pre-implantation (day 4) and decidualisation (day 5-8) of pregnancy, revealed HIF-1α was present in the luminal epithelium prior to implantation and throughout the epithelium and stroma during decidualisation and implantation (Daikoku *et al.*, 2003). HIF-2α was seen in the stroma on day 4 and limited to cells surrounding the blastocyst on day 5. The authors suggested that HIF-1 was involved in maintaining oxygen homeostasis and that HIF-2 was driving the angiogenesis necessary for successful implantation.

Various concerns have been raised about using HIF as a hypoxic surrogate marker. Transient hypoxic events can be too brief to stabilise HIF for immunohistochemical detection (Wang *et al.*, 1995). Antibody unreliability is an added factor, which is compounded by the fact that tissue collection and fixation can also affect HIF detection (Zhang & Salamonsen, 2002)**.** Furthermore, HIF stabilisation can be induced by NF-κB-driven cytokine production in a non-hypoxic dependent manner and hypoxia can exert downstream effects independently of HIF signalling (Lin & Simon, 2016). Alongside detection of pimonidazole and HIF, hypoxia-inducible factor downstream targets may indicate a hypoxic response in the mouse menstrual endometrium**.** HIF-1α-mediated induction of the angiogenic factors vascular endothelial growth factor (VEGF) and the chemokine receptor CXCR4 was increased during menstruation and endometrial repair (Fan *et al.*, 2008; Chen *et al.*, 2015; Cousins, Murray, *et al.*, 2016; Maybin *et al.*, 2018).

Xenograft mouse model

The xenograft mouse model provides an alternative model for study of menstrual physiology and pathology (extensively reviewed in (Kuokkanen, Zhu & Pollard, 2017)). Human functional endometrium is transplanted into immunodeficient mice (**Fig. 1c**). This is usually collected during the proliferative phase and can be transplanted as (i) small fragments (1-2 mm^3) of endometrial tissue

 Accepted Manuscript published as REP-20-0268.R1. Accepted for publication: 03-Aug-2020

(Guo *et al.*, 2011; Coudyzer *et al.*, 2013) or (ii) dissociated endometrial cells from epithelial and stromal fractions that are mixed before implantation (Masuda *et al.*, 2007; Polotsky *et al.*, 2009). The recipient mice are selected to limit xenograft tissue rejection, but the immunodeficient strain used can vary. The most commonly used in xenograft menstruation models is the severe combined immunodeficiency (SCID) mouse, which has T and B cell deficiencies (Gaide Chevronnay *et al.*, 2009; Guo *et al.*, 2011; Coudyzer *et al.*, 2013)**.** The best engraftment results are achieved with the non-obese diabetic (NOD)/SCID/γc^{null} mice (NOG), which also have defective NK cell activity (Matsuura-Sawada *et al.*, 2005; Masuda *et al.*, 2007).

Generally, the patches of endometrial tissue are placed subcutaneously in mice (Guo *et al.*, 2011; Coudyzer *et al.*, 2013) with a survival time of 4 weeks, whereas the dissociated endometrial cells are implanted below the kidney capsule and survive up to 10 weeks (Masuda *et al.*, 2007). This latter mode of implantation allows extension of the duration of experiments, making this the method of choice for studies of the proliferation kinetics of the endometrium after pharmacological treatments (Polotsky *et al.*, 2009).

Xenograft menstruation studies mainly focus on endometrial regeneration and the role of ovarian steroids in orchestrating the process (Gaide Chevronnay *et al.*, 2009; Guo *et al.*, 2011; Coudyzer *et al.*, 2015) and use the endometrial fragments model variant**.** To date, this mouse model has only been employed once to study the presence of hypoxia during menstruation (Coudyzer *et al.*, 2013). In 2013, Coudyzer et al. subcutaneously implanted endometrial patches on SCID female mice and tested for signs of hypoxia in the resulting xenograft using several methods. Firstly, they directly measured the local partial oxygen pressure (pO_2) using electron paramagnetic resonance and OxyLite fluorescent probes. They also studied the presence of pimonidazole staining and HIF-1α using immunohistochemistry (IHC). The authors did not detect hypoxia during endometrial breakdown or repair using any of these methods. These results contrast with findings in the mouse

model of simulated menses and may be partially explained by the xenograft model itself. Endometrial tissue architecture and vasculature is severely compromised following transplantation and may impair vasoconstriction and prevent endometrial hypoxia. Moreover, endometrial breakdown and repair are considered inflammatory events, as they involve pro-inflammatory cytokine production and myeloid leukocyte recruitment (Finn, 1986). Therefore, the necessary immunosuppressed state of the recipient mice may alter physiological menstrual endometrial events. The SCID model aims to suppress T and B-cell mediated transplant/xenograft rejection without substantially affecting the innate immune response and may be more relevant than other immunocompromised recipient mice (Guo *et al.*, 2011; Donoghue *et al.*, 2012).

Spiny mouse

The common spiny mouse (*Acomys cahirinus*) is, to date, the only known rodent to display spontaneous decidualisation and natural menstruation (Bellofiore *et al.*, 2017, 2018). Although anatomically different, the spiny mouse uterus has physiological similarities to the human endometrium. For example, the spiny mouse displays spiral arteriole remodelling in the perimenstrual phase (Bellofiore *et al.*, 2018). In addition, endometrial decidualisation is tightly controlled, not compromising the structural integrity of the endometrial glands or the myometrium, as observed in other mouse models (Bellofiore *et al.*, 2018). Hypoxia has not been examined in this rodent to date and these studies are awaited with interest.

Macaque models

Macaques have morphologically similar uteri to humans, a similar length of menstrual cycle and they display spontaneous decidualisation (Brenner & Slayden, 2012). Macaques also experience menstrual abnormalities (e.g. heavy menstrual bleeding (HMB)) and can be fitted with tampons, hence they are exceptional candidates for evaluating therapies for menstrual disorders (reviewed in

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(Brenner & Slayden, 2012)). Despite menstruating naturally, macaques are routinely ovariectomized and treated with oestradiol and progesterone to create artificial menstrual cycles and enable accurate timing of endometrial sampling. However, the need for larger experimental groups, longer experimental times and the increased cost of these experiments has meant many researchers are now preferentially using rodent models to study menstrual physiology.

As previously mentioned, the first indication of endometrial tissue hypoxia was observed in endometrial explants transplanted to the eye of rhesus macaques in the 1940s (Markee, 1940). Rather than hypoxia, Markee observed pulses of intense vasoconstriction in the spiral arterioles that he associated with localised hypoxic ischemia. This hypothesis was later supported by the detection and increased expression of HIF-1α in the functional layer of the macaque endometrium during menstruation (Brenner & Slayden, 2012), consistent with the presence of endometrial hypoxia.

Ex vivo human endometrial studies

HIF-1α protein has been identified, both by western blot and IHC , in human endometrial biopsies collected during the late secretory and menstrual phases (Critchley *et al.*, 2006; Maybin *et al.*, 2018). HIF-1α staining was localised in the glandular and stromal cells in the functional endometrium, whereas in the basal layer HIF-1α staining was restricted to the glands.

