The Endometrial Lymphatic Vasculature: Function and Dysfunction

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Abstract

The endometrium has a complex and dynamic blood and lymphatic vasculature which undergoes regular cycles of growth and breakdown. While we now have a detailed picture of the endometrial blood vasculature, our understanding of the lymphatic vasculature in the endometrium is limited. Recent studies have illustrated that the endometrium contains a population of lymphatic vessels with restricted distribution in the functional layer relative to the basal layer. The mechanisms responsible for this restricted distribution and the consequences for endometrial function are not known. This review will summarise our current understanding of endometrial lymphatics, including the mechanisms regulating their growth and function. The potential contribution of lymphatic vessels and lymphangiogenic growth factors to various endometrial disorders will be discussed.

1.   Introduction

The endometrium has a complex and dynamic blood and lymphatic vasculature which undergoes regular cycles of growth and breakdown. These cyclic changes reflect variations in circulating sex steroids and uterine blood flow and result in cyclic patterns in tissue oxygenation, haemostasis, nutrient supply, fluid balance and leukocyte distribution. Appropriate growth and functioning of the vasculature is essential for normal endometrial function, including preparation for potential embryo implantation and subsequent pregnancy. Conversely, inappropriate endometrial vascular growth and/or function is associated with several endometrial disorders including various forms of abnormal uterine bleeding. In this review, we will discuss recent advances in our understanding of normal and abnormal endometrial vascular function with a specific focus on lymphatic vessels and lymphatic regulatory factors.

2.   The endometrium has a complex and dynamic blood and lymphatic vasculature

Building on early pivotal studies (eg. [1,2]), we now have a detailed picture of the blood vasculature in the endometrium. Arriving from the myometrium, the radial arterioles differentiate into the straight and specialised 'spiral' arterioles of the endometrium. The straight arterioles supply the basal layer of the endometrium and also run alongside the spiral arterioles into the functional layer. The specialised spiral arterioles are characteristic of the human endometrium and have central roles in the menstruation process and in placental formation [3]. They develop during the secretory phase of the menstrual cycle under the influence of progesterone, coiling through the basal layer into the functional layer to supply a discrete region of the sub-epithelial capillary plexus [1]; the plexus subsequently drains into

venous sinuses. For more in-depth information regarding the endometrial blood vasculature, readers are directed to recent review papers and the references therein [4-8].

While there is now a clear picture of the endometrial blood vasculature and recognition of its importance in normal and abnormal endometrial function, our understanding of the lymphatic vasculature in the endometrium is limited. This is not a new problem; studies as early as Hogan and Hogan [9] bemoaned the lack of useful information regarding endometrial lymphatics and the erroneous results arising from limited methodology. More recently, there has been resurgence in interest in the lymphatic vasculature stemming from advances in the understanding of the regulatory mechanisms responsible for lymphatic vessel growth [10-15]. This knowledge now needs to be applied to the uterine lymphatic vasculature.

In early studies, which used various methodologies including routine histology, dye tracking techniques, and electron microscopy, a distinctive network of variable sized myometrial lymphatics was consistently observed [16,17]. However, variable patterns of endometrial lymphatics were described with some studies reporting lymphatics in the functional layer of the human endometrium [18], while others only observed lymphatic vessels in the basal layer [16]. More recently, the development of specific lymphatic endothelial cell markers (eg. vascular endothelial growth factor [VEGF] receptor-3 [VEGF-R3] [19]; lymphatic endothelial hyaluronan receptor-1 [LYVE-1] [20]; podoplanin [21]; prospero-related homeobox-1 (PROX1) [22] has facilitated the identification of lymphatic vasculature. In studies utilising an antibody directed against podoplanin (D2-40), lymphatic vessels have been identified in the myometrium and both the functional and basal layers of the endometrium [23,24]. The lymphatics in the functional layer were small and sparsely distributed (Fig 1A). In comparison, the lymphatics of the basal layer were larger (Fig 1B),

and of note, sometimes closely associated with the specialised endometrial spiral arterioles [23]. While the density of lymphatic vessels did not change across the menstrual cycle, significantly fewer lymphatic vessels were present in the functional layer relative to the basal layer and myometrium [23,24] (Fig 1A-C). Lymphatic vessel density was further reduced in subepithelium relative to the rest of the functional layer [24]. Donoghue et al. [23] estimated that 13%, 43% and 28% of all vessels were lymphatics in the functional layer, basal layer and myometrium, respectively.

