THE DEPARTMENTS
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IDIOPATHIC MENORRHAGIA: STUDIES OF ANGIOGENESIS AND SURGICAL THERAPY

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Du ska tacka dina gudar,
om de tvingar dig att gå
där du inga fotspår
har att lita på.

Den som tvingas ut i vildskog
ser med nyfödd syn på allt,
och han smakar tacksam
livets bröd och salt.

Du ska tacka dina gudar,
när de bryter bort ditt skal.
Verklighet och kärna
Blir ditt enda val.

Karin Boye, ur ”Gömda Land”

Abstract

Background Excessive menstrual bleeding, menorrhagia (i.e. > 80 ml loss of blood) is a common gynecological problem in women of reproductive age, accounting for over 20% of visits to gynecology outpatient clinics. The disorder may not only cause iron deficiency anemia but also considerable social discomfort and reduction in the quality of life.

Although commonly associated with fibroids and carcinoma, approximately 50% of patients with menorrhagia do not show any evidence of uterine pathology. This suggests a defect in the cellular processes and regulatory mechanisms of menstruation.

Historically, many women with heavy menstrual bleeding were advised to undergo hysterectomy, which was the only way of enduring a "cure". Hysterectomy is an effective treatment of menorrhagia, but it is associated with substantial postoperative morbidity and convalescence. In the early 1990s, endometrial resection or ablation became a well-established outpatient alternative for the surgical treatment of menorrhagia.

The aim of this thesis This work has mainly been focused on two aspects: firstly, the analysis of transcervical resection of the endometrium (TCRE) as a surgical option for treatment of menorrhagia and, secondly, on the involvement of the vascular endothelial growth factor (VEGF) family in the regulation of angiogenesis in the human endometrium in healthy women and those with idiopathic menorrhagia. In particular, we investigated if the vessel wall anatomy was abnormal and related findings to the expression of VEGF and VEGF receptors in the blood vessels.

Results The general clinical outcome in the present studies (*papers I and II*) showed favorable results with low peroperative and postoperative complication rates: fluid overload occurred in 4% and perforation in 1% of the patients.

Most of women who underwent TCRE found this surgery acceptable and approximately 80% of these women have avoided hysterectomy. Second-look hysteroscopy in women after TCRE showed signs of regenerative endometrium.

In order to determine why the endometrium regenerates and what regulates this process, we have investigated the expression and distribution of VEGF and its receptors as well as vessel morphology in normal and menorrhagic endometrium (*papers III-V*).

Our data suggest an up-regulation of the agonist-receptor pathway of VEGF in idiopathic menorrhagia: the vascular expression of VEGF-A, VEGFR1, -2, -3 in capillaries was 1.8-, 1.8-, 2.0-, and 1. 6-fold higher, respectively, in the menorrhagia group.

Since VEGF-A not only stimulates migration and survival of endothelial cells but also induces vascular permeability, we have addressed this aspect by analyzing of vessel morphology.

We found that vessels in patients with menorrhagia displayed an unusual morphology with focal regions, gaps. The relative size of the gaps was significantly larger in menorrhagia samples than in controls (P=0.000002). Moreover, the sizes of the gaps correlated positively to the number of endometrial blood vessels expressing VEGF-A (P=0.0002) and VEGFR1 (P=0.03).

To our knowledge, this is the first study that demonstrates the presence of endothelial gaps in menorrhagic endometrium and as a part of a specific disease process.

Conclusions TCRE provides a minimally invasive technique for treatment of menorrhagia with good clinical results: about 80% of women have the possibility of avoiding hysterectomy. Therefore, endometrial resection/ablation should be offered as a surgical option to all women with idiopathic menorrhagia who have completed their families.

Normal endometrial angiogenesis is perturbed in idiopathic menorrhagia with an up-regulation of the agonist-receptor pathway of VEGF-A, which leads to anatomical differences in blood vessels, manifested inter alia as gaps.

Our novel observations may be of significance in order to explain some of the underlying mechanisms that contribute to idiopathic menorrhagia and will provide novel opportunities for therapeutic intervention in the future.

Key words: menorrhagia, TCRE, endometrium, angiogenesis, VEGF, VEGFR1, -2, -3, gaps.

List of publications

I. Miriam Mints, Arne Rådestad, Eva Rylander
Follow up of hysteroscopic surgery for menorrhagia
Acta Obstet Gynecol Scand 1998; 77: 435-438

II. Miriam Mints, Harald Almström, Eva Rylander, Arne Rådestad
Ultrasonographic and hysteroscopic follow up after transcervical resection of the endometrium
Gynaecological Endoscopy 1999; 8: 213-217

III. Miriam Mints, Bo Blomgren, Christian Falconer, Jan Palmblad
Expression of the vascular endothelial growth factor (VEGF) family in human endometrial blood vessels
J Clin Lab Invest 2002; 62: 167-176

IV. Miriam Mints, Bo Blomgren, Christian Falconer, Aino Fianu-Jonasson, Jan Palmblad
Vascular endothelial growth factor-A and its receptors in endometrial blood vessels in menorrhagia
Manuscript

V. Miriam Mints, Eva Zetterberg, Bo Blomgren, Christian Falconer, Rick Rogers, Jan Palmblad
Vascular abnormalities in the endometrium of menorrhagia patients
Manuscript

Contents

Abbreviations

Ang-1	angiopoetin 1
Ang-2	angiopoetin 2
bFGF	basic fibroblast growth factor
BL	basal lamina
DUB	dysfunctional uterine bleeding
Ebaf	endometrial bleeding associated factor
EC	endothelial cells
EGF	epidermal growth factor
EG-VEGF	endocrine-gland-derived vascular endothelial growth factor
ELA	endometrial laser ablation
ELISA	enzyme-linked immunosorbent assay
EPC	endothelial progenitor cells
ER	estrogen receptors
ET-1	endothelin-1
FEAT	first-generation endometrial ablation techniques
FGFs	fibroblast growth factors
GnRh	gonadotropin-releasing hormone
HIF-1a	hypoxia-inducible transcription factors
HPF	high-power microscopic fields
HRT	hormone replacement therapy
IGF-1	insulin-like growth factor-1
LNG-IUS	levonorgestrel intrauterine system
MISTLETOE	Minimally Invasive Surgical Techniques-Laser, EndoThermal Or Endoresection survey
MMPs	matrix metalloproteinases
MVD	microvascular density
NK	natural killer
NOS	nitric oxide synthase
NRP-1	neuropilin
NSAIDs	non-steroidal anti-inflammatory drugs
PAI-1	plasminogen activator inhibitor
PBAC	pictorial blood loss assessment charts
PC	pericytes
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PECAM-1	platelet endothelial cell adhesion molecule
PGs	prostaglandins
PlGF	placenta growth factor
PR	progesterone receptors
SEAT	second-generation endometrial ablation technologies
SMA	smooth muscle actin
TAF	tumor angiogenesis factor
TCRE	transcervical resection of the endometrium
TF	tissue factor
TGFß	transforming growth factor ß
Tie-2	receptor tyrosine kinase
TIMPs	tissue-localized inhibitors of metalloproteinases
tPA	tissue plasminogen activator
TVS	transvaginal sonography
uPA	urokinase plasminogen activator
VE	vascular endothelial -cadherin

VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VPF	vascular permeability factor
vSMS	vascular smooth muscle cells,
VVOs	vesiculovacuolar organelles
VWF	von Willebrand factor

Introduction

1 Angiogenesis

The organization of the vascular network has fascinated scientists for more than two millennia. Aristotle was one of the first to describe it: "The system of blood vessels in the body may be compared to those of watercourses which are constructed in gardens: they start from one source, or spring, and branch off into numerous channels, and then into still more, and so on progressively, so as to carry a supply to every part of the garden."

In the embryo, the first vessels arise by *de novo* differentiation of multipotent mesenchymal cells into angioblasts, the progenitor of endothelial cells (EC). The process by which these endothelial progenitor cells (EPC) migrate and differentiate at discrete locations to assemble into endothelial cords, albeit not in the adult, is referred to as vasculogenesis[1]. Once a primitive vascular plexus has been formed by vasculogenesis, it grows and transforms into a mature and structured network through a process called angiogenesis[1].

1.1 The structure and function of blood vessels

The vasculature consists mainly of three types of cells: endothelial cells (EC), forming the inner tube, surrounded by mural cells (pericytes, PC, and vascular smooth muscle cells, vSMS) and fibroblasts[2]. Arteries and arterioles are comprised of multiple layers of vSMC, whereas veins and venules have a thin coat of mural cells. The capillary network consists only of endothelial cells (EC), linked by tight junctions, their associated basal lamina (BL) and surrounding pericytes. The latter, which coat blood vessels, serve multiple functions: modulation of blood flow and vascular permeability, regulation of the growth of blood vessels and the provision of signals to the endothelium and matrix[3]. The basal lamina (BL), which is a specialized form of extracellular matrix, containing collagen IV, laminin 8/10, perlecan, nidogen and fibronectin[4], contributes to the growth, differentiation and permeability of vessels. Since BL is distributed throughout many cells, any changes in its integrity or composition will modify the behavior of other groups of cells. Thus, disruption of the BL could contribute to endometrial vascular fragility[5].