In contrast, HIF-2α is present exclusively during the early-mid secretory phase (Maybin *et al.*, 2018). Downstream targets of HIF, such as VEGF and carbonic anhydrase IX (CA-IX) have also been shown to be increased during the menstrual and proliferative phases (Stephen Charnock-Jones *et al.*, 1993; Sharkey *et al.*, 2000; Punyadeera, 2006; Maybin, Hirani, *et al.*, 2011) .

In vivo human endometrial studies

Detection of human endometrial hypoxia *in vivo* has been largely via measurements of perfusion, initially investigated using thermal heat dissipation (Prill & Götz, 1961) and later by a Xenon-133 clearance technique (Fraser *et al.*, 1987) **(Fig. 2).** Both methods are invasive and results were conflicting as suffering from variable calibration and poor spatial and temporal resolution respectively. The introduction of Doppler ultrasound allowed perfusion measurements in individual spiral arterioles (Kupesic & Kurjak, 1993), but this showed an increase in flow the day before ovulation, in contrast with the ^{133}Xe clearance study which found a fall at this time. Laser Doppler fluxmetry was able to assess endometrial perfusion using a fibre optic probe (Gannon, Carati & Verco, 1997), finding blood flow peaks in the early proliferative and early secretory phase, but spatial resolution was limited. The more sensitive three-dimensional power Doppler angiography (3D-PDA) was also used in spiral arterioles (Raine-Fenning, 2004) and revealed a significant pre-ovulatory peak in perfusion, followed by a post-ovulatory fall and gradual increase through early to mid-secretory phases. In general, there has been little consensus regarding changes in endometrial blood flow over the menstrual cycle and how to measure such changes. Magnetic resonance imaging (MRI) methods may now offer a better alternative, although there has been little work on the application of these techniques to detect endometrial hypoxia.

To our knowledge, functional investigation of the normal endometrium has been limited to MR spectroscopy (Sarac *et al.*, 2004; Celik *et al.*, 2005). This technique detects the presence of specific metabolites in the body by examining the resonant frequencies of the hydrogen protons within them. In particular, lactate is a product of anaerobic respiration (and therefore a marker of hypoxia) and has been detected in normal secretory and proliferative endometrium (Sarac *et al.*, 2004; Celik *et al.*, 2005). Although lactate is arguably a more direct marker of hypoxia than measurement of

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perfusion, analysis and acquisition of spectroscopy data is technically challenging (Lange *et al.*, 2006) and spatial resolution tends to be poor.

Dynamic contrast-enhanced (DCE) MRI is a technique that can detect hypoxia indirectly by measuring perfusion using an exogenous gadolinium-based contrast agent (CA) (Sourbron, 2010). Passage of the CA through the tissue can be modelled to allow perfusion to be estimated as part of a model-fitting process (Sourbron & Buckley, 2012). The technique has been applied in the normal endometrium (Majd *et al.*, 2017) but showed no differences between the secretory and proliferative phases. The advantage of DCE-MRI for hypoxia imaging is its good spatial resolution, but imaging and analysis can be complex (Brix *et al.*, 2004, 2009; Michaely *et al.*, 2008) and there is no gold standard for validation of the technique. Use of DCE-MRI to detect a reduction in perfusion related to hypoxia in the menstrual cycle would require a specialised imaging protocol and robust data analysis using a complex model, including estimation of parameter uncertainties.

Other existing MRI techniques could be applied to measure endometrial hypoxia (**Fig. 2**). T2* is a characteristic tissue relaxation time that depends on inhomogeneities in the main magnetic field produced by the scanner as well as rapidly-changing inhomogeneities induced by the presence of other nearby molecules. Detection of a reduction in T2* is commonly assumed to be due to the presence of deoxyhaemoglobin and therefore tissue hypoxia. This technique has been used in the myometrium (Kido *et al.*, 2007; Imaoka *et al.*, 2012) and has the high spatial resolution necessary to investigate the endometrium. T2* can change for a number of other reasons, (e.g. local haematocrit, hemosiderin, calcification and tissue iron deposition) therefore changes should be interpreted with caution. Similarly, a non-invasive perfusion technique known as arterial spin labelling (ASL) (Ferré *et al.*, 2013) could be extended from existing work in the myometrium (Takahashi *et al.*, 2016) to the endometrium, though it can be technically challenging. Finally, the extensive work on hypoxia measurements in cancer (Horsman *et al.*, 2012) could be applied in the endometrium. Oxygen-

enhanced (OE) MRI (O'Connor, Robinson & Waterton, 2019) allows a change in the tissue relaxation time T1 as a result of the patient breathing 100% oxygen through a mask to be related to the oxygen status of the tissue (O'Connor *et al.*, 2016). These minimally invasive MRI techniques may provide key information on the presence of human endometrial hypoxia throughout the menstrual cycle, with potential diagnostic and therapeutic benefits for women.

ROLE OF HYPOXIA THROUGHOUT THE MENSTRUAL CYCLE

Mice have the experimental advantage of genetic or pharmacological alteration to assess the role of hypoxia in endometrial function. HIF-1α heterozygote mice have revealed that HIF-1α is required for normal menstruation, and decreased HIF-1α delays endometrial repair (Maybin *et al.*, 2018). Pharmacological stabilisation and inhibition of HIF-1α in mice has confirmed this role (Chen *et al.*, 2015; Maybin *et al.*, 2018). Mice placed in hyperoxic chambers (75% O_2) during menses had reduced local endometrial hypoxia at menstruation and delayed endometrial repair (Maybin *et al.*, 2018). HIF-2α deficiency restricted to uterine stromal cells in a mouse implantation model revealed a key role in decidualisation, endometrial receptivity, embryonic implantation and survival (Matsumoto *et al.*, 2018).

This emerging evidence for the presence and important role of hypoxia and HIF in endometrial function presents an exciting and developing research area (**Fig. 3**). The effects of hypoxia on the important menstrual processes of inflammation, proliferation and tissue remodelling remains to be elucidated.

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Impact of hypoxia on inflammation

Inflammation is a key event during implantation, at menstruation and the subsequent endometrial repair. There is a peri-menstrual influx of leukocytes into the endometrium, in particular neutrophils and macrophages (Armstrong *et al.*, 2017). Interactions between the inflammatory response and hypoxia are well described at other tissue sites (Cramer *et al.*, 2003; Taylor, 2008; Taylor *et al.*, 2016) but the impact of hypoxia on the endometrial inflammatory response is less well characterised.