In contrast to the observations outlined above, lymphatic vessels were not observed in the endometrium when LYVE 1 was used as a specific lymphatic endothelial cell marker [25-27]. Further, podoplanin-positive lymphatics exhibited limited and variable immunostaining with LYVE1 in human endometrial biopsies (placental bed) at all stages of pregnancy except term [28]. Although LYVE1 is a specific and widely used lymphatic endothelial cell marker, its heterogeneity in endometrial vasculature suggests functional differences in comparison to other vascular beds. LYVE1 is a trans-membrane receptor that binds to the glycosaminoglycan hyaluronan, sharing homology with the leukocyte hyaluronan receptor CD44 [29,30]. Its expression is limited almost entirely to lymphatic vessels and the endothelium of lymph node sinuses. Although the functional role of LYVE1 and its mechanism of action remain elusive, it is hypothesised to participate in lymphatic hyaluronan metabolism and hyaluronan-mediated leukocyte trafficking within the lymphatics [29,30]. Whether the unexpected patterns of LYVE1 immunoexpression in human endometrial tissues reflect hyaluronan activity or other functions is not known. Clearly, the functional roles of LYVE1 in particular and the endometrial lymphatics more generally warrant further investigation.

3. **We do not fully understand the function of endometrial lymphatic vessels.**

Maintaining fluid homeostasis is a key function of lymphatic vasculature. Excess protein-rich fluid is removed from tissues via lymphatic vessels for return to the blood circulation [14]. In parallel, lymphatics have key roles in immune surveillance transporting both soluble antigens and antigen presenting cells from peripheral tissues to the lymph nodes [15]. Presumably, similar functions can be ascribed to the endometrial lymphatics; however, this has not yet been specifically addressed (Table 1). The consequences of reduced lymphatic density in the functional layer of the endometrium have not yet been examined, although reduced lymphatic drainage would provide a functional explanation for the oedema that is characteristic of cycling endometrium [23]. It could be that reduced endometrial lymphatic drainage facilitates the establishment of pregnancy and survival of the fetal allograft, however confirmation of this hypothesis requires further mechanistic studies (discussed further below; [23]). As well as tolerating the presence of sperm and fetal allograft, it should be remembered that the endometrium must also be able to protect against various infectious agents [31]; the contribution of lymphatics to immune function during the menstrual cycle has not been investigated.

A novel interaction observed by Donoghue et al. [23] was the close association of some lymphatic vessels with the spiral arterioles within the basal layer of the endometrium. This association means lymphatic endothelium is often directly adjacent to the VSMCs of the spiral arterioles. This suggests that lymph fluid draining from the functional layer of the endometrium can come into close contact with the vascular smooth muscle cells of the spiral arteriole wall, thus providing a mechanism by which signalling from the superficial layers of the endometrium can be directly specifically to the spiral arterioles. Donoghue et al. [23]

hypothesised that this intimate association may influence blood flow through the arterioles via either vasoconstriction or dilation; whether lymphatics have a regulatory role in the vasoconstriction associated with menstruation is still to be examined.

The interaction between lymphatics and spiral arterioles was further illustrated by Volchek et al. [32], who examined the distribution of lymphatic vessels in the human endometrium during pregnancy (placental bed). Podoplanin-positive lymphatic vessels were commonly seen in the non-decidualised hypersecretory endometrium where they were commonly found encircling the spiral arterioles (Fig 1D-E). Lymphatics were also seen adjacent to endometrial glands. In complete contrast to the hypersecretory endometrium, the more superficial decidual tissues were largely devoid of lymphatic vessels [32] (Fig 1F-G). On occasions, lymphatic vessels that appeared to be regressing were observed at the boundary of non-decidualised hypersecretory and decidualised endometrium. Of note, lymphatic vessels were absent from spiral arterioles that were surrounded by decidualised stromal cells (Fig 1G), regardless of whether these vessels had or had not been transformed by extravillous trophoblast cells.