1.2 The molecular basis of angiogenesis

Angiogenesis, the process by which new microvessels develop from existing blood vessels, has a molecular basis often characterized as a step-wise progression[6], (Figure 1). It is initiated by vasodilatation of existing vessels and an increase in vascular permeability. VEGF, transcriptionally up-regulated in part by hypoxia via HIF-1a, mediates an increase in vascular permeability, accomplished by means of redistribution of intercellular adhesion molecules, including platelet endothelial cell adhesion molecule (PECAM)-1 and vascular endothelial (VE)-cadherin, and through alterations in the structure of the cell membrane via induction of a series of kinases[7]. Increased microvascular permeability results in leakage of plasma proteins, including fibrinogen and other clotting proteins.

It is a matter of debate as to what pathways the plasma proteins and other circulating macromolecules follow in extravasating from vessels. Dvorak *et al.* postulated that in response to VEGF- A, macromolecules cross the endothelial barrier, predominantly by means of vesiculovacuolar organelles (VVOs), interconnected chains of uncoated cytoplasmic vesicles and vacuoles. These VVOs interconnect with each other and the endothelial plasma membrane by stomata which are normally closed by thin diaphragms[8].

Another group showed that macromolecules cross the cell membrane through intercellular openings, i.e. the result of loosening up intercellular bonds[9].

Extravasation is accompanied by degradation of the extracellular matrix[10], which involves an array of proteinases. Not only does this provide "room" for the migrating endothelial cells, but it also results in the liberation of growth factors, including bFGF, VEGF and insulin-like growth factor-1 (IGF-1) from cell surface glycopoteins. Matrix-metalloproteinases (MMPs) play a central role in the degradation of extracellular membranes and basement membrane structures[11].

As the physical barriers are dissolved, proliferating endothelial cells are free to migrate to distant sites. At this stage, interplay between the various forms of VEGF, angiopoetins, FGFs and their receptors is responsible for mediating angiogenesis[12]. Fibroblast growth factors stimulate endothelial cell growth and recruit mesenchymal and/or inflammatory cells, producing several angiogenic factors[13]. Furthermore, the proliferation and migration of the endothelial cells, directed in part by signalling through integrins a_v ß$_3$ and a_5 ß$_1$, PECAM-1, enhances contact with other cells[14,15].

Subsequently, the endothelial cells migrate into the tissues, where they proliferate and differentiate to form new vessels. In order to attract supporting cells, e.g. pericytes, the endothelial cells produce specific growth factors, such as platelet-derived growth factors PDGF and TGF-ß[16]. As a result, a new, highly specific basement membrane is produced. Vessels surrounded by a basement membrane and pericytes are considered mature.

The formation of more complex vessels, i.e. arterioles and venules, is mediated by angiopoetins (Figure 2)[17]. All EC in adult tissues express the receptor tyrosine kinase, Tie-2, whose cardinal function is the formation and maintenance of the vascular network during angiogenesis[18]. It has been observed that Tie-2 null mutant mice display severe vascular abnormalities and die at midgestation[19].

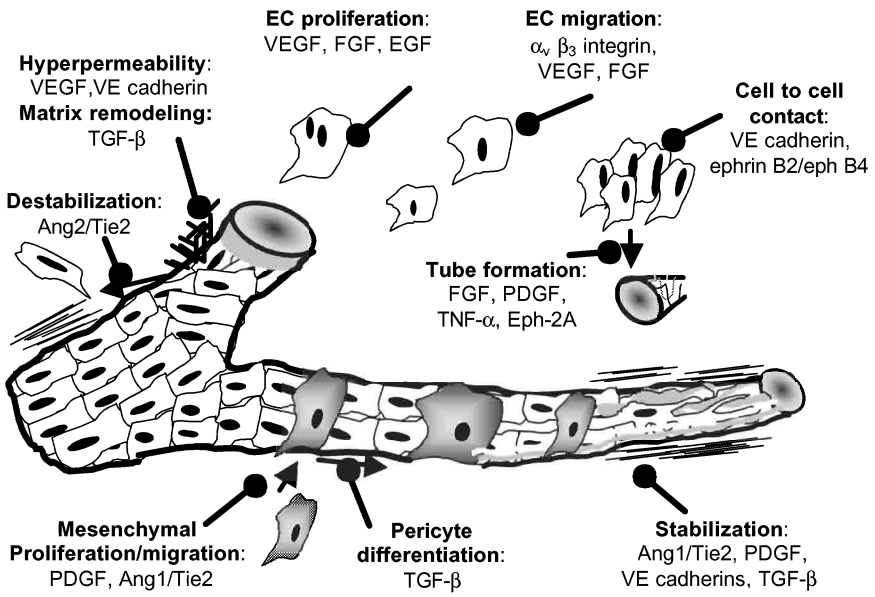

Figure 1. Mechanism of physiological angiogenesis (after ref. 6).

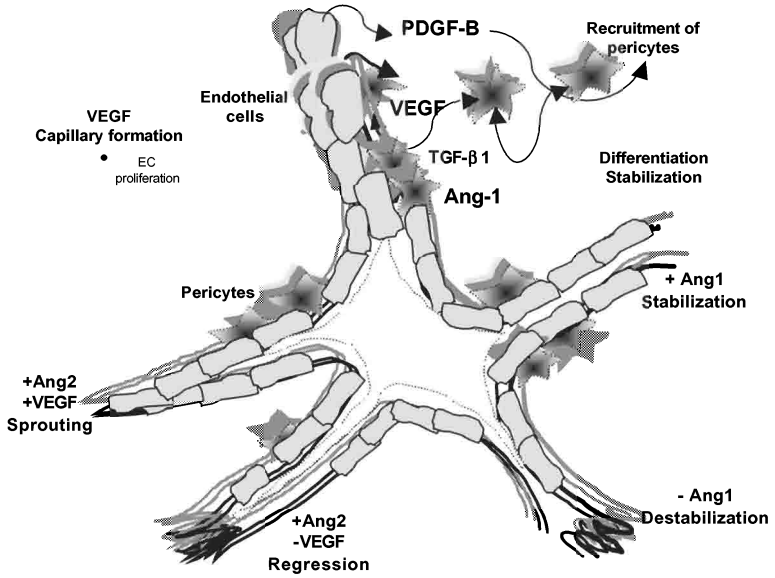

Figure 2. The multifactorial regulation of vessel assembly (after ref. 17).

The ligands Ang-1 and -2 bind to Tie-2 and are also expressed by vascular cells. It has been postulated that Ang-1 has angiogenic potential *in vitro*, but the constitutive expression *in vivo* in adult vessels would rather suggest involvement in stabilization of the vessels. Moreover, in the absence of Ang-1, vascular complexity is decreased. Thus, Ang-1 promotes the health of the blood vessels. Angiopoetin 2 (Ang-2) acts as a natural antagonist of Tie-2, but its functions remain controversial. Previous studies support a model whereby the consequence of Ang-2 stimulation, anti-or-pro-angiogenic, depends on the presence of VEGF. It was demonstrated that in the presence of VEGF-A, Ang-2 promotes a rapid increase in the diameter of the capillaries, remodelling of the basal

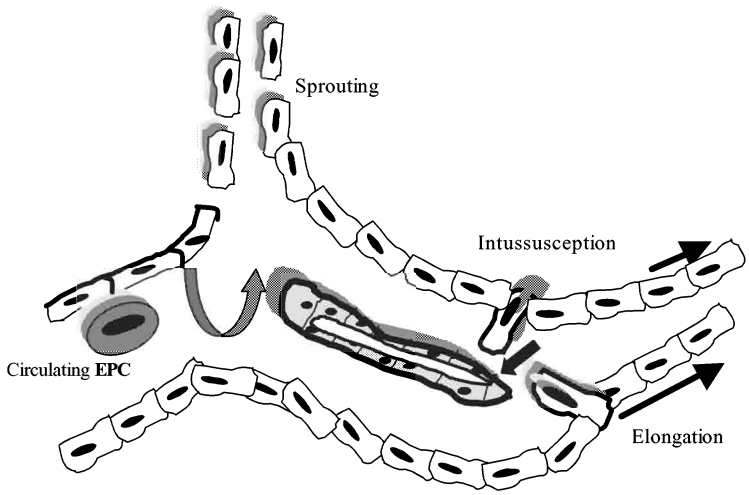

Figure 3. Adult vessel formation. The different angiogenic processes whereby vessels can be formed (after ref. 21).

lamina, proliferation and migration of EC and in sprouting of new vessels[20]. However, if the activity of VEGF is inhibited, Ang-2 induces regression of the vessels[21].

New growth of vessels can occur in the adult by four different mechanisms (Figure 3): sprouting, intussusception, elongation of vessels and by incorporation of circulating endothelial progenitor cells into growing vessels.

Sprouting angiogenesis is important when neovascularization of vascular tissue occurs, such as during the rapid growth of the corpus luteum after ovulation. Intussusception is the process in which the lumen of a vessel is divided internally into two as proliferating endothelial cells migrate inwards, producing a network of interlocking vessels or an arcade of parallel vessels, as seen in the developing lung[1]. Elongation and widening of vessels probably occur in growing tissues as existing vessels are constantly undergoing transformation in response to the metabolic demands of the surrounding cells, a process also known as remodelling. Recently, a small proportion of circulating mononuclear cells were identified as endothelial cell progenitors, which have the capacity to incorporate into growing but not quiescent vessels[22].