Impact on neutrophils

Neutrophils comprise up to 15% of the total endometrial cell numbers during menstruation (Poropatich, Rojas & Silverberg, 1987; Salamonsen & Lathbury, 2000)**.** Their influx is tightly regulated, displaying a rapid, short lasting induction**,** which coincides with the upregulation of chemokines and cytokines. This temporal dynamic has been observed in both the mouse model of simulated menses and in human endometrial samples (Armstrong *et al.*, 2017). Neutrophils are important mediators of endometrial breakdown, which has been confirmed by their depletion in the mouse model of menstruation (Kaitu'u-Lino, Morison & Salamonsen, 2007b). However, the depleting agent used in this study also affects the monocytic cell lineage. Activated neutrophils release enzymes such as neutrophil elastase and cathepsin G. These enzymes activate MMPs produced by endometrial stromal cells and cause degradation of the extracellular matrix (Salamonsen & Lathbury, 2000). In airway inflammation, hypoxia boosts neutrophil degranulation and protease release (Hoenderdos *et al.*, 2016). It would be informative to determine whether hypoxia has similar effects in the endometrial environment during menses.

Neutrophils also produce reactive oxygen species (ROS) that might participate in endometrial breakdown. The potential role of ROS in menstruation has been reported (Sugino *et al.*, 1996), suggesting that free oxygen radicals may contribute to endometrial shedding by causing tissue

damage. Indeed, the inhibition of ROS generation in the mouse model of simulated menstruation has been shown to abrogate endometrial breakdown (Wu *et al.*, 2014).

Neutrophil depletion in mouse models also affected endometrial regeneration (Kaitu'u-Lino, Morison & Salamonsen, 2007b)**.** Little is known about the impact of hypoxia on neutrophils during endometrial repair. The concept that hypoxia has an effect on neutrophil number and function is derived from studies of tumour biology. In a mouse model of endometrial carcinoma there was spatiotemporal correlation between hypoxia and neutrophil infiltration within the tumour (Blaisdell *et al.*, 2015). Accumulation of pimonidazole and nuclear staining of HIF-1α was detected slightly prior to neutrophil infiltration. These results are consistent with those observed in the mouse model of simulated menses, where pharmacological inhibition of HIF-1α decreased the number of endometrial neutrophils present during active bleeding (Maybin *et al.*, 2018)**.** The role of hypoxia in promoting neutrophil recruitment in endometrial carcinoma was confirmed by placing mice in hyperoxic chambers (60% O_2) (Mahiddine *et al.*, 2019). This resulted in a dramatic reduction in neutrophil influx within the tumour and also improved the ability of these cells to oppose tumour growth through increased activation and expression of several MMPs and ROS production. This is consistent with hypoxia not only affecting the recruitment of neutrophils, but also their function. Determining the effects of hypoxia on neutrophil number and phenotype in the normal endometrium would be of great interest to advance our understanding of menstrual physiology.

Effects of hypoxia on neutrophils have also been observed in benign tissues. Airway inflammation studies have revealed that hypoxia, via HIF-1α and HIF-2α, prolonged neutrophil lifespan by inhibiting apoptosis (Walmsley *et al.*, 2005; Thompson *et al.*, 2014)**.** Glucocorticoids have also been shown to delay neutrophil apoptosis *in vitro*, but this did not occur in the presence of hypoxia (Marwick *et al.*, 2013). Neutrophil apoptosis has been identified in the menstrual endometrium of mice, when hypoxia is present (Armstrong *et al.*, 2017). In addition, glucocorticoids have been

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identified as having an important role in the human menstrual endometrium (McDonald *et al.*, 2006; Rae *et al.*, 2009)**.** The impact of hypoxia on endometrial myeloid apoptosis has not been examined to date.

Impact on macrophages

Macrophages have been detected in the endometrium throughout the menstrual cycle, both close to the endometrial glands and in the stromal compartment (Bonatz *et al.*, 1992). They show a peri-menstrual peak in number, reaching up to 15% of the cell total number at the time of menses (Salamonsen & Woolley, 1999). Like neutrophils, it is proposed that macrophages play a critical role in the onset of endometrial breakdown via production and release of MMPs (reviewed in (Critchley *et al.*, 2001; Thiruchelvam *et al.*, 2013)). There are also indications of their involvement in glandular remodelling (Garry *et al.*, 2010) and endometrial regeneration (Maybin *et al.*, 2012; Cousins, Kirkwood, *et al.*, 2016)**,** including the regulation of angiogenesis (Thiruchelvam *et al.*, 2016).

Macrophages are remarkably plastic cells, capable of shifting towards different phenotypes by sensing the surrounding microenvironment (Martinez, 2008).Thus, their microenvironment may affect their recruitment and function. Historically, macrophage polarisation has been categorised as classical (M1) or alternative (M2). M1 phenotype is associated with microbicidal properties and M2 reflects a more regulatory, anti-inflammatory phenotype. More recently, macrophage polarisation is understood to be a dynamic spectrum of macrophage transition in response to environmental cues (Martinez & Gordon, 2014). As there is mounting evidence for hypoxia in the local endometrial environment at menstruation (Cousins, Murray, *et al.*, 2016; Maybin *et al.*, 2018), it is important to determine its effect on endometrial macrophages.

Under physiological conditions M2 macrophages are involved in angiogenesis and cellular clearance, hence promote wound healing. However, tumour-infiltrating macrophages (TAMs) are often correlated with poor cancer prognosis (Kawanaka *et al.*, 2008)**.** TAMs have been shown to be retained in hypoxic regions of tumours through the Sema3A/Neuropilin-1signaling axis, which is regulated by HIF-2α (Casazza *et al.*, 2013). The influence of hypoxia on TAMs is not only limited to macrophage number but also influences their phenotype. Indeed, specific TAM phenotypical subsets have been reported depending on intra-tumoral oxygen levels (Laoui *et al.*, 2014).

Non-tumoral studies have also linked HIF to changes in macrophage phenotype. In a model of endotoxemia, HIF-1α and HIF-2α were differentially expressed in M1 and M2-macrophages respectively (Takeda *et al.*, 2010). In addition, in the context of obesity and adipose tissue inflammation, HIF-1α has been proven to promote inflammation and insulin resistance through M1 macrophage polarisation whereas HIF-2α ameliorated the effects via M2-macrophage induction (reviewed in (Lin & Simon, 2016)). Interestingly, HIF-1α was found to be decreased in mouse adipose tissue when glucocorticoid activation was suppressed, suggesting a crucial role of glucocorticoids in HIF-dependent macrophage polarisation (Chapman *et al.*, 2013). Thus, different research fields converge around the concept that HIF-1α may be required for M1 polarization of macrophages, while HIF-2α might promote M2 polarization.

The menstrual endometrium presents a unique model of transient, physiological hypoxia in which to study macrophage number and phenotype. HIF-2α may have a role in the recruitment and function of macrophages during implantation, when endometrial HIF-2α was found to be present (Maybin *et al.*, 2018)**.** However, a recent study of mice with a targeted deletion of HIF-1α in myeloid cells resulted in decreased pregnancy rates and increased miscarriage rates, suggesting that HIF-1α dependent pathways in myeloid cells are also important for maintenance of pregnancy (Köstlin-Gille

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et al., 2019). It would be informative to establish if the balance between HIF-1α/HIF-2α determines the pro-inflammatory or anti-inflammatory fate of the endometrium.