Decidualisation is a process by which endometrial stromal cells differentiate to form a support structure that envelops the developing fetus and placenta [33,8]. The decidua acts to ensure haemostasis during pregnancy and is responsible for the production of various growth factors and cytokines needed to regulate endometrial function and embryo development during pregnancy. The decidua also has hypothesised roles in maternal tolerance of the fetal allograft. During pregnancy, the mother's immune system is modulated ensuring continued protection against infection while allowing and supporting implantation of the conceptus. The decidua contributes to this complex immune response. It is the initial tissue layer

through which the extravillous trophoblast cells must migrate. The decidualised cells also interact with the large number of immune cells (eg. Macrophages, uterine natural killer cells, regulatory T-cells) that traffic into the decidua, each affecting the others behaviour and function [34-36]. For instance, recent studies suggest that decidua may act to immobilise certain leukocyte populations [37]. In studies with mice, dendritic cells present at the maternal/fetal interface became trapped during decidualisation and were unable to migrate to the lymphatic vessels of the uterus and thus the draining lymph nodes [37]. Further studies examining similar mechanisms involving decidualised cells, immune cells and endometrial lymphatics in humans would increase our understanding of maternal tolerance of the fetal allograft. When considering the role of lymphatics in maternal/fetal tolerance, it will also be important to consider the contribution of extravillous trophoblasts, which travel beyond the decidua into the hypersecretory region of the endometrium and underlying myometrium, both regions that contain plentiful lymphatic vessels. In the study by Volchek et al. [32], trophoblast cells were seen in close contact with lymphatic vessels of the hypersecretory zone, but were never seen invading or being incorporated in the walls of lymphatic vessels. Further, conditioned media collected from trophoblasts increased migration of LYVE1 positive human skin lymphatic endothelial cells [27]. Future studies will also need to consider the functional consequences of a lack of decidual lymphatics on fluid homeostasis in the decidua itself, as well as across the maternal and fetal component of the placenta. The transport of water across the placenta is a complex process reflecting the current status of the fetus (Faber and Anderson, 2010). How the distribution of endometrial lymphatics in the decidua versus the hypersecretory zones contributes to the process has not been considered.

In humans, decidualisation begins in the mid-late secretory phase of the menstrual cycle in response to circulating progesterone. The process originates in the stromal cells surrounding

the spiral arterioles and spreads out through the endometrium during early pregnancy. The impact of decidualisation on the endometrial lymphatic distribution adjacent to spiral arterioles in the secretory phase has not been specifically examined. Curiously, however, decidualisation is characterised by transient oedema, which may reflect a localised reduction in the lymphatic population. If implantation does not occur, circulating oestrogen and progesterone levels drop and the endometrium is shed at menstruation. The proximate mechanism thought to be responsible for menstruation is tissue destruction in the functional layer, which is associated with vascular breakdown and bleeding (see [38-41] for review). The bleeding events occurring during menstruation are focal and thought to be arrested by vasoconstriction of the spiral arterioles at the level of the basalis. Repair of the endometrium begins while bleeding is still in progress. This vascular breakdown and repair presumably involves the lymphatic vessels, although studies have yet to examine lymphatic vessels in menstrual phase tissues. It could also be hypothesised that disrupted communication between the lymphatic vessels and spiral arterioles caused by the decidualisation process may be a factor in the initiation of menstruation.

Menstruation incorporates both inflammatory and repair process such as tissue oedema, inflammatory cell recruitment and associated inflammatory cytokine release [38-41]. Lymphangiogenesis is known to occur during inflammation and wound healing, and also facilitates the resolution of tissue oedema [11,15]. Lymphatics are involved in clearing leukocytes from areas of resolving inflammation. Whether endometrial lymphatics are involved in clearing leukocytes and menstrual debris from the endometrium is not known (but see discussion of endometriosis below). It could be hypothesised that the inability of endometrium to efficiently clear tissue debris, particularly from areas of decidualised cells, could partially explain the need for menstrual shedding rather than remodelling. However,

the observation that the rodent endometrium regularly remodels despite the lack of lymphatic vessels does not support this hypothesis.