1.3 The mechanism of the" angiogenic switch"

In the normal adult mammal, the vasculature is quiescent, except for highly orderly processes in the female reproductive cycles. The endothelial cells are among the longest living in the body outside the central nervous system; in normal adult vessels, only one in every 10 000 endothelial cells (0.01%) is in the cycle of cell division at any given time. In a normal adult, most vasculature is quiescent, with only 0.001% of endothelial cells

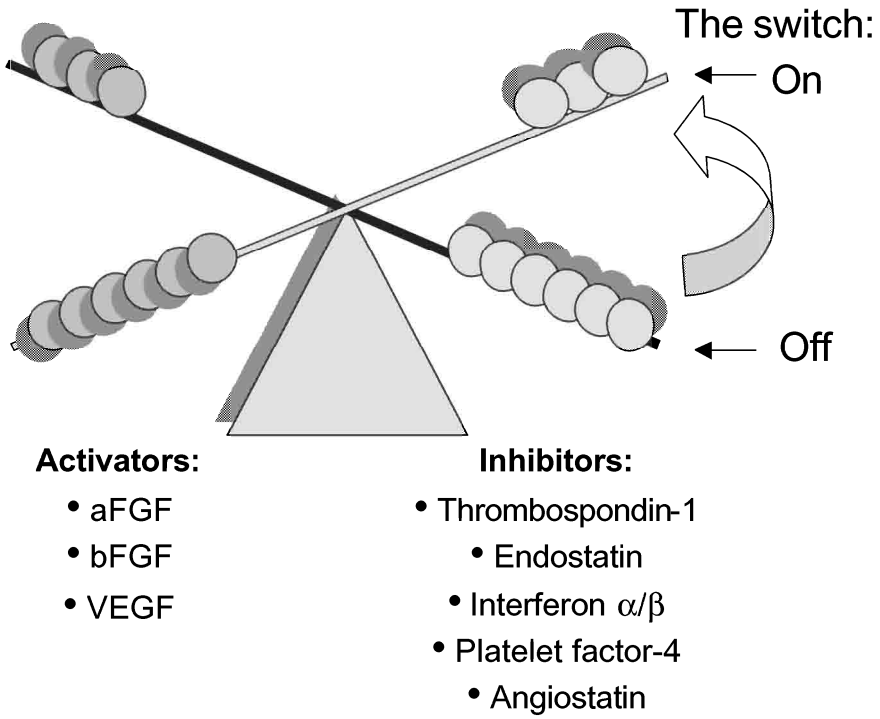

Figure 4. Judah Folkman and Douglas Hanahan hypothesized that changes in the relative balance of inducers and inhibitors of angiogenesis can activate the switch (after ref. 25)

undergoing division, presumably reflecting a process of cell turnover to maintain tissue[23]. Angiogenesis is regulated both by inducers and inhibitors of endothelial cell proliferation and migration. In response to an appropriate stimulus, the quiescent vasculature can become activated to develop new vessels.

In the early 1970`s, Judah Folkman and his colleagues at Children's Hospital Medical Centre in Boston discovered that tumours secrete a substance known as tumor angiogenesis factor, TAF[24] which induced the growth of existing vessels and infiltrated the tumor, providing it with necessary nutrients for growth. Recently, Folkman and his colleagues formulated a hypothesis in which the primary tumor can stimulate angiogenesis in its own vascular bed while inhibiting angiogenesis in the vasculature of a metastasis or secondary tumor. Even though this angiogenic inhibitor was produced by the primary tumor, it did not inhibit angiogenesis therein due to the excess of local stimulators. O`Reilly *et al.* identified this angiogenic inhibitor from the serum and urine of mice afflicted with Lewis lung carcinoma and termed it angiostatin[25]. Angiostatin is an internal fragment of plasminogen and it inhibits endothelial cell proliferation *in vitro* whereas *in vivo,* it suppresses the growth of several tumors and their metastases.

These findings led to the formulation of the hypothesis by Judah Folkman and Douglas Hanahan, in which changes in the relative balance of inducers and inhibitors of angiogenesis can activate the angiogenic switch, resulting in the growth of new blood vessels[26] (Figure 4). In recent years, it was discovered that hypoxia activates hypoxia-inducible transcription factors (HIFs), which function as master switches to induce the expression of several angiogenic factors, including VEGF, nitric oxide synthase (NOS), PDGF, Ang-2, etc. Moreover, apart from stimulating angiogenesis, hypoxia can cause vascular remodelling.

Excessive or insufficient vascular growth or abnormal remodelling contributes to numerous non-neoplastic disorders[27].

1.4 The vascular endothelial growth factor (VEGF) family

The vascular endothelial growth factors constitute a family of closely related cytokines, which play a crucial role in vasculogenesis and in both pathological and physiological angiogenesis. VEGF denotes a family of dimeric glycoproteins that belong to the platelet-derived growth factor (PDGF), the superfamily of growth factors. The VEGF family currently includes VEGF-A, -B, -C, -D, -E and the placenta growth factor (PlGF).

The original VEGF (currently known as VEGF-A) was first described as a vascular permeability factor (VPF)[8,28], but later defined as an endothelial cell mitogen[29, 30]. Intense research has established the role of VEGF-A in vascular development and as an inducer of the migration and survival of endothelial cells. The expression of VEGF-A, which has been detected in most of the organs in mice and humans, is enhanced in areas of hypoxia that are characterized by active angiogenesis, but is also expressed around quiescent microvessels, implying that VEGF-A is a survival factor for differentiated endothelium[31]. VEGF-A exists in five isoforms: 121, 145, 165, 189 or 206, each appearing to have unique biological functions. $VEGF_{165}$ is the predominant isoform, produced by a variety of normal and transformed cells. Whereas $VEGF_{121}$ is a freely released protein, the other isoforms of VEGF show an amplified affinity for heparin sulphate proteoglucans with increasing molecular weight. Although $VEGF_{165}$ is also released, a fraction thereof remains bound to the extracellular matrix; in contrast, $VEGF_{189}$ and $VEGF_{206}$ are almost exclusively sequestered in the extracellular matrix.

VEGF-B is expressed in most tissues, although most prominently in the heart, brown fat and spinal cord. VEGF-B knockout mice are viable but have small hearts, suggesting that this cytokine has a role in the development of coronary arteries[32, 33].

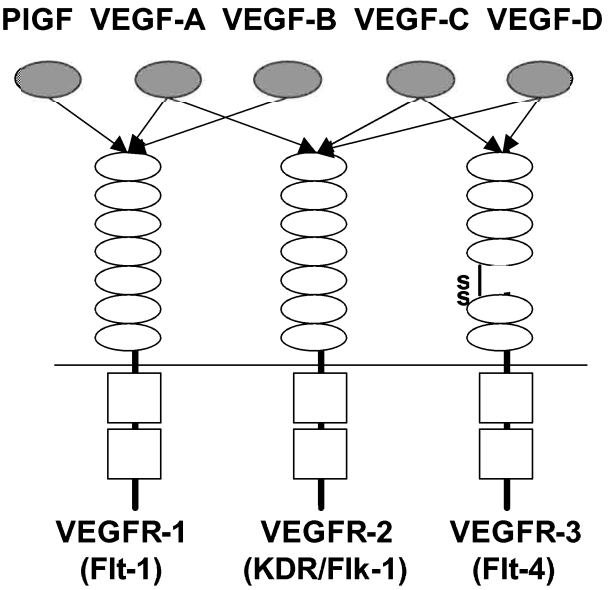

Figure 5 VEGF ligand-receptor specificity (after ref. 40).

VEGF-C, which has been detected in many tissues and is expressed in lymphatic endothelium, has a role in lymphangiogenesis. VEGF-D, prominent in the lungs and skin, is structurally related to VEGF-C and is an endothelial cell mitogen[34].

PlGF is strongly expressed in the placenta and thought to have an accessory role in pathological angiogenesis by serving to potentiate the activity of VEGF-A[35].

As shown in figure 5, the VEGF family proteins bind to three structurally related receptor tyrosine kinases denoted VEGFR1, -2, -3. Furthermore, VEGF family members bind with relatively high affinity to neuropilins[36].

Studies on gene targeting have demonstrated that both VEGFR1 and-2 are essential for the development of the embryonic vasculature in mice[37]. There is evidence that VEGFR1 and-2 have different properties of signal transduction and possibly mediate various functions[38]: VEGFR-2 mediated mitogenesis and chemotaxis in response to VEGF-A, but the signalling steps mediated through VEGFR-1 have not been characterized as explicitly. VEGFR-1 may have an independent role in stimulating cell motility and may also dampen certain signalling pathways and biological effects (e.g. cell proliferation), mediated by VEGFR-2[39,40]. It has been demonstrated that neuropilin (NRP-1) potentiates the binding of VEGF-A to VEGFR-2[36]. Recently, it has been noted that inactivation of the genes for VEGF-A and VEGFR-2 results in embryonic death due to the lack of endothelial cells. However, inactivation of the genes encoding VEGFR-1 leads to an increasing number of endothelial cells. Moreover, inactivation of VEGFR-3 results in abnormally organized vessels and subsequent cardiac failure[41].