Impact of hypoxia on proliferation

After 'injury', fibroblasts must migrate and proliferate in the damaged area, where they produce extracellular matrix (ECM) components that contribute to repair (Gonzalez *et al.*, 2016). This production must be tightly regulated to prevent excessive ECM growth, scar formation and fibrosis (Ruthenborg *et al.*, 2014). In dermal tissue, hypoxia has been shown to stimulate macrophage growth factors that may contribute to fibroblast proliferation and tissue repair (Murdoch, Muthana & Lewis, 2005). Macrophage production of platelet-derived growth factor (PDGF) enhances fibroblast mitosis, while transforming growth factor β (TGF-β) promotes the formation of the ECM (Ruthenborg *et al.*, 2014). In addition, hypoxia has been proven to induce the transcription of VEGF, connective tissue growth factor and adrenomedullin in endometrial stromal tissue (Maybin, Battersby, *et al.*, 2011; Maybin *et al.*, 2012). Hence, hypoxia may induce a pro-repair environment by modifying the secretome of endometrial cell populations.

To complete tissue restoration, reepithelialisation of the affected area must take place. In the skin, this is achieved through the migration and proliferation of keratinocytes towards the injury site (Ruthenborg *et al.*, 2014). Stabilisation of HIF-1α in a mouse model of skin wound healing revealed its role in promoting keratinocyte proliferation and migration to the injured area, accelerating wound closure (Kalucka *et al.*, 2013). This is consistent with the findings of delayed endometrial repair with decreased HIF-1α (Maybin *et al.*, 2018).

Impact of hypoxia on vascular remodelling and angiogenesis

Angiogenesis and vascular remodelling are crucial events in the endometrium throughout the menstrual cycle. Optimal vascular function is necessary to support the repair of the functional endometrial layer and to supply the thickened endometrium required for successful implantation and placentation.

VEGF is a key mediator of both physiological and tumoral angiogenesis and may be induced by hypoxia (Carmeliet, 2005)**.** VEGF mRNA and protein have been detected during all phases of the menstrual cycle, both in the stromal compartment and the glandular epithelium (Stephen Charnock-Jones *et al.*, 1993; Shifren *et al.*, 1996; Punyadeera, 2006) but was maximal during menses (Sharkey *et al.*, 2000; Graubert *et al.*, 2001; Maybin, Hirani, *et al.*, 2011). Studies in mouse and macaque models of menstruation have shown that blocking VEGF dramatically decreases reepithelialisation and new blood vessel formation in the endometrium (Fan *et al.*, 2008)**,** consistent with an essential role for VEGF in endometrial angiogenesis and repair.

Hypoxia has been detected in the mouse model of simulated menses (Chen *et al.*, 2015; Cousins, Murray, *et al.*, 2016; Maybin *et al.*, 2018) and coincides with increased VEGF mRNA (Cousins, Murray, *et al.*, 2016). Hypoxia and VEGF have also been detected in human perimenstrual endometrial biopsies (Punyadeera, 2006) highlighting their possible interrelation. *In vitro* studies have also shown that subjecting endometrial and epithelial stromal cells to hypoxia increases VEGF mRNA and protein (Popovici *et al.*, 1999; Sharkey *et al.*, 2000; Graubert *et al.*, 2001) and that silencing of HIF-1α abrogates this hypoxia-induced VEGF expression (Maybin, Hirani, *et al.*, 2011; Chen *et al.*, 2015). Through a chromatin immunoprecipitation (ChIP) assay, Chen *et al.* detected the direct binding of HIF-1α to the VEGF promoter, which was maximal during endometrial breakdown of the mouse model of menses (Chen *et al.*, 2015). Inhibition of HIF-1α using 2-methoxyestradiol (2-

 Accepted Manuscript published as REP-20-0268.R1. Accepted for publication: 03-Aug-2020

ME) significantly suppressed VEGF levels during menses. Therefore, hypoxia, and more specifically HIF-1α, seems to promote endometrial VEGF during menses.

In addition, VEGF expression is induced by different cytokines and chemokines (Li *et al.*, 1995; Stavri *et al.*, 1995; Zagzag *et al.*, 2006), some of which contain hypoxic response elements. Optimal blood vessel formation requires the trafficking of endothelial progenitors cells through the interaction of the chemokine CXCL12 with its receptor CXCR4 (Ruthenborg *et al.*, 2014). Both ligand and receptor have been found to be upregulated by HIF-1α, contributing to angiogenesis and blood vessel repair partly through VEGF (Ceradini *et al.*, 2004; Zagzag *et al.*, 2006). CXCL12 and CXCR4 have been described in the human endometrium (Ruiz *et al.*, 2010) and endometrial CXCR4 was found to be decreased in patients with heavy menstrual bleeding (Maybin *et al.*, 2018)**.** Hence, the interactions between hypoxia pathways and inflammatory processes may significantly influence endometrial vascular function.

During decidualisation there is *in vitro* evidence that endometrial stromal cells increase VEGF mRNA and protein (Popovici *et al.*, 1999; Matsui *et al.*, 2004) and that hypoxia induced further increases in VEGF (Popovici *et al.*, 1999)**.** This VEGF production may be responsible for macrophage recruitment and polarisation towards a pro-angiogenic M2 phenotype (Wheeler *et al.*, 2018). Thus, the responsiveness of the decidualised stroma to hypoxia suggests a possible role in the preparation of the endometrial vasculature for implantation. Uterine HIF2-α deficiency has been shown to impair decidualisation in mice, revealing a downregulation of prolactin-related factors which can compromise the maintenance of the corpus luteum and therefore endometrial receptivity (Matsumoto *et al.*, 2018).

When studying implantation in mice, HIF factors were found to be differentially expressed at the time of peri-implantation: HIF-1α was detected in the luminal epithelium, whereas HIF-2α

556 expression was limited to the stromal compartment and neither correlated with VEGF expression
557 (Daikoku *et al.*, 2003). Therefore, HIF effects on implantation seem to be more versatile than simply
558 contributing to vessel formation, playing a substantial role in decidualisation, endometrial
559 receptivity and embryo survival (Matsumoto *et al.*, 2018). After implantation, HIF-1α was found in
560 the luminal epithelium and the decidual layer. However, the strongest signal came from HIF-2α,
561 whose expression was localised to stromal cells surrounding the blastocyst. This post-implantation
562 HIF-2α expression was correlated with VEGF induction, switching to a proangiogenic stimulus once
563 implantation had taken place (Daikoku *et al.*, 2003)**.**
564
565

566 THE ROLE OF HYPOXIA IN ENDOMETRIAL PATHOLOGY

567 As outlined above, the literature regarding the influence of hypoxia on inflammation, proliferation
568 and vascular function is increasing (**Fig. 3**). The influence of oxygen levels on implantation,
569 placentation and disorders such as pre-eclampsia has been comprehensively reviewed within this
570 series by Burton et al. (Burton, 2009). The impact of hypoxia on embryo function has been covered
571 in detail by Dunwoodie et al. (Dunwoodie, 2009). Therefore, this section is focused on the role of
572 endometrial hypoxia during menstruation and its potential in the identification of novel diagnostic
573 and therapeutic strategies.
574

575 Abnormal uterine bleeding

576 Abnormal uterine bleeding (AUB) affects 20-30% of pre-menopausal women and over 800,000
577 women seek treatment in the UK each year **(National Heavy Menstrual Bleeding Audit, 2011)**.
578 Available medical treatments are often discontinued due to side effects or lack of efficacy. Research
579 in this area was previously hindered by lack of a consistent classification system for the diagnosis of

 Accepted Manuscript published as REP-20-0268.R1. Accepted for publication: 03-Aug-2020

causes of AUB. This was rectified by the development of the FIGO classification system of structural and non-structural causes (Munro, Critchley & Fraser, 2011, 2018) (**Fig. 4**).