4. There is overlap and interaction between the signalling pathways regulating blood and lymphatic vessel growth.

The endometrium is unusual amongst adult tissues in undergoing repeated cycles of growth and tissue breakdown. This means that both the endometrial blood and lymphatic vasculature is constantly remodelling through the menstrual cycle [4-8]. After the functional layer is shed at menstruation, the blood vessels and presumably lymphatic vessels remaining in basal layer tissues must be repaired. During the subsequent proliferative phase, the blood and lymphatic vasculature develops rapidly as the functional layer re-grows. During the secretory phase, the specialised spiral arterioles grow and coil and the sub-epithelial capillary plexus matures. How the interaction between lymphatics and spiral arterioles develops has not been considered.

Vascular remodelling is regulated by a large suite of factors acting directly and indirectly on blood and lymphatic vessels. There is considerable overlap in the regulatory factors responsible for blood versus lymphatic vessel growth and many of the signalling pathways are shared [42]. It is also worth noting that many of the vascular signalling pathways are shared with those regulating nerve growth [43-45]. It is interesting to speculate on the regulatory mechanisms present in the endometrium considering this tissue contains a restricted population of both nerves and lymphatics. If we are to progress our understanding of endometrial vascular remodelling, our research needs to take a broader perspective addressing these interacting regulatory mechanisms.

4.1   VEGF-C and VEGF-D are key regulatory proteins

It is beyond the scope of this review to discuss in detail the multitude of factors interacting to regulate blood and lymphatic endometrial vascular growth. Instead we will focus on Vascular Endothelial Growth Factor (VEGF) family members VEGFC and VEGFD (c-FOS induced growth factor/FIGF) and their associated signalling pathways. The VEGF family members, which also include VEGFA and placental growth factor, are key regulators of vascular growth and function. The family members have variable interactions with the VEGF receptors VEGR1 (FLT1), VEGFR2 (FLK1, KDR) and VEGFR3 (FLT4), as well as co-receptors such as the axon guidance receptors neuropilin (NRP) 1 and NRP2.

VEGFC and VEGFD are best known for their roles in lymphatic vascular growth and function; however, both proteins are also regulators of blood vessel growth [11,46-48]. VEGFC and VEGFD interact with receptors VEGFR3 and VEGFR2, which are found predominantly on lymphatic and blood endothelial cells, respectively. Unlike other VEGF family members, VEGFC and VEGFD are produced with pro-peptides at the N- and C-termini of the conserved VEGF homology domain. Proteolytic cleavage of these pro-peptides results in multiple smaller peptides with increasing affinity for VEGFR2 and VEGFR3.

Although studies have shown that both VEGFC and VEGFD are expressed by the endometrium, there have been few mechanistic studies considering how these growth factors function in uterine tissues. Studies to date have reported variable levels of VEGFC and VEGFD immunostaining in the vasculature, stroma and epithelial tissues of human

endometrium [23,49-52] and low levels of constitutively expressed VEGFC mRNA in endometrial biopsies [53]. Studies have also shown that various different VEGFC (58, 41, 31and 21 kDa) and VEGFD (56, 41, 31 and 21 kDa) peptides are present in the human endometrium [23]. These different peptides include full length, partially processed and fully processed forms illustrating that both VEGFC and VEGFD are produced and processed within the endometrium.

Of note, no differences in immunostaining of either VEGF-C or VEGF-D were noted between functional and basal layers of the endometrium, despite the reduced density of lymphatic vessels in the functional layer [23]. The regulatory mechanisms responsible for the differential lymphatic distribution will need further consideration. Studies will need to include analysis of the expression and function of specific VEGFC and VEGFD peptides in different cell types/layers of the endometrium and at different stages of the menstrual cycle, which will have consequences for interactions of these factors with endometrial VEGF receptors and co-receptors. The need for attention of specific isoforms/peptides/splice variants and their regulation in different cell types is illustrated by studies examining VegfA isoform expression in mouse uterus. The different VEGFA splice variants have variable interactions with heparin, the extracellular matrix, and VEGF receptors [54-57]. While $VEGFA_{121}$ is freely soluble, $VEGFA_{145}$ and $VEGFA_{188}$ are bound to the extracellular matrix. The common isoform $VEGFA_{165}$ has intermediate properties. In mouse uterus, the various isoforms are differentially regulated. For instance, $VegfA_{120}$ and $VegfA_{164}$ mRNA significantly increase in uterine tissues in early pregnant mouse uterus (prior to implantation); however, there was no significant change in $VegfA_{188}$ mRNA [58]. In addition, $VegfA_{188}$ protein expression was consistently higher than other isoforms. VegfA splice variant expression was also examined in the epithelium, muscle and stroma of mouse uterus in