1.5 Biological activities of the VEGF-family

The most distinctive and foremost biological activity *in vivo* of VEGF-A is increased microvascular permeability. Not only does VEGF-A induce endothelial fenestration, when the interconnections between VVO vesicles and vacuoles are fully opened, but it is also a multifunctional cytokine that has a multitude of effects on vascular endothelium, including striking changes in cell morphology and stimulation of endothelial cell mi-

gration and division. Moreover, VEGF-A serves as an endothelial cell survival factor, protecting endothelial cells against apoptosis[42]. Although acting primarily on vascular endothelium, VEGF-A also interacts with other types of cells that express VEGF receptors. Thus, VEGF-A stimulates monocyte/macrophage chemotaxis[43].

There is evidence that VEGF-B plays a role in the degradation of extracellular matrix via activation of plasminogen.

VEGF-C was shown to stimulate angiogenesis in the adult, although its endogenous role in pathology remains undefined. Its receptor, VEGFR-3, is required for vascular remodelling and angiogenesis[44].

Recent studies have confirmed the identification of an angiogenic mitogen selective for one type of endothelial cell, endocrine gland endothelium (EG-VEGF). This molecule, known as endocrine-gland-derived vascular endothelial growth factor, induces proliferation, migration and fenestration in capillary endothelial cells derived from endocrine glands[45].

How do blood vessels grow in the endometrium and what regulates them? In order to answer this question, we need to examine the various sites where this process occurs in the endometrium.

2 The endometrium

The main functions of the human endometrium are the provision of a hormone, defined as implantation window,[46,46] the ability to trigger its own destruction in the absence of pregnancy, and protection against invading pathogens. It is composed of both a basal (basalis) and functional (functionalis) layer. The former is adjacent to the myometrium and undergoes limited changes during the menstrual cycle, while the superficial layer is highly responsive to cyclic variations in estrogen and progesterone levels and is subsequently discharged at menstruation[47]. The human endometrium has a mucosal epithelial surface, comprised of surface and glandular epithelium, a heterogenic stroma and a characteristic vascular system. The stroma of human endometrium consists of fibroblasts, some macrophages and T cells[48]. Populations of large granular lymphocytes and neutrophils appear in the late secretory phase. The epithelial surface of the endometrium has a dual function in that it prepares for implantation and provides defense against infection. Furthermore, endometrial cells are the major source of several vasoactive substances, such as prostaglandins[49] and endothelins,[50] which have a role in menstruation.

2.1 Endometrial vasculature

The endometrium has a well-developed vasculature. Arterial blood reaches the uterus via the uterine and ovarian arteries (Figure 6). Basal arteries supply the basal endometrium and larger spiral arterioles, which run towards the functionalis, each supplying about 9 mm^2 of endometrium. The capillaries arising from the spiral arterioles form a subepithelial plexus, which empties into a venous plexus within the functional layer. These veins subsequently drain into the inner myometrium[51].

2.2 The menstrual cycle

Most of the data on endometrial vascular changes during the normal menstrual cycle emanates from *in vivo* studies conducted by Markee[52]. He observed that the late pre-ovulatory phase is accompanied by a five-fold increase in the length of the spiral arterioles, leading to vascular coiling. Distal spiral arterioles connect to the sub-epithelial capillary plexus. During the secretory phase, the spiral arterioles increase in size and the capillaries dilate. Two to six days before the onset of bleeding, there was shrinkage of the stromal edema and a subsequent increase in the coiling of the spiral arterioles and vascular stasis.

This was followed by a period of vasodilatation and perivascular bleeding from the wall of a capillary or arteriole, and 24 hours later, by intense vasoconstriction and tissue necrosis[53]. Approximately 70% of the blood loss occurs through vessel walls, 5% by diapedesis and 25% by reflux from veins through previous disruptions. Each bleeding episode is focal and normally lasts for only several minutes until the spiral artery constricts in the basalis[54].

A prerequisite for hemorrhage of the endometrium is the breakdown of both the endometrial blood vessels and surface epithelium. Although circulating sex steroids may exert some overall control over these vessels, local factors are likely to be of primary importance[51]. There are a number of molecular mechanisms that may be involved in the occurrence of normal uterine bleeding. In response to falling levels of progesterone, lysosomes in the premenstrual endometrium activate and release proteolytic enzymes, which could contribute to tissue breakdown, bleeding and remodelling[55]. There is also evidence that matrix metalloproteinases (MMPs) have a crucial role in the breakdown of tissue at menstruation and that together with lysosomes have the specificities to cause degradation of all of the components of both interstitial matrix and basement membranes[56]. Endometrial leukocytes are known to be involved in this process. Menstruation is preceded by a marked increase in the number of endometrial stromal granulated lymphocytes, T lymphocytes and macrophages. Moreover, studies conducted on this topic showed that polymorphonuclear leukocytes only appear in uterine tissues at the onset of tissue breakdown and are often located near blood vessels in the endometrium[57,58].

Intense research has supported a role for prostaglandins as pressors in menstruation. Baird *et al.* demonstrated that PGs occur in the endometrium and menstrual fluid in high concentrations; PGF_{2a} causes vasoconstriction, whereas PGI_2 causes vasodilatation[59]. In addition, the most potent vasoconstrictor, endothelin-1 (ET-1), is produced and released by the human endometrium and can act on epithelial and endothelial cells of the endometrium. Of further interest is the increased production of ET-1 around menstruation and that ET-1 is closely opposed to the spiral arterioles[60].

It is assumed that the cessation of bleeding occurs due to the formation of intravascular platelet fibrin plugs at the ends of the vessels facilitated by the intense vasoconstriction that occurs at this time. However, in contrast to haemostatic mechanisms in other body tissues, menstrual blood does not show persistent clotting[61].

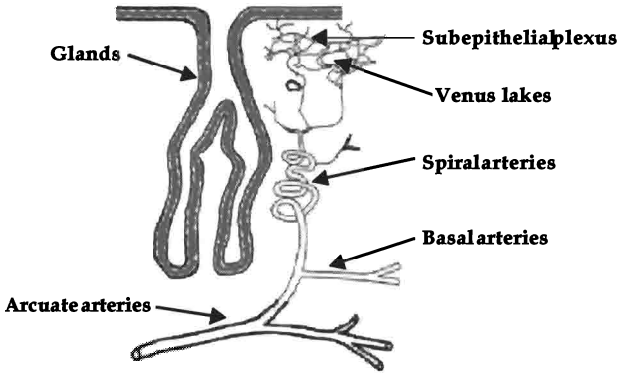

Figure 6. Vasculature of primate endometrium (after ref. 50).

2.3 Angiogenesis in the endometrium

Repair of the endometrium begins as early as 36 hours after the onset of menstrual bleeding. Within a period of 5-6 days, the old lining is removed and a new one is regenerated, without any evidence of scarring. This is a remarkable example of controlled tissue remodelling, unparalleled in other organs.

Angiogenesis is an essential component of the regeneration of the endometrium. However, the timing and mechanism of the formation of new vessels in the endometrium during the menstrual cycle are still widely unknown. Maas *et al.*, using the chorioallantoic membrane of the chick embryo as an *in vivo* assay for angiogenesis, demonstrated that the endometrium has angiogenic potential throughout the menstrual cycle[62]. Furthermore, studies by Rogers *et al.* suggested that there are three distinct stages during angiogenesis: a) repair of the vascular bed during menstruation, b) rapid endometrial growth in the proliferative phase and c) endometrial growth in the secretory stage, where spiral arterioles show significant growth and coiling[63].

The immunohistochemical identification of the a_v ß$_3$ integrin, a marker of sprouting endothelium, has been observed only within existing vessel profiles[64]. Consequently, in 2001, Gargett and Rogers hypothesized that growth of vessels in the human endometrium occurs by non-sprouting mechanisms. Specifically, during the proliferative phase, the growth of vessels in the functionalis endometrium occurs by elongation, whereas during the proliferative and early secretory stages, growth of the subepithelial capillary plexus is mediated by an intussusceptive mechanism[65]. Two peaks of endothelial cell migratory activity have been discovered: in the early and mid/late proliferative phases of the cycle[66]. However, endothelial cell proliferation shows no correlation to the stage of the cycle[67].

There is still controversy over the presence of steroid receptors in the vessels. Early immunostaining indicated the presence of estrogen (ER) and progesterone (PR) receptors in smooth muscle cells and perivascular cells of uterine vessels, but an absence thereof in endothelial cells[68]. In one particular study, estrogen receptors were identified in endometrial endothelial cells, although their type was not reported. Cultures of endometrial endothelial cells were treated with 17ß-estradiol and progesterone at the same time as the cells were treated with growth factors[69]. A recent study has confirmed the presence of PR on endothelial cells in human endometrium. Proliferation of endothelial cells in culture was down–regulated by progesterone, and this effect was blocked by the administration of RU486, a potent PR antagonist[70].

In spite of the direct effects of estrogen and progesterone on endothelial cells, the presence of numerous growth factors and cytokines in the endometrium play a major role in regulating endometrial angiogenesis. Not only does the endometrium express all of the growth factors known to induce angiogenesis, but it also expresses many of those that inhibit angiogenesis[71,72].