Structural causes of AUB

Structural causes of AUB can be detected on examination or imaging of the uterus, e.g polyps, adenomyosis, leiomyoma (fibroids) and malignancy (Munro, Critchley & Fraser, 2011, 2018). These conditions have previously been under-diagnosed, with clinicians often treating the symptom of AUB without identifying the underlying cause. This has limited our knowledge on why these conditions develop and why they result in AUB.

Adenomyosis is the presence of ectopic endometrial glands and stroma within the myometrial layer of the uterus. It occurs in 7-27% of reproductive aged women and presents with painful, heavy menstrual bleeding (Naftalin *et al.*, 2012; Mavrelos *et al.*, 2017). The impact of the adenomyotic lesions on the eutopic endometrium and the mechanisms causing AUB are not well understood. AUB due to adenomyosis (AUB-A) is particularly challenging as it is often resistant to medical treatment and surgical options (ablation or hysterectomy) are unacceptable to those wishing to preserve their fertility.

There is some evidence that the hypoxic response is aberrant within adenomyotic lesions. A study of hysterectomy samples from 14 women with adenomyosis and 9 without revealed increased VEGF protein in the eutopic endometrium of women with adenomyosis and increased VEGF and HIF-1α protein in ectopic versus eutopic endometrium (Goteri *et al.*, 2009). This suggests that a hypoxic environment in the adenomyotic lesions could contribute to increased vessel formation. In endometriosis, where ectopic endometrium implants outside of the uterus, HIF-1α was also found to be increased in ectopic versus eutopic endometrium (Wu *et al.*, 2007; Young *et al.*, 2014).

Inhibition of HIF-1 in a mouse model of endometriosis suppressed growth of lesions (Becker *et al.*, 2008), identifying the hypoxia pathway as a potential therapeutic target. The peritoneum is a common site for implantation of ectopic endometrial deposits in endometriosis. Women with endometriosis have been shown to have increased HIF-1α in non-affected peritoneum compared to peritoneum from women without disease (Young *et al.*, 2014), consistent with a role of the hypoxia pathway in the development of peritoneal disease. Studies examining the non-affected myometrium in women with adenomyosis are not yet available, but similar alterations in hypoxic response would highlight hypoxia pathways as a potential target for preventative and therapeutic interventions.

Leiomyomas (uterine fibroids) are common, benign tumours of the myometrium that form as a consequence of the proliferation of uterine smooth muscle cells and collagen matrix. They occur in approximately 70% of women (Stewart *et al.*, 2017) and are extremely heterogeneous in size, location and pathophysiology. Leiomyoma are symptomatic in approximately 50% of women (Day Baird *et al.*, 2003) and may cause symptoms of AUB, pressure, pelvic pain and be associated with subfertility.

Genome wide association studies have identified genetic subgroups that may predispose to leiomyoma formation (reviewed in (Stewart *et al.*, 2016)) but local mechanisms regulating their development remain an area of active research. Uterine leiomyomas contain broad avascular areas and HIF-1α protein was found to be increased in leiomyoma nuclear protein extracts when compared to adjacent myometrium (Ishikawa *et al.*, 2019). However, it is not yet clear whether hypoxia is necessary for leiomyoma development and/or growth. In contrast, an *in vivo* study of women with leiomyoma using DCE-MRI has revealed increased K^{trans} (a combination of perfusion and permeability) in fibroids compared with normal uterus (Majd *et al.*, 2017) which does not support the presence of hypoxia within fibroids**.** There is evidence that treatment of leiomyomas with gonadotrophin releasing hormone (GnRH) analogues, often used pre-operatively to reduce fibroid

 Accepted Manuscript published as REP-20-0268.R1. Accepted for publication: 03-Aug-2020

size and decrease AUB, lead to a decrease in perfusion parameters (Munro *et al.*, 2014). These contrasting *in vitro* and *in vivo* findings may reflect the heterogeneity of leiomyomas and it remains unclear if altered perfusion is associated with AUB.

The cause of AUB experienced by a proportion of women with leiomyomas is not understood. Vasoconstriction may be impaired at the time of menstruation in women with fibroids, with leiomyoma tissue expressing altered levels of endothelin receptors and prostaglandin F2α when compared to normal myometrium (Pekonen, Nyman & Rutanen, 1994; Miura *et al.*, 2006). A small decrease in spiral arteriole vasoconstriction can significantly increase menstrual blood flow, causing heavy menstrual bleeding. A greater understanding of the role of hypoxia in leiomyoma formation and growth may identify new, specific treatments to reduce their presence, size and symptoms.

Endometrial cancer. The importance of hypoxia in the tumour microenvironment is well established, including its influence on immune cell populations, angiogenesis, tumour progression and metastasis (De Bock, Mazzone & Carmeliet, 2011; Casazza *et al.*, 2014; Schito & Semenza, 2016; Semenza, 2016). The accuracy of translation of these principles to patients with endometrial cancer is less well determined. In a quest to identify a robust biomarker that would predict tumour behaviour, Chang *et al* identified an eight gene set of lymphocyte and tumour hypoxia markers and validated its performance in predicting overall survival in six cancers, including 370 women with endometrial cancer (Chang, Forde & Lai, 2019). They found a superior performance over current tumour staging parameters, highlighting the importance of hypoxia in determining risk and aiding clinical decision making.

Assessment of endometrial tissues from 386 patients with endometrial carcinoma using CAIX as a hypoxia marker and CD34 to determine vascular density, revealed that patients with the presence of both hypoxia and high vascular density (16.4%) had reduced disease-specific survival and distant

disease-free survival (Reijnen *et al.*, 2019). *In vivo* imaging with DCE-MRI revealed that a poor prognosis was associated with low microvascular blood flow to the endometrial tumour (Haldorsen *et al.*, 2013, 2014; Berg *et al.*, 2016). This was thought to reflect disorganised angiogenesis with coexisting vascular proliferation and hypoxia. These studies highlight normalisation of the vasculature to limit hypoxia as a potential therapeutic target in endometrial cancer.