response to estradiol-17β using laser capture microscopy [59]. Whereas stromal and myometrial VegfA$_{188}$ mRNA expression were significantly reduced in E2-treated mice relative to controls (24 hours post treatment), epithelial VegfA$_{188}$ mRNA expression had increased. Similarly, VegfA$_{120}$ decreased in endometrial stroma and myometrium, but remained unchanged in the epithelium. Unlike the splice variants of VEGFA, however, which can be examined by analysing mRNA expression, the processing of VEGFC and VEGFD occurs post-translation necessitating analysis of multiple closely-related peptides.

Consistent with studies examining VEGFA, the strongest VEGFC and VEGFD immunoreactivity is observed in the endometrial epithelium. Decidualised cells also have an epithelial like phenotype, which may help explain why lymphatics do not grow in this tissue. Most epithelial VEGFA is secreted into the uterine lumen [60] and recent studies have suggested that VEGFA secreted from the endometrium is involved in implantation [61]. Based on co-culture studies with endometrial epithelial, stromal and microvascular endothelial cells, there are also potential paracrine effects of VEGFA on vascular remodelling. Whether VEGFC or VEGFD have similar roles has not yet been explored. Decidualised cells have an increased expression of VEGFD protein relative to non-differentiated stromal cells, particularly of the biologically active 21 kD form [28]. No such increase in VEGFC protein expression was observed. Whether increased expression of decidual VEGFD is responsible for the extensive lymphatic vasculature in the underlying hypersecretory zone of the placental-bed endometrium is unknown. Growth factors such as VEGFC and VEGFD are also produced by extra-villous trophoblasts [62-65] and leukocytes such as uterine natural killer cells [66,67]; roles for these growth factors in the facilitation of immune tolerance and in the endometrial vascular remodelling associated with the menstrual cycle and pregnancy have been postulated.

It is difficult to understand why a highly vascularised tissue with constitutive expression of VEGFC and VEGFD might have a reduced lymphatic vessel density, particularly as the luminal and glandular epithelium also express these growth factors. This question is further illustrated by the mouse uterus, which largely lacks endometrial lymphatics despite expressing VEGFD [68]. [To our knowledge, VEGFC expression has not been examined in the rodent uterus]. VEGFD immunoexpression was observed in the epithelium, stroma and myometrium of mouse uterus, although expression was generally low. In a mechanistic study, administration of VEGFD over-expressing 293EBNA tumour cells did not induce growth of lymphatic vessels into the mouse endometrium [68], although increased proliferation and vessel size of myometrial lymphatics was observed. In addition, there was increased proliferation and vessel size of endometrial, but not myometrial, blood vessels. The functional roles of VEGFC and VEGFD in endometrial vascular remodelling warrants further investigation.

4.2   VEGFC and VEGFD can interact with both VEGFR2 and VEGFR3

VEGFC and VEGFD are capable of activating several different signalling pathways [7,48,54, 69-70]. They bind with and activate VEGFR3, which is found predominantly on lymphatic endothelial cells. Proteolytic processing of VEGFC and VEGFD also allows binding with VEGFR2, which is found on blood endothelial cells as well as a subpopulation of lymphatic endothelial cells. VEGFR2 and VEGFR3 form homodimers, but are also capable of forming heterodimers; whether specific signalling pathways are associated with heterodimers is not clear. VEGFC and VEGFD are also able to bind with the co-receptor neuropilin 2 (NRP2).

In combination, therefore, VEGFC and VEGFD are capable of impacting on both angiogenesis and lymphangiogenesis.