2.4 VEGF-A and its receptors in the endometrium

The role of VEGF as a mediator of angiogenesis during the period of menstrual repair has been investigated intensively during the last decade[73]. Immunohistochemistry and in-situ hybridization demonstrate the presence of VEGF-A in the luminal and glandular epithelium. Most studies on the expression of VEGF in the endometrium have shown that there is an increased expression in the glands compared to stroma, as well as in the secretory compared to the proliferative phase[73, 74]. However, previous studies on the presence of VEGF in the endometrial vessels have yielded contradictory results. Zhang *et al.*[75] observed a moderate staining of VEGF-A, independent of the menstrual cycle, in small blood vessels, some endothelial cells in the arterioles and in cells in the muscular wall of the arterioles at the endometrial and myometrial junction. This is further emphasized

by Lau *et al.*, who found that blood vessels were largely negative to VEGF-A staining, although approximately 25% of the samples showed varying degrees of VEGF-A staining that did not appear to be cycle specific. These authors also detected VEGF-A staining in individual stroma cells, unrelated to the menstrual cycle. A more recent study reported the presence of VEGF-A immunoreactive vessel-associated neutrophils[65].

Despite the variation in levels of VEGF in the endometrium during the menstrual cycle, there are also controversial results regarding correlations between immunostaining of VEGF and endothelial cell proliferation. According to Gargett *et al.*, neither total glandular nor total stromal production of VEGF correlated with endothelial cell proliferation[76]. However, a recent study noted a peak in endothelial cell proliferation during the late menstrual and early proliferative phases regulated by the presence of VEGF-A[77]. In addition, Nayak and Brenner demonstrated that endothelial cell proliferation was significantly correlated with the amount of stromal VEGF[78].

The presence of VEGFR1-2 in cycling human endometrium has been investigated, including by Meduri *et al.*, who found that immunostaining for VEGFR-2 was maximal in the proliferative phase with a second peak of staining occuring in the mid-secretory phase. Moreover, the authors reported that the proportion of capillaries expressing VEGFR-2 is much higher in the proliferative phase.

They also examined VEGFR-1 and found that the most intense staining occurred in the mid-secretory phase[79]. RNA for both receptors has been detected in the cycling human endometrium, and was expressed at constant levels throughout the cycle. Moreover, levels of soluble-VEGFR-1, a secreted biologically inactive VEGFR-1, were found to peak in the mid- to late proliferative phase, and were down-regulated in the secretory phase[80].

Together, these findings indicate that VEGF and its receptors contribute to the onset of angiogenesis and endothelial repair in the human endometrium.

A critical feature of menstruation is the vasoconstriction of the spiral arterioles, which precedes the onset of bleeding. Under these circumstances, the oxygen tension in the tissue declines rapidly and consequently up-regulates VEGF by hypoxia-inducible factor (HIF)-1a.

A potential additional effect of VEGF on the vasculature is that of vasodilatation. VEGF stimulates the release of nitric oxide as well as prostacyclins, another vasodilating agent, from endothelial cells, possibly modulating the contractility of the spiral arterioles and thus altering the volume of menstrual blood loss[81, 82].

Furthermore, VEGF has an effect on coagulation by increasing the expression of tissue plasminogen activator (tPA) and urokinase plasminogene activator (uPA), but this is tempered by co-induction of plasminogen activator inhibitor (PAI-1)[83].

2.5 Other growth factors in the endometrium

Endometrial *EGF* has a special function in endometrial growth, since it can itself replace estradiol and reproduce all of its effects[84,85,86,87]. A well-known mitogenic agent in endometrial tissue, EGF is expressed by both the epithelial and stromal cells of the endometrium as well as in the secretory phase, where it reportedly modulates endometrial differentiation both *in vitro* and *in vivo*[88, 89,90,91].

TGFβ 1-3 have been identified in the endometrial epithelium and stroma throughout the menstrual cycle[92,93].

In the endometrium of ovulating women, *bFGF* is present in a low concentration. It has been found in endometrial glands and is a potent mitogen for cultured endometrial stroma cells. Synergism between VEGF and bFGF may be of importance during angiogenesis[94].

3 Menorrhagia

The concept of monthly menstruation is a natural phenomenon of the modern world. In primitive civilizations, women during the reproductive lifespan would experience only a few menstrual cycles between pregnancies[95]. In contrast, women in the 20th century, who have an average of two children, have only a couple of years during their period of fertility when they do not menstruate.

The concept of "normal menstruation" was derived from population studies[96]. By carefully measuring total menstrual loss, Hallberg *et al.* found that the 90th percentile for blood loss during menstruation was 80 ml and that anemia was significantly increased in women with a loss greater than 60 ml[2, 97]. These studies have established international standards, in which ***menorrhagia*** is defined as the loss of more than 80 ml of blood per menstrual episode. However, this is of little value to the clinician, as the quantity of blood loss cannot usually be measured. In order to assess the amount of menstrual blood loss semi-quantitatively, Higham *et al.* described the use of pictorial blood loss assessment charts (PBAC). They assessed the degree of staining of each pad/tampon and the number of pads used. A pictorial chart score higher than 100 was diagnostic for menorrhagia with a specificity and sensitivity of > 80%[98].

This degree of blood loss can cause disturbances of the woman's social, occupational or sexual life, as well as medical problems, such as chronic iron deficiency anemia. Excessive bleeding is the main complaint women present with when referred to gynecologists; it accounts for over 20% of gynecology outpatient visits. In a Swedish population investigated in the years 1963 to1964, more than 11% of the women in the age range of 15-50 years suffered from menorrhagia[97]. Hence, menorrhagia is considered an important problem in healthcare[99].

A broad spectrum of conditions can be associated with menorrhagia and categorized into three main groups: a) pelvic pathology, b) systemic diseases and c) "unexplained" menorrhagia.

3.1 Pelvic pathology and menorrhagia

Fibroids

Approximately 30% of women with fibroids have been reported to have menstrual disorders, usually in the form of menorrhagia[100]. The degree of severity of menorrhagia is generally determined by the relationship of the largest myoma to the endometrium[101]. It has been suggested that excessive bleeding may be due to increased vascularity of the uterus. Prostaglandins may also contribute to menorrhagia due to a disturbance in normal myometrial contractility[102].

Endometrial polyps

Menorrhagia occurs in about 12 per cent of women with endometrial polyps. The etiology of endometrial polyps and the mechanism by which they cause excessive bleeding are unknown[103].

Endometrial carcinoma

It has been reported that about 40% of women suffering from endometrial carcinoma prior to menopause present with regular and excessive heavy periods[104].

3.2 Systemic disorders and menorrhagia

Menorrhagia is caused by systemic disorders, most frequently by coagulation disorders, and occasionally by hypothyroidism, severe hepatic cirrhosis, chronic renal diseases and systemic lupus[105].

Coagulation disorders

Twenty-five per cent of adolescents with excessively heavy periods and a hemoglobin count of less than 100 g/l, as well as one-third of those who require a blood transfusion for menorrhagia, have underlying coagulation defects[106]. Von Willebrand`s disease is the most common coagulopathy associated

with menorrhagia. Congenital hemostatic disorders, such as the coagulation deficiencies of factors V, VIII, IX, X, XI, and XIII, and severe thrombocytopenia are infrequent causes of heavy menstrual loss[107]. In 1998, Kadir *et al.* showed that 26 of 150 women (17%) with a median age of 39 years, referred for investigation of menorrhagia, were diagnosed as having an inherited bleeding disorder[108].

3.3 Idiopathic menorrhagia

Although various pathologies have been implicated in menorrhagia, in 50 percent of the cases of objective menorrhagia, no pathology is found at hysterectomy[109]. Excessive bleeding with no pelvic pathology is often termed dysfunctional uterine bleeding (DUB). The definition of DUB, which has been endorsed by the European Society for Human Reproduction and Embryology, is "excessive bleeding (excessive, heavy, prolonged or frequent) of uterine origin which is not due to demonstrable pelvic disease, complications of pregnancy or systemic disease"[110,111]. *Anovulatory DUB* is recognizable as irregular, prolonged and usually excessive bleeding, caused by a disturbance in the function of the hypothalamic-pituitary-ovarian axis. It is most commonly seen in polycystic ovary syndrome and the extremes of female reproductive life, namely the perimenarchal and perimenopausal years. However, *ovulatory DUB* or *unexplained* menorrhagia is characterized by regular episodes of heavy menstrual loss during the first three days of normal menstruation. There is no disturbance of the hypothalamic–pituitary-ovarian axis and indeed the gonadotropin and steroid hormone profiles are no different to those seen in the normal menstrual cycle[112].

In recent years, research has focused on the possible role of focal uterine factors in the control of blood loss during menstruation. In previous studies, it was observed that idiopathic menorrhagia is associated with a shift in the ratio of endometrial vasoconstricting PGF_{2a} to vasodilatory prostacyclin and PGE_2[113] and with an increase in the total endometrial concentration of PG. Endometrial tissues may be more responsive to the action of vasodilatory prostaglandins via increased concentrations of receptors in women with menorrhagia[114].

Adequate and timely vasoconstriction of the spiral arterioles is an essential component of menstrual hemostasis. Deficient production of endometrial endothelins may prolong or increase menstrual bleeding and facilitate menorrhagia and breakthrough bleeding[60].

Lysosomal enzyme activity in the endometrium intensifies in women with idiopathic menorrhagia[115].

Macrophages and other migratory leukocytes may be involved in the control of menstrual blood loss and contribute to the mechanisms of excessive blood loss. Endometrial granulated lymphocytes and natural killer (NK) cells secrete perforins, which may cause a degradation of endometrial vascular and other cellular structures and thus promote bleeding.