Non-structural causes of AUB

These non-structural disorders are not usually identified by routine pelvic imaging. They include coagulopathies, ovulatory dysfunction, endometrial and iatrogenic causes (Munro, Critchley & Fraser, 2011, 2018). Evidence for a role of hypoxia in these disorders is limited but its contribution to AUB of endometrial origin (AUB-E) is discussed below.

AUB-E includes disorders of local endometrial haemostasis, vascular function and/or inflammation (**Fig. 4**). Women with objectively defined HMB (>80ml/cycle) had reduced levels of HIF-1α protein and downstream target genes in menstrual phase endometrial biopsies when compared to those from women with normal blood loss (Maybin *et al.*, 2018). Examination of endometrial repair in mice where hypoxia was prevented during simulated menses, or where HIF-1α was pharmacologically or genetically reduced, revealed delayed repair (Maybin *et al.*, 2018). This is consistent with hypoxia having a key role in the rapid endometrial repair necessary to limit menstrual blood loss. The delayed repair in a non-hypoxic mouse menstruation model could be rescued with a pharmacological compound that stabilises HIF-1, identifying a potential non-hormonal therapeutic target for women with AUB-E.

The cause of the endometrial tissue hypoxia observed at menstruation is unknown. It is likely that spiral arteriole vasoconstriction limits blood supply to the functional layer of the endometrium

 Accepted Manuscript published as REP-20-0268.R1. Accepted for publication: 03-Aug-2020

following progesterone withdrawal (Markee, 1940). Hence, factors that limit the ability of the specialised endometrial arterioles to constrict will have a significant impact on the presence of endometrial hypoxia. Women with the symptom of HMB have been shown to have significantly decreased smooth muscle myosin heavy chain in their spiral arterioles and also reduced vascular smooth muscle cell proliferation during the mid-late secretory phase compared to those with normal menstrual blood loss (Abberton *et al.*, 1999). Another study showed that endometrial vessel wall circumference and endothelial cell focal discontinuities were both significantly larger in women with HMB compared to normal controls (Mints *et al.*, 2007). Furthermore, calponin (a vascular smooth muscle cell contractile protein) was found to be significantly lower in endometrial blood vessels in women with HMB (Biswas Shivhare *et al.*, 2014). This evidence is all consistent with an aberrant vasculature within the pre-menstrual endometrium of women with AUB-E, leading to a suboptimal hypoxic response during menstruation.

CONCLUSIONS

Herein, we have reviewed the mounting evidence for the presence of endometrial hypoxia and its potential impact on endometrial function. Furthering our understanding of hypoxia in endometrial physiology and pathology using the tools described in this review may provide novel preventative and therapeutic strategies for those suffering from endometrial disorders, including abnormal uterine bleeding (AUB). Furthermore, a complete understanding of optimal endometrial physiology may inform the management of other disorders where aberrant hypoxia is a prominent feature, such as tumour biology and chronic obstructive pulmonary disorder. Addressing the gaps in our knowledge of how hypoxia influences endometrial function represents an exciting area with huge translational potential.

ACKNOWLEDGEMENTS

We are grateful to Alison Murray for manuscript review and Sheila Milne for assistance with manuscript formatting. Some aspects of studies described herein were undertaken in the MRC Centre for Reproductive Health and have been supported by funding from the Medical Research Council: MRC Project Grant G0600048 and Centre Grants G1002033 and MR/N022556/1. This work was also supported by Wellcome Trust Grants 209589/Z/17/Z and 100646/Z/21/Z; Wellbeing of Women Grant RG1820; and Academy of Medical Sciences Grant AMS-SGCL13

Disclosure: RM-A, LK, JJR and JAM have nothing to disclose. HODC has clinical research support for laboratory consumables and staff from Bayer AG and provides consultancy advice (but with no personal remuneration) for Bayer AG, PregLem SA, Gedeon Richter, Vifor Pharma UK Ltd, AbbVie Inc; Myovant Sciences GmbH. HODC receives royalties from UpToDate for article on abnormal uterine bleeding. Jackie Maybin is a Guest Editor of this special issue of Reproduction. Jackie Maybin was not involved in the review or editorial process for this paper, on which she is listed as an author.

Author contributions: RM-A, LK and JAM wrote and edited the manuscript. JJR and HODC modified the manuscript. All authors have read and approved the final version of this manuscript.

 Accepted Manuscript published as REP-20-0268.R1. Accepted for publication: 03-Aug-2020

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1131

1132 FIGURE LEGENDS

1133 **Figure 1. Rodent models of simulated menstruation. (a) An exogenous hormone mouse model of simulated**
1134 **menstruation.** Female mice are ovariectomized (ovex) and allowed to recover for 7-14 days before being given
1135 subcutaneous injections of oestradiol (E_2). A progesterone (P_4) implant is subcutaneously inserted and lower
1136 dose E_2 injections administered. The decidualisation stimulus (oil) is intracervically administered. In order to
1137 induce a *menstrual-like* event, the P_4 implant is subsequently removed (T_0). This triggers a menstrual like bleed
1138 (8h after P_4 withdrawal-T_8) and subsequent endometrial regeneration (24h after P_4 withdrawal-T_{24}). **(b) A**
1139 **pseudopregnancy mouse model of simulated menstruation.** Female mice are mated with vasectomized males
1140 to induce pseudopregnancy. Three to four days after the formation of the vaginal plug, decidualisation is
1141 externally induced via uterine oil injection. Two days after the decidualisation stimulus, mice are
1142 ovariectomised (ovex) to trigger P_4 withdrawal (T_0). Using this approach, endometrial breakdown is apparent

 Accepted Manuscript published as REP-20-0268.R1. Accepted for publication: 03-Aug-2020

12-16h after P_4 withdrawal (T_{12}) and re-epithelialisation can be detected 24h after P_4 withdrawal. Optionally, mice can receive daily subcutaneous injections of E_2 to prevent atrophy of the uterus following ovariectomy. **(c) Xenograft mouse model.** Female immunodeficient mice are ovariectomized (ovex) and allowed to recover for 7-14 days before the implantation of the endometrial patches/dissociated endometrial cells. At the time of implantation or shortly after the formation of the xenografts, mice are treated with E_2 and P_4 for 21-28 days to induce the menstrual cycle. When the P_4 pellet is removed, menstruation and successive regeneration takes place in the xenograft for the next 4-8 days.

Figure 2. In vivo methods with the potential to detect markers of endometrial hypoxia in women. Left: previous *in vivo* work to assess human endometrial hypoxia, shown with structural MRI of the uterus and surrounding tissues. Right: potential non-invasive imaging methods for translation from other body areas. [+] indicates advantages of each technique, [-] indicates disadvantages. Relevant references shown for each. DCE-MRI = Dynamic contrast-enhanced MRI, MRI = Magnetic resonance imaging.