As recently reviewed [7], VEGFR2, VEGFR3 and NRP2 are all expressed by endometrial tissues, particularly by the endometrial vasculature, and their expression is hormonally regulated [50-52,71-80]. However, studies have yet to examine the types and patterns of endometrial VEGFC and VEGFD receptor binding during the menstrual cycle. Information about the downstream signalling initiated by VEGFC and VEGFD (VEGFR2: phospholipase C (PLC) g/protein kinase C (PKC) pathway, RAF–MEK–MAP-kinase cascade, phosphoinositide 3-kinase/AKT pathway; VEGFR3: PLC g/PKC pathway; see [70, 81-82] and references therein) in endometrial tissues is also lacking.

4.3   Numerous other regulatory factors impact on lymphatic growth and function

In addition to VEGFC and VEGFD, there are numerous other factors known to be involved in lymphangiogenesis and the regulation of lymphatic function. An example is the calcitonin gene peptide superfamily member adrenomedullin and its G protein-coupled receptor calcitonin receptor-like receptor (CALCRL). Adrenomedullin has a number of biological functions in various tissues including promotion of cellular growth and survival, vasodilation, and regulation of blood and lymphatic vessel growth [83]. A recent study demonstrated that adrenomedullin expression peaks at the time of menstruation in human endometrium [84]. Using endometrial cell culture and explant studies, endometrial adrenomedullin expression was shown to be regulated by hypoxia (mediated by HIF-1α) and prostaglandin pathways. The receptor CALCRL was expressed by both blood and lymphatic endothelial cells; antagonism of CALCRL abolished PGF2α-induced blood and lymphatic endothelial cell

proliferation and tube formation *in vitro*. Other lymphatic regulatory factors, such as the angiopoietin-TIE (Tyr kinase with Ig and epidermal growth factor homology domains, or TEK) system, are known to be expressed by the endometrium [85-88], but their role in regulating endometrial lymphangiogenesis is not known. Still others, such as the ephrins and their Eph tyrosine kinase receptors, or the forkhead transcription factor FoxC2 (involved in lymphatic vessel valve development; [48]), have not been examined in endometrial tissues. It is clear that our understanding of the mechanisms regulating endometrial lymphatic vessel growth is still in its infancy.

5. Contribution of Lymphatics to Endometrial Dysfunction

Little is known about the mechanisms regulating growth and function of endometrial lymphatic vessels. Even less is known about the potential contribution of endometrial lymphatics to various gynaecological pathologies, or the role played by various growth factors and regulatory proteins which impact on lymphatic growth. Not surprisingly, it is endometrial cancer that has received the most attention. The lymphatic vasculature is a well known conduit for cancer, with cells moving from primary tumours to form secondary tumours in lymph nodes or distant tissues. This is not a passive process; extensive research now illustrates the interaction of tumour cells with local lymphatic vessels as part of the metastatic process (See [10-11,13,15] and references therein).

The uterine lymphatic vasculature is also known to be important in the progression of endometrial cancer. In endometrial adenocarcinoma, lymphatic vessel density was significantly increased peri-tumour in the basal layer relative to normal basal layer and myometrium [23]. Other studies report that increased lymphatic vessel density in the area

around endometrial tumours is a marker of higher grade tumours with less favourable prognosis [89-90]. Another feature of endometrial adenocarcinoma is lymphovascular space invasion [91-94]. Donoghue et al. [23] observed lymphatic vessel space invasion in all high grade tumours they examined. Vessels associated with tumours exhibited mixed blood and lymphatic vessel phenotype. This was also true of vessels affected by lymphovascular space invasion; some vessels contained erythrocytes suggesting potential blood vessel origin. Whether vessels affected by lymphovascular space invasion were originally blood or lymphatic vessels was not further investigated in this study. VEGFC and VEGFD expression has been observed in several reproductive tract cancers and is associated with lymph node metastasis [95]. In endometrial adenocarcinoma, VEGFC and VEGFD protein expression was increased in tumour relative to normal cycling endometrium [23]. The increased expression of VEGF-D is also thought to be an independent prognostic factor for progression of endometrial cancer and may also predict invasion into the myometrium and lymph node metastasis [52]. VEGF-C expression was also associated with metastasis of endometrial adenocarcinoma, but not with surgical stage or histological grade [96]. In a mouse model of endometrial cancer, lymph node metastasis was increased in mice receiving intrauterine injections of HEC1A cells over-expressing VEGFC in comparison to animals that received untreated HEC1A cells [97].