Mast cells degranulate premenstrually to secrete heparin, histamine, tryptase and possibly a range of other substances[56]. Heparin stimulates endometrial fibrinolysis via secretion of tissue plasminogen activator (tPA) and MMPs. In idiopathic menorrhagia, endometrial secretion of heparin-like substances is also increased[116].

The occurrence of fibrinolysis is correlated to the balance of plasminogen activators, plasminogen inhibitors and plasmin. Excessive menstrual blood loss is associated with an increase in endometrial fibrinolysis. An increased concentration of tissue plasminogen activators (tPA) has been observed in women with idiopathic menorrhagia[117]. Over-activation of the fibrinolytic system may disrupt the balance of the hemostatic system.

3.4 Pathological angiogenesis and menorrhagia

In several recent studies, it has been suggested that disorders of angiogenesis may be a feature of menorrhagia.

In 1996, Kooy *et al.* observed an increase in endothelial cell proliferation in the endometrium in patients with menorrhagia compared to the endometrium in the controls[118]. A recent study demonstrated that the proliferative index of endothelial cells is higher in women with excessive menstrual blood loss. However, within the same group, there were no differences in vascular smooth muscle proliferation between women with menorrhagia and those with a normal blood loss[119].

Changes in superficial endometrial vascular morphology have been observed in women using progesterone contraceptives. Vascular dilatation, subepithelial bleeding, and neovascular formations, as seen at hysteroscopy, suggest that vascular growth and development are altered in these women[120]. Rogers *et al.* demonstrated that perivascular smooth muscle alpha actin is reduced around the endometrial vessels in women who use Norplant and suffer from menorrhagia compared to those with no bleeding problems[121]. Furthermore, abnormal expression and activation of MMMs and their tissue inhibitors (TIMPs) has been observed in the endometrium of these women with increased activity at the start of bleeding episodes[122,123].

Recently it has emerged that angiopoetins may coordinate angiogenesis by providing signals that result in the stabilization of newly formed blood vessels. Hewett *et al.* demonstrated that in the endometrium of patients suffering from menorrhagia, there was a marked down-regulation of both Ang-1 mRNA and protein while the expression of Ang-2 increased or remained at similar levels, resulting in a substantial decrease in the ratio of Ang-1 to Ang-2[124].

However, it is unclear how often combinations of any or all of these abnormalities occur in the same women. The overall effect of these vascular changes, together with a greater influx and activity of local molecules, may have the potential to break down vessels. In conjunction with the hemostatic mechanism, this may induce and perpetuate menorrhagia.

Recent research has also begun to focus on the activation of specific genes that may be associated with abnormal uterine bleeding. A novel gene called *"endometrial bleeding associated factor" (ebaf)* has recently been demonstrated to be transient in the endometrium during normal menstruation, and much more strongly expressed when bleeding is abnormal[125]. This gene is located on chromosome 1 and codes for a member of the TGFß superfamily. Its exact function is not clear. The concept of multigene activation patterns is currently being explored as a possible explanation for complex functional disturbances of idiopathic menorrhagia[126].

4 Treatment of idiopathic menorrhagia

The aims of therapy are to decrease blood loss, reduce the risk of anemia and improve the quality of life.

4.1 Medical treatment

Medical treatments for menorrhagia can be divided into two main classes: non-hormonal and hormonal treatment.

Non-hormonal treatment

Non-steroidal anti-inflammatory drugs (NSAIDs). The discovery of the relationship of endomyometrial prostaglandins to the genesis of menorrhagia has provided an opportunity to evaluate therapy with cyclooxygenase inhibitors. NSAIDs reduce levels of endometrial prostaglandins by inhibiting cyclooxygenase, the enzyme largely responsible for conversion of arachidonic acid to prostaglandins[127]. A reduction in menstrual loss has been documented and depending on the agent and dosage used,

varied from 25 to 47%[128]. Another beneficial effect is that these drugs alleviate menstrual pain.

Antifibrinolytics. Tranexamic acid, a synthetic derivate of the amino acid lysine, exerts its antifibrinolytic effects through the reversible blockade of plasmin[129] and thereby reduces menstrual blood loss by up to 50%[130]. Comparative studies have shown tranexamic acid to be superior to NSAIDs in reducing menstrual blood loss[131,132].

Hormonal treatment

Among methods of hormonal treatment, the levonorgestrel intrauterine system (LNG-IUS) (Mirena, Schering) will be named. It is superior to oral progestogens in reducing menstrual bleeding and reduces menstrual blood loss by up to 96%[133], [134]. Twenty percent of women using this device are reported to be amenorrheic after one year[135]. Furthermore, the LNG-IUS has been compared to transcervical resection of the endometrium (TCRE) and resulted in a smaller mean reduction in menstrual blood loss than TCRE (67% and 90% respectively). However, the LNG-IUS is reversible and has no operative hazards[136]. In addition, the LNG-IUS was evaluated in comparison to hysterectomy. In a trial from Finland, a cohort of 56 women scheduled for hysterectomy was randomized for continuation of their medical treatment or levonorgestrel-releasing IUD. In the latter group, 64% of the women decided to cancel surgery compared to 14 % in the medical group[134]. In conclusion, LNG-IUS can now be considered an alternative to both traditional medical and surgical management of essential menorrhagia since it preserves fertility (while providing contraception), reduces morbidity and is more economical than surgery[137].

4.2 Surgical treatment of menorrhagia

Surgery is indicated when the patient does not respond to or tolerate medical treatment or when it is a matter of choice.

4.3 Hysterectomy

Hysterectomy is the most widely performed, major gynecological operation: approximately 20% of the female population have undergone the procedure by 60 years of age, about 40% for menorrhagia with no gynecological pathology[138].

The effectiveness of hysterectomy in curing menorrhagia is indisputable. However, hysterectomy is accompanied by significant morbidity, low mortality and a lengthy recovery period. The mortality rate for women undergoing hysterectomy for a benign disease is approximately one per 1000 cases. Major complications, including pulmonary embolism, hemorrhage, anesthesia, visceral injury, urinary retention, and peripheral nerve injuries occur in approximately 8%[139].

Unfortunately, the long-term consequences of hysterectomy are rarely considered. Bowel obstruction resulting from adhesions as a delayed complication of hysterectomy, has been described in 1.6%of cases[140]. Other reports confirm that women who have undergone hysterectomy for reasons other than stress incontinence or prolapse run an increased risk of requiring subsequent surgery. Moreover, Olsen *et al.* found that 37% of patients who underwent a primary procedure for urinary incontinence or pelvic organ prolapse had a history of hysterectomy for reasons other than prolapse[141]. Another reason for dissatisfaction following hysterectomy may be sexual dysfunction[142]. In addition, there has been no advantage in performing laparoscopic-assisted techniques with standard abdominal or vaginal hysterectomy[143].

4.4 Endometrial ablation techniques

Surgery that selectively destroys the endometrium is generally known as endometrial ablation. The aim of the various methods is to destroy the basal layer of the endometrium. The techniques employed are hysteroscopic and non-hysteroscopic endometrial ablation (first- and second-generation endometrial ablation).

4.5 First-generation endometrial ablation techniques (FEAT)

In hysteroscopically directed endometrial ablation, laser, radiofrequency, electrical or thermal energy is used directly to trigger coagulation or vaporization of the endometrial tissue. Though in 1981 Goldrath *et al.* pioneered endometrial laser ablation (ELA)[144], which has been employed by Davis in Glasgow since 1985[145], it was only in 1989, when Magos *et al.* introduced transcervical resection of the endometrium (TCRE) in Britain, that the procedures became established[146].

The surgeon requires three principal components to undertake a FEAT: a) an operative hysteroscope, b) a power source for endometrial ablation. This may be laser or electrical energy, delivered via a rollerball or resector loop, c) a uterine distention medium. Standard electrosurgical operative hysteroscopy requires the use of electrolyte-free, low-viscosity solutions for distension of the endometrial cavity.

Complications that may arise from these methods include uterine perforation and haemorrhage, pelvic sepsis and fluid overload syndromes[147,148]. If there is excessive absorption of the distension medium, fluid overload may occur, which results in hyponatremia, pulmonary and brain edema and can lead to death[147].

The first randomized trials comparing TCRE and ELA to hysterectomy for the treatment of menorrhagia showed similar short-term results: a satisfaction rate of 84% after TCRE compared to 93% for hysterectomy[149,150]. A crucial point is that in both studies, the upper limit of uterine size corresponded to that of 12 weeks of pregnancy. Furthermore, it was shown that hysterectomy did not lead to 100% satisfaction among the patients. Therefore, the role of ablation as an alternative to hysterectomy was firmly established.

As a surgical alternative to hysterectomy in the treatment of menorrhagia, endometrial ablation is considered a safe procedure, with an overall complication rate of 1.25-4.58%. In the MISTLETOE study, which examined over 10 500 endometrial ablations performed by 690 surgeons over an 18-month period, the risk of fluid overload was reported to be approximately 4%. The risk of perforation has been reported as 0.65-2.47%, higher for transcervical resection of the endometrium than with either ELA or rollerball ablation[151]. The risk of intraoperative hysterectomy is 1%[152]. Mortality from endometrial ablation, as determined in the MISTLETOE study, is two in 10 000 for ELA or rollerball ablation and three in 10 000 for loop TCRE.