Figure 3. Overview of the presence and role of hypoxia in endometrial physiology. (a) Hypoxia during implantation. Endometrial stromal cells undergo decidualisation under the influence of progesterone. Hypoxia inducible factor (HIF)-2α in these uterine stromal cells supports decidualisation, embryo invasion and survival. Endometrial blood vessels undergo dynamic remodelling that may be influenced by hypoxia/HIF. **(b) Hypoxia during endometrial breakdown.** Vasoconstriction of the endometrial vessels may limit blood loss during menstruation and cause transient endometrial hypoxia to stabilise HIF-1α. The endometrial leukocyte population may be altered in number and/or function by hypoxia/HIF. **(c) Role of hypoxia during endometrial repair.** Hypoxia is not detected in endometrial areas that have reepithelialised, while those areas undergoing active regeneration remain hypoxic. This hypoxia is thought to promote endometrial VEGF (alongside other factors), which is responsible for reepithelialisation and new blood vessel formation. P_4 = progesterone, G = glands, BV = blood vessel, VEGF = vascular endothelial growth factor, HIF = hypoxia-inducible factor.

Figure 4. Abnormal uterine bleeding (AUB) and the potential role of hypoxia. Abnormal uterine bleeding may be due to structural (Polyps, Adenomyosis, Leiomyoma, Malignancy) or non-structural (Coagulopathy, Ovulatory, Endometrial, Iatrogenic or Not otherwise classified) disorders. The role of hypoxia in AUB is

unknown but its potential role in four disorders is illustrated. (**I**) Leiomyoma (fibroids): the decreased levels of endothelin and PG2Fα receptors may compromise endometrial vasoconstriction and increase menstrual blood flow. (**II**) Malignancy: tumour hypoxia leads to disorganised angiogenesis and increased metastasis. (**III**) Endometrial disorders: endothelial cell focal discontinuities and impairment of vascular smooth muscle cells may influence vasoconstriction. This may decrease HIF-1α and prevent optimal post-menstrual repair. (**IV**) Adenomyosis: VEGF and HIF-1α overexpression may contribute to increased vessel formation and AUB. G = glands, BV = blood vessels, Myo = myometrium, E = endometrium, VEGF = vascular endothelial growth factor, HIF = hypoxia-inducible factor.

Accepted Manuscript published as REP-20-0268.R1. Accepted for publication: 03-Aug-2020

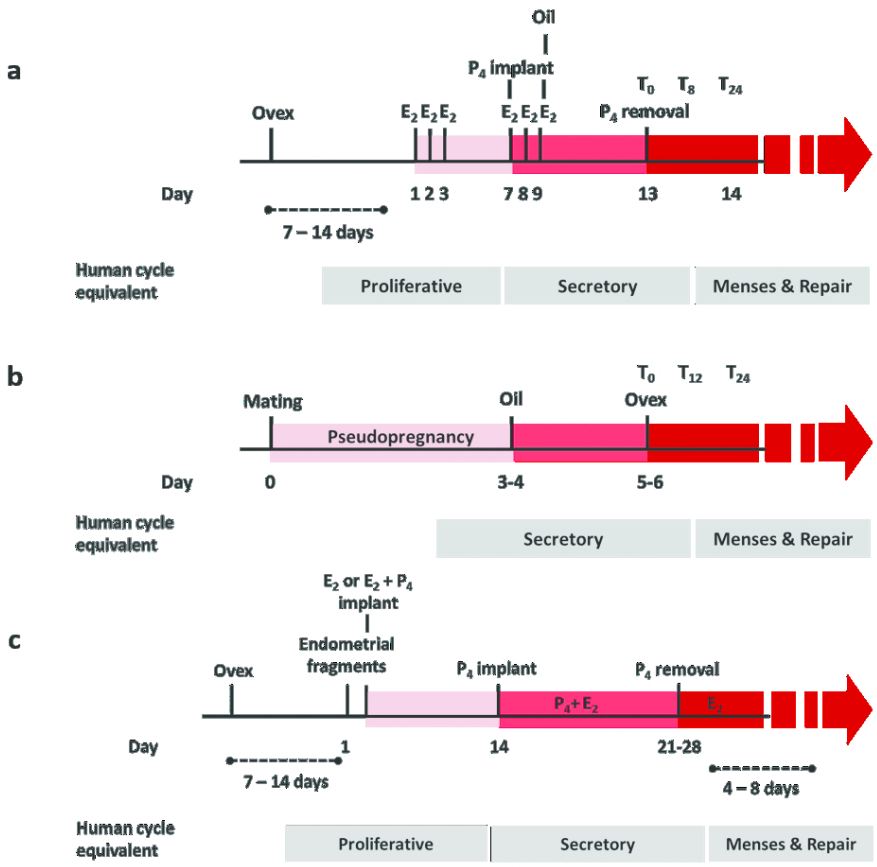

Figure 1. Rodent models of simulated menstruation. (a) An exogenous hormone mouse model of simulated menstruation. Female mice are ovariectomized (ovex) and allowed to recover for 7-14 days before being given subcutaneous injections of oestradiol (E2). A progesterone (P4) implant is subcutaneously inserted and lower dose E2 injections administered. The decidualisation stimulus (oil) is intracervically administered. In order to induce a menstrual-like event, the P4 implant is subsequently removed (T0). This triggers a menstrual like bleed (8h after P4 withdrawal-T8) and subsequent endometrial regeneration (24h after P4 withdrawal-T24). (b) A pseudopregnancy mouse model of simulated menstruation. Female mice are mated with vasectomized males to induce pseudopregnancy. Three to four days after the formation of the vaginal plug, decidualisation is externally induced via uterine oil injection. Two days after the decidualisation stimulus, mice are ovariectomised (ovex) to trigger P4 withdrawal (T0). Using this approach, endometrial breakdown is apparent 12-16h after P4 withdrawal (T12) and re-epithelialisation can be detected 24h after P4 withdrawal. Optionally, mice can receive daily subcutaneous injections of E2 to prevent atrophy of the uterus following ovariectomy. (c) Xenograft mouse model. Female immunodeficient mice are ovariectomized (ovex) and allowed to recover for 7-14 days before the implantation of the endometrial patches/dissociated endometrial cells. At the time of implantation or shortly after the formation of the xenografts, mice are treated with E2 and P4 for 21-28 days to induce the menstrual cycle. When the P4 pellet is removed, menstruation and successive regeneration takes place in the xenograft for the next 4-8 days.