There is also a hypothesised role for lymphatics in endometriosis [98]. It was suggested by Sampson [99-100] that endometrial cells may be transported around the body via lymphatic or blood vessels, which may account for the presence of endometriotic lesions in distant sites such as the brain or lungs. However, these sites of endometriosis are rare and it is thought unlikely that vascular transport is responsible for most endometriosis. Recent studies also suggest that regional lymph node involvement is common in women with endometriosis with

endometriotic foci identified in mesorectal and pelvic sentinel lymph nodes (eg. [101-103] and references therein). It is interesting to note that lymphatic vessels grow into peritoneal and deep infiltrating endometriotic lesions and that the density of lymphatics is higher in the lesions than in surrounding peritoneal tissues [104-105]. VEGFC and VEGFD immunoexpression has also been reported in lesions with the greatest expression in ectopic epithelium relative to stroma [104-105]. It has been suggested that the lymphatic vessels growing into endometriotic lesions may provide the route for dissemination of endometrial cells to lymph nodes. The endometriotic cells transported to lymph nodes may then be a source for disease reoccurrence [106]. However, if this is the case, how are endometriotic cells returned to the peritoneal cavity from the lymphatics and lymph nodes, and why are lesions not found more frequently in distant sites away from the peritoneal cavity?

Progestin-only therapies provide effective, safe and cheap forms of contraception, as well as being used in the treatment of various different gynaecological disorders [107-109]. However, progestin use is associated with irregular endometrial bleeding that is not part of the normal menstrual process (breakthrough bleeding, [110-111]); this symptom is the leading reason why women chose to discontinue use of these therapies. There are a series of morphological changes in progestin-exposed endometrium, which include pseudo-decidualisation, epithelial regression and development of a population of abnormally large, irregular shaped thin walled vessels [112-118]. It has been postulated that these dilated thin-walled vessels are more fragile and may contribute to breakthrough bleeding [119]. However, the mechanisms responsible for the appearance of these large vessels are not known. In a recent study, the population of large, dilated thin-walled vessels characteristic of endometrium from progestin-treated endometrium was found to include both blood and lymphatic vessels [28]. While it is unlikely that lymphatic vessels contribute to breakthrough

bleeding, these observations raise important questions about the mechanisms responsible for abnormal changes in <u>both</u> blood and lymphatic vessels.

VEGFD can cause increases in both blood and lymphatic vessel diameters. In previous studies examining rabbit hind limb skeletal muscle, VEGFA caused enlargement of blood microvessels. However, the proteolytically processed 21kD isoform of VEGFD caused enlargement of both blood and lymphatic vessels. Vessel enlargement in response to VEGFD has also been demonstrated in mouse hind limb [23,31], rat cremaster muscle [32], pig heart [33], mouse skin [34] and mouse uterine horn [35]. Increased VEGF-D immunoexpression occurs in the decidualised cells from progestin-treated endometrium. Increased VEGFD protein expression, particularly of the proteolytically processed 21kD isoform, was also observed in decidualised stromal cells *in vitro* relative to non-decidualised stromal cells. In a xenograft model in which human uterine samples were grafted under the skin of NOD SCID mice, progestin treatment caused pre-decidual changes, increased VEGFD expression and vessel dilation. In combination, these observations suggest that VEGFD produced by decidualising cells may be responsible for the abnormally dilated vessels present in progestin-treated endometrium. However, the link between dilated vessels and breakthrough bleeding remains elusive.