The occurrence of both intrauterine and ectopic pregnancies following endometrial ablation have been reported[153], and therefore patients should be encouraged to continue taking contraceptives. There have also been isolated reports of malignancy following ablation[154].

In the most extensive material with the longest follow-up period, the clinical outcomes were similar to all of the first-generation procedures. Following TCRE, the rate of amenorrhea has been reported to range between 26 and 40%[155], with an improvement of 85% in those continuing to menstruate. At a five-year follow-up, 91% of the women had avoided hysterectomy and only 20% required any additional surgery. Failure of the treatment appears to peak at about three years following the initial surgery. Furthermore, there were no differences in the outcome measured as rates of satisfaction (90%), amenorrhea (45%) or hysterectomy (20%) between FEAT procedures[156].

Another long-term follow-up study (mean 5.1 years) from Aberdeen showed that hysterectomy was avoided in 76% of the

women, with no overall difference in the satisfaction rates between the hysterectomy and ablation groups[157]. Within five years of treatment, approximately 15% of the women will undergo a second ablation and 20%, a hysterectomy[158].

Currently, there is evidence concerning prognostic factors for successful ablation[159]. The age of the patient appears to be significant, with younger women having a lower satisfaction rate than older. This is concordant with findings from the Scottish Audit of Hysteroscopic Surgery, in which satisfaction in women under 40 years of age was 79% compared to 88% in women over 40[160].

Davis *et al.* found that hysterectomy, indicated due to failure of ablation, was associated with the finding of fibroids in 30% and adenomyosis in 27%[161].

4.6 Second-generation endometrial ablation technologies (SEAT)

In an effort to overcome the problems with the technical skills required and complications of hysteroscopic surgery, less invasive techniques have been developed.

Following Neuwirth´s description of balloon ablation in 1994, a number of new technologies have emerged to ablate the endometrium[162], including balloon technologies[163], microwave endometrial ablation[164, 165], bipolar technology, diode laser[165] and photodynamic therapy[166]. In addition, there is one technique using freely circulating hot water that has hysteroscopic control[167]. Several trials have demonstrated that some of these techniques are equivalent to FEAT in randomized studies[168,169], but hitherto not one has shown a significant improvement in either the outcome or complication rate.

The advantage of these techniques is their ease of use. In fact, some may even be performed in an outpatient setting with local anesthesia and analgesia. However, most of these methods seem to be restricted to a normal uterine cavity.

4.7 Hysterectomy versus ablation

A number of studies have compared hysterectomy and endometrial ablation, with results suggesting that both treatments are efficacious and have high satisfaction rates among the patients. In the short term, endometrial ablation offers a reduction in operative and recovery time, period of hospitalization and the number of postoperative complications. However, in the long term, women undergoing endometrial ablation have an increased risk for further surgery, though 76% of the patients have avoided hysterectomy[170].

Aims of the present study

To analyze clinical short- and long-term results of TCRE.

To investigate morphological changes in the uterine cavity after TCRE.

To provide further evidence on idiopathic menorrhagia as a disorder of angiogenesis.

To provide further evidence on the role of the VEGF-family in the regulation of angiogenesis in the human endometrium in healthy women and those with idiopathic menorrhagia.

Material and methods

The present studies are based on an analysis of two groups of patients.

Group nr 1 (papers I & II) includes 104 women who underwent TCR-E/M between 1990 and 1995 at the Departments of Obstetrics and Gynecology at Karolinska Hospital and Danderyd Hospital. Eligible patients were between 34 and 55 years of age (mean, 44.6 years) and suffered from severe menorrhagia.

All women had benign endometrial histology. The uterine cavity was evaluated by hysteroscopy. Ultrasound measurement of the uterus never exceeded 11 cm; 37% of the women had one or more submucous fibromas of < 3 cm in diameter.

Medical treatment had failed to alleviate their symptoms sufficiently. Since none of the women wished to bear more children, they accepted the therapeutic goal of achieving amenorrhea.

Group nr 2 (papers III-V) includes 24 normally ovulating women (ages 35-50) with a history of menorrhagia and 18 healthy, age-matched ovulating age-matched women (ages 35-51). The former lost more than 80 ml of blood per menstrual episode, according to pictorial blood loss assessment charts (PBAC).

All of the women were non-smokers and had not used drugs, hormonal or intrauterine contraception for at least three months prior to the biopsy sampling. Preoperative blood samples showed normal values for platelets, activated prothrombin thromboplastin time, INR, bleeding time and von Willebrand factor.

Transcervical resection of the endometrium and myom (TCRE/M)

Timing of TCRE

Endometrial thinning was induced in 85 women (81%) by treatment for six weeks with a GnRh-agonist (n=70) or a gestagen (n=15) before TCR-E/M. The remaining women underwent TCR-E/M in the proliferative phase of the menstrual cycle.

Surgical technique

The cervical canal was dilated up to Hegar 11 and Olympus resectoscope Ch 28, attached to a video camera, was passed into the uterine cavity. Glycine (1.5%) was infused for uterine dilatation and irrigation of the uterine cavity. An electronic fluid pump (Hysteromat) with an intrauterine "working-pressure" below 100 mm Hg was used for careful monitoring of fluid absorption during the entire surgical procedure. In some cases, when ethyl-glycine was employed as the fluid-distension media, an alcometer was used. It was our policy to stop the operation if the amount of fluid absorbed exceeded 1500 ml. A mixed diathermy current of 120W (80% cutting/20% coagulation) was routinely used. A current of 50W was applied for electrocoagulation.

All procedures were carried out in an outpatient surgical clinic. Four gynecology surgeons, who had considerable experience in the use of this method, performed the resections.

Anesthesia

Eighty-nine patients (83%) were given spinal anesthesia, 15% general anesthesia and two (1.9%), a paracervical block with infiltration of a local anesthetic in the uterine wall near the tubal ostiae. No prophylactic treatment with antibiotics was given.

Questionnaire

All follow-up patients (104 women) completed a questionnaire, which focused on the menstrual cycle: manifestation of bleeding, the number of days of bleeding, the interval between menses, the date of the last menstruation and a subjective assessment of each day's maximum bleeding. The degree of satisfaction with the result of the TCRE/M was defined by a scoring scale from one to ten, i.e. a score of 1-3 indicated not satisfied, 4-7 satisfied, and 8-10 very satisfied. Other questions concerned dyspareunia and dysmenorrhea before and/or after treatment. Furthermore, we determined the use of contraceptives or hormone replacement therapy and whether the women had

undergone any surgical procedures after their TCR-E/M

Transvaginal sonography (TVS) and hydrosonography

The uterus was scanned to identify echoes from the uterine cavity and to detect abnormalities in the uterine wall. The echo from the uterine cavity was measured as the total depth, "double layer", in sagittal sections and any abnormal findings in the myometrium were described. An echo less than 5mm was defined as atrophy whereas an echo exceeding this value was definitively regarded as residual endometrium.

Immunohistochemistry

In papers III-V the avidin-biotin method was used. Briefly, 5 µm thick endometrial sections from formaldehyde fixed, paraffin embedded sections were stained with a primary antibody. Primary antibodies against VEGF-A, VEGFR1, -2, -3 as well as against CD 34, CD31, and VWF were replaced. Secondary antibodies were decorated with either Chromogen Fast-red or DAB. For all the antibodies used, negative controls were run without the primary and secondary antibodies. These procedures resulted in negative staining.

Sections from a biopsy of endometrial carcinoma were used as a positive control since it comprised numerous blood vessels, including arterioles, capillaries and venules.

Adjacent sections were used in order to obtain information on co-localization of vessels identified with the endothelial marker CD34 and those stained by antibodies against the VEGF family.

After coding of the slides, they were examined by three independent observers, each of whom examined the slides on two different occasions. The staining was graded as follows: 0 = no detectable staining, + = weak staining pattern, ++ = moderate staining pattern, and +++ = marked (strong) staining pattern. Differences in opinion between the observers were resolved by discussion at the microscope.

Vessels were classified as follows: Arterioles: vessels with a clearly visible cuff of one to two layers of smooth muscle cells in the tunica media. Capillaries: thin-walled vessels, consisting of a single layer of endothelial cells without any smooth muscle cells. Venules: vessels larger than the capillaries, lined with endothelial cells and occasional pericytes or smooth muscle cells.

Double staining

The same procedure as that employed for immunohistochemistry can also be used for confocal microscopy. The difference is that the second antibody is provided with a fluorescent label.

Microvascular density (MVD) and computer-assisted stereological analysis of immunoassayed blood vessels

Blood vessels, stained for CD34, were analyzed in "hot spots" and in randomly chosen areas in five high-power microscopic fields (HPF), using 400 x ocular magnification.

In paper III, we could not detect discrete areas of high vessel density. However, in papers IV and V, the "hot spot" areas were clearly visualized. This discrepancy may be explained by the material present in papers IV and V being substantially larger. However, when MVD in "hot spots and randomly chosen areas were analyzed, we did not find any differences between theses variables. Subsequently, we continued our analyses in random fields only.

Thereafter, we assessed the number of vessels within a defined area that expressed the VEGF family members. Using an unbiased counting frame proposed by Gundersen[171], we performed two-dimensional quantitation of the different immunoassayed blood vessel profiles.

The number of blood vessels was given as numerical density, i.e. the number of profiles per area.