178x156mm (150 x 150 DPI)

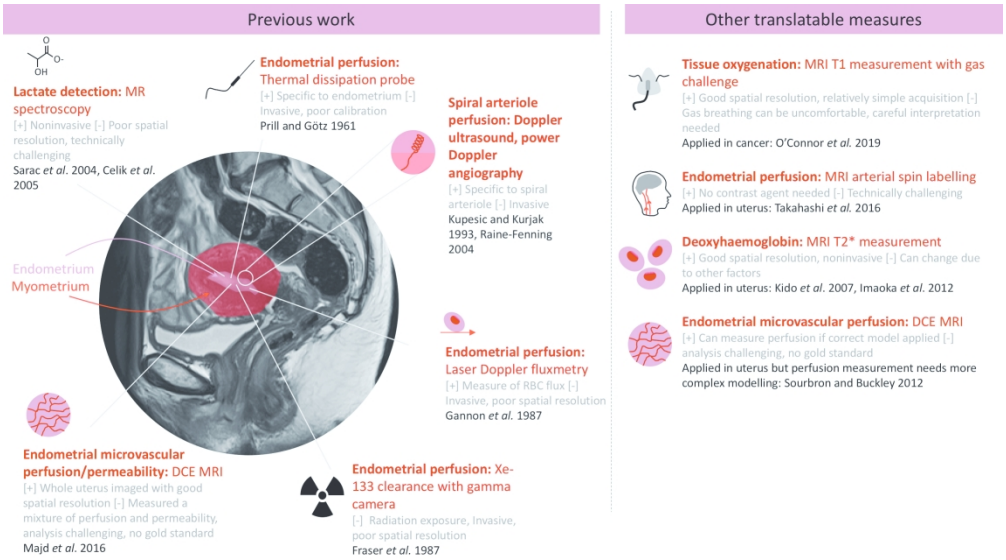

Figure 2. In vivo methods with the potential to detect markers of endometrial hypoxia in women. Left: previous in vivo work to assess human endometrial hypoxia, shown with structural MRI of the uterus and surrounding tissues. Right: potential non-invasive imaging methods for translation from other body areas. [+] indicates advantages of each technique, [-] indicates disadvantages. Relevant references shown for each. DCE-MRI = Dynamic contrast-enhanced MRI, MRI = Magnetic resonance imaging.

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Accepted Manuscript published as REP-20-0268.R1. Accepted for publication: 03-Aug-2020

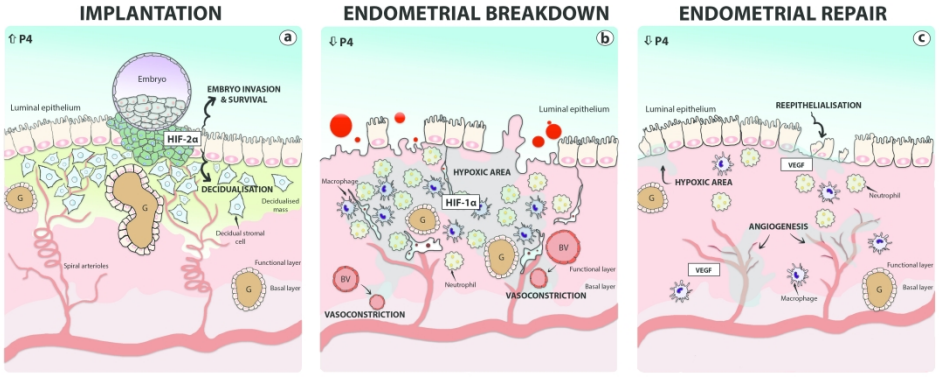

Figure 3. Overview of the presence and role of hypoxia in endometrial physiology. (a) Hypoxia during implantation. Endometrial stromal cells undergo decidualisation under the influence of progesterone. Hypoxia inducible factor (HIF)-2α in these uterine stromal cells supports decidualisation, embryo invasion and survival. Endometrial blood vessels undergo dynamic remodelling that may be influenced by hypoxia/HIF. (b) Hypoxia during endometrial breakdown. Vasoconstriction of the endometrial vessels may limit blood loss during menstruation and cause transient endometrial hypoxia to stabilise HIF-1α. The endometrial leukocyte population may be altered in number and/or function by hypoxia/HIF. (c) Role of hypoxia during endometrial repair. Hypoxia is not detected in endometrial areas that have reepithelialised, while those areas undergoing active regeneration remain hypoxic. This hypoxia is thought to promote endometrial VEGF (alongside other factors), which is responsible for reepithelialisation and new blood vessel formation. P4 = progesterone, G = glands, BV = blood vessel, VEGF = vascular endothelial growth factor, HIF = hypoxia-inducible factor.

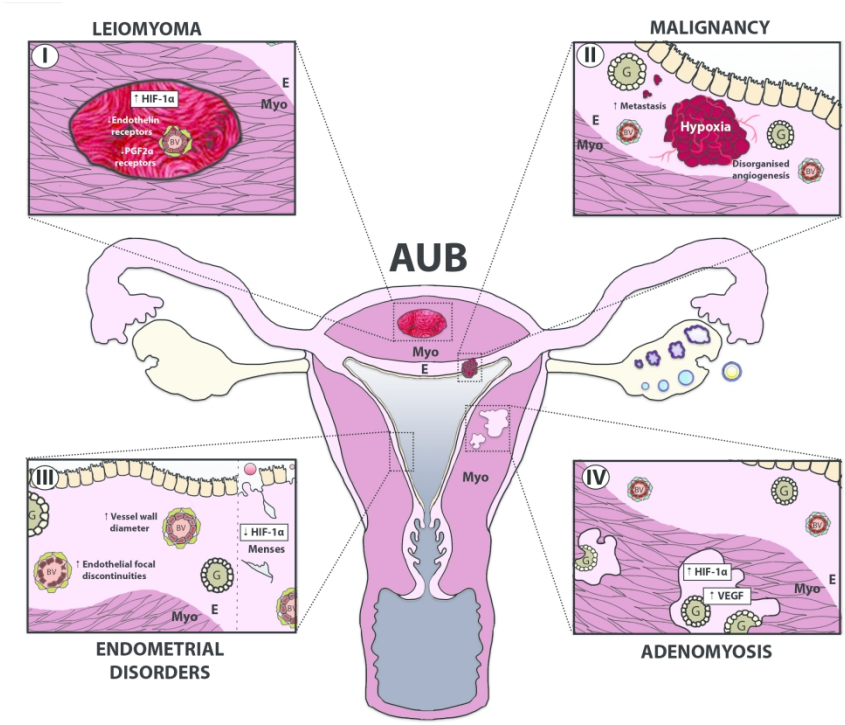

Figure 4. Abnormal uterine bleeding (AUB) and the potential role of hypoxia. Abnormal uterine bleeding may be due to structural (Polyps, Adenomyosis, Leiomyoma, Malignancy) or non-structural (Coagulopathy, Ovulatory, Endometrial, Iatrogenic or Not otherwise classified) disorders. The role of hypoxia in AUB is unknown but its potential role in four disorders is illustrated. (I) Leiomyoma (fibroids): the decreased levels of endothelin and PG2Fα receptors may compromise endometrial vasoconstriction and increase menstrual blood flow. (II) Malignancy: tumour hypoxia leads to disorganised angiogenesis and increased metastasis. (III) Endometrial disorders: endothelial cell focal discontinuities and impairment of vascular smooth muscle cells may influence vasoconstriction. This may decrease HIF-1α and prevent optimal post-menstrual repair. (IV) Adenomyosis: VEGF and HIF-1α overexpression may contribute to increased vessel formation and AUB. G = glands, BV = blood vessels, Myo = myometrium, E = endometrium, VEGF = vascular endothelial growth factor, HIF = hypoxia-inducible factor.