Whether lymphatic growth factors, or the lymphatic vessels themselves, contribute to various other types of abnormal uterine bleeding have still to be investigated in any detail. However, increased immunoreactivity for VEGFR2 and VEGFR3 has been reported in endometrium from women with non-structural heavy menstrual bleeding [76,120]. There is also the potential that VEGFC and VEGFD impact on the various vascular abnormalities associated with pregnancy (eg: pre-eclampsia, recurrent miscarriage, preterm birth). As discussed

above, VEGFC and VEGFD are known to be expressed variously by uterine natural killer cells, decidualised cells and cytotrophoblast and likely impact on spiral arteriole remodelling and other endometrial changes required for successful pregnancy. Pregnancy-related disorders will not be discussed further here, but readers are directed to the recent review by Andraweera [121], which discusses current information about the role of VEGF family members and their receptors in various pregnancy disorders.   It is clear that lymphangiogenic growth factors have the potential to impact on both endometrial blood and lymphatic vessels. Investigations of the regulatory activity of these growth factors may identity additional pathways useful in the treatment of key gynaecological and obstetric disorders.

6.   Conclusion

An appropriately developed endometrial vasculature is essential for normal endometrial function through the menstrual cycle and in pregnancy. The importance of the dynamic endometrial blood vasculature has long been recognised, but our knowledge of the lymphatic vessels in this tissue is still in its infancy. Considerable effort is still required to understand the functional roles of lymphatics within the endometrium, and the regulatory mechanisms that control their growth and activity. The restricted lymphatic distribution in functional versus basal endometrial layers has now been illustrated, as has the lack of lymphatics in areas of decidualised endometrium. However, the functional consequences of these differential distributions are not known. The regulatory pathways responsible for lymphatic and blood vessel growth interact and there are commonalities in signalling pathways. The roles of lymphangiogenic growth factors VEGFC and VEGFD, and their downstream signalling pathways, require further examination. A role for VEGFD has already been postulated in the development of abnormally dilated blood and lymphatic vessels in

progestin-treated endometrium. Whether VEGFC and VEGFD and associated downstream

signalling molecules contribute to other gynaecological and obstetric disorders warrants

investigation.

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Table 1 Summary of postulated roles of lymphatic vessels in human endometrium: consequences of reduced lymphatic distribution

Main functions of lymphatic vessels throughout body

- Maintenance of fluid homeostasis: removal of excess protein-rich fluid from tissues via lymphatic vessels for return to blood circulation.

- Immune surveillance: transport of both soluble antigens and antigen presenting cells from peripheral tissues to lymph nodes.

Human endometrium during menstrual cycle

- Reduced lymphatic density may explain oedema characteristic of cycling endometrium.

- Role in infection prevention during the menstrual cycle?

- Potential interaction with spiral arterioles: role in vasoconstriction or dilation of endometrial blood vessels?

- Involved in clearing leukocytes and menstrual debris during menstruation?

- Role in sperm tolerance?

Human endometrium during pregnancy

- Reduced endometrial lymphatic drainage may facilitate establishment of pregnancy and survival of fetal allograft?

- Reduced distribution of lymphatics in decidua (versus hypersecretory zone) may contribute to tolerance and survival of fetal allograft?

- Extravillous trophoblasts in close proximity to lymphatics of hypersecretory zone: potential interaction?

- The functional consequences of lack of decidual lymphatics on fluid homeostasis in decidua and fetal membranes are unknown.

Figure Captions

<u>Fig 1</u>

Photomicrographs illustrating the distribution of lymphatic vessels in human endometrium during the menstrual cycle (A-C) and pregnancy (D-G). During the menstrual cycle, there is a reduced density of lymphatic vessels in the functional layer (A) of the endometrium relative to the basal layer (B) and myometrium (C). (A-C) Blue colour illustrates lymphatic vessels immunostained with an antibody against podoplanin (D2-40). During pregnancy, there are a large number of lymphatic vessels in the (D-E) hypersecretory zone of the human endometrium (placental bed). Notice the close association of lymphatic vessels with the wall of a spiral arteriole (D, black arrows). (D-E) Brown colour illustrates lymphatic vessels immunostained with an antibody against podoplanin (D2-40); blue colour illustrates blood vessels immunostained with an antibody against the pan-endothelial cell marker CD31. In contrast, lymphatic vessels are largely absence from the hypersecretory zone (F-G). (F) Blue colour illustrates blood vessels immunostained with an antibody against CD31. (G) Blue colour illustrates vascular smooth muscle cells immunostained with an antibody against α-smooth muscle actin.