Micrographs were obtained in a light microscope and stored electronically. In the image analysis program, for each specimen and each staining method, five images were taken in a uniform way, always starting on the upper left side of the specimen. In each image, we placed an unbiased counting frame over the computerized image and counted the number of stained arteries, veins and capillaries[172].

Three-dimensional reconstructions of vessels

Biopsies were also sliced into 35 µm thick sections, processed as specified[173] and stained for CD34. Three-dimensional reconstructions of the structure of the vessels were rendered using ImageSpace (Molecular Dynamics, Sunnyvale, CA) and VoxelView 2.5.1 (Vital Images, Fairfield, IA). We quantified five vessels per subject.

Morphometric analyses

In paper V, we used the image analysis system Leica Q550IW with a color video camera Leica DM RXA (Wetzlar, Germany) for light microscopy together with a software system for measurements of the characteristics of the blood vessels, developed with the Leica QWin Image Analysis. Ten randomly selected and crosscut vessels per slide, with a clearly visualized lumen, were captured with a 63x oil immersion objective in order to evaluate the vascular diameter and perimeter. The perimeter was assessed by manually tracing the inner (luminal) CD34 staining. The length of the gaps in the vessel wall is given as percentages of the total vessel perimeter.

Statistics

Data are given as median values and the 95 percent confidence interval (CI). Kruskal-Wallis, Mann-Whitney and Spearman tests were performed with the Statistica® software package.

Results

Paper I In Paper I, we present a retrospective study of short- and long- term (up to five years) results of transcervical endomyometrial resection for menorrhagia.

In this study, the incidence of amenorrhea was 21% and that of minimal menstrual bleeding, 51%. There was no difference between women with hypomenorrhea or amenorrhea with regard to the mean age or presence of fibroids.

The rate of incidence of the following short-term complications was low: fluid overload in four patients, uterine perforation in one case and major bleeding in one patient. The long-term complications included three cases of hematometra.

However, we observed that 13% of the women underwent hysterectomy, generally within one year after TCRE, due to persistent menorrhagia or pain. Histopathology of the removed uteri from these women revealed signs of adenomyosis in three cases, fibroids in four cases and adenomyosis with fibroids in three cases. In a long-term follow-up, we also observed that 11% of the women, eight of whom with amenorrhea, suffered from recurrent episodes of pain.

Paper II In this study, we continued to follow-up the women after transcervical resection of the endometrium (TCRE); in particular, we evaluated the uterine endometrium and cavity.

The latter was investigated by second-look hysteroscopy (including biopsies), hydrosonography, and transvaginal ultrasound examination (TVS). The examinations were performed in 61 women, 19 to 67 months (mean, 40) after transcervical endometrial resection.

At the follow- up, most of the women expressed satisfaction with the endometrial resection: three women scored their satisfaction with the treatment as grades 0-3 (unsatisfactory), 12 women as grades 4-7 (satisfied) and 46 women as grades 8-10 (very satisfied).

Regarding the bleeding pattern following surgery, 26 women still had amenorrhea, 15 had minimal bleeding and 17 had normal withdrawal bleeding. However, three women had polymenorrhea despite the treatment and three women developed dysmenorrhea post-operatively.

Furthermore, of the 40 women with dysmenorrhea before treatment, 17 still had cyclic pain afterwards. In three women, dysmenorrhoea developed post-operatively.

TVS was performed in all of the women except for two. In 14 cases, it showed echoes from the uterine cavity, interpreted as residual endometrium. However, due to stenotic, rigid and stiff uterine cavities, hydrosono-

graphy could not be evaluated in 35 women. In another 15 women, the investigation had to be interrupted due to pain caused by the infusion. Hydrosonography revealed uterine crypts and occlusions in all of the 11 women in whom the technique could be accomplished.

Hysteroscopy was performed in all of the women except for one. Residual endometrium was observed in 52 women. The uterine cavity generally appeared as a partly occluded, narrow sac with synechiae. Tubal ostiae were identified in two cases only. Intrauterine synechiae were present in all of women included in our study. Regarding the extension of intrauterine synechiae, there were no apparent differences between younger and older women, or between women with amenorrhea and those with regular bleeding.

According to the histopathological examination, endometrial tissue was present in 67% of the women. There were no cases with atypia or malignant changes. We found that there was no difference concerning the presence of residual endometrium between younger and older women who use HRT.

In our study, hysteroscopy revealed the presence of residual endometrium in 85% of the women, but according to the histological examination, residual endometrium existed in only 67%. This discrepancy can mainly be attributed to difficulties in obtaining representative material at curettage due to fibrosis and synechiae.

Paper III In this study, with the use of immunohistochemistry and computerized image analysis, we examined the distribution and modulation of several VEGF family members (VEGF-A,-B,-C), as well as the high-affinity receptors (VEGFR1-3) in endometrial vessels.

We demonstrated that there is a marked expression of VEGF-A, as well as VEGFR2 and -3 in capillaries, whereas these vessels stained moderately for VEGFR1 and VEGF-C, and only weakly for VEGF-B. Arterioles expressed VEGF-B, VEGFR1, -2, and -3 moderately, but VEGF-A, weakly. In contrast, VEGF-C was not expressed in arterioles. Venules expressed only VEGFR3 markedly, whereas VEGF-A, VEGF-B, VEGFR1 and -2, moderately. However, the expression of VEGF-C was only weak in venules.

In all of these vessels, we observed staining for VEGF-A in the endothelial cells. Moreover, we noted occasional staining for VEGF-A in individual stromal cells that could not be associated with vessels. We did not attempt further identification of those cells; they have been assumed to be macrophages, mast cells, neutrophils and NK cells[174].

Staining for VEGF-B was mainly localized to endothelial cells; however we also detected some staining in perivascular cells in arterioles.

It is of interest that we observed occasional staining for VEGF-C in larger, thin-walled vessels, assumed to be lymph vessels since they did not stain for CD34.

We observed staining for VEGFR1-3 in endothelial cells, whereas staining for VEGFR2 was also seen in many stromal cells, not associated with vessels or glands.

In addition, we were unable to detect any significant difference in the staining for any of the assessed variables that were related to the menstrual cycle.

Paper IV In this study, we tested the hypothesis that menorrhagia is associated with an aberrant expression and distribution of VEGF-A, VEGFR1, -2 and -3 in the endometria of women with menorrhagia. Furthermore, we assessed if this resulted in a divergence in the number of vessels per unit area, i.e. the microvascular density (MVD).

MVD, analyzed as the number of vessels per HPF or randomly chosen fields, did not differ between the patients or controls. The menstrual phase did not affect the MVD in the endometrium in controls (P=0.42).

Despite the various levels of intensity, we observed, in both groups, endothelial expression of VEGF-A and VEGFR1-3 in capillaries, arterioles and venules in the proliferative and secretory phases, with no marked differences between the patients and controls.

However, when we assessed the vascular expression of VEGF-A as the number of capillaries stained for this growth factor per unit area, we observed a two-fold higher number of vessels stained positively for VEGF-A in the menorrhagia group compared to the controls (P=0.001).

Furthermore, there were a 1.5-fold higher number of vessels stained positively for VEGFR1 in the menorrhagia group compared to the controls (P=0.019). These differences were not related to the menstrual cycle. Similarly, we observed that there was a two-fold increase in the number of VEGFR2 positive vessels in the menorrhagia group compared to the controls (P=0.003), but only in the secretory phase.

Recently, we noted a 1.6-fold increase in the number of capillaries, which were VEGFR3 positive, in the menorrhagia group compared to the controls. This difference was significant (P=0.003) and also related to the menstrual cycle since there were significant differences in the proliferative (P=0.04) and in the secretory phases (P=0.015). We also observed that patients with menorrhagia had a significantly higher number of VEGFR-3 positive arterioles in the secretory phase than the controls (P=0.03).

With regard to venules, there were no differences for any of these variables, mainly due to the fact that we found very few venules.

When the statistical correlations between MVD and the number of stained vessels, which were VEGF-A, VEGFR1, -2 and -3 positive, were analyzed there were no significant relations between the variables. Thus, a higher number of vessels, which were VEGF-A and/or VEGFR1, -2 and -3 positive in the menorrhagia-group, did not translate into a difference in MVD counts, suggesting that vessels in patients with menorrhagia might display other differences, e.g. anatomical, than those in controls.

However, we observed a statistically significant positive correlation between the number of capillaries which were VEGF-A positive and those which were VEGFR1 or VEGFR2 positive (r=0.6, P=0.0002 and r=0.5, P=0.012, respectively).

Thus, up-regulation of VEGF-A is associated with an increased expression and distribution of VEGFR1, -2 and -3, but with vessel-specific differences.

Paper V The aim of this study was to identify abnormalities in the structure of the endometrial vessels in patients with menorrhagia.

We found that these vessels had a larger perimeter than those in controls (p=0.0007) and displayed an unusual morphology with focal regions, gaps, where we did not detect vessel wall CD31, CD34 or VWF but observed abluminal tie-2 immunoreactivity. The relative size of the gaps was significantly larger for menorrhagia samples than for controls (median and 95 % CI values 9.02±2.48 % and 4.64±1.56 % of the perimeter of the vessel wall, respectively; P=0.000002).

Moreover, there was a significant positive correlation between the number of VEGF-A positive vessels and the relative size of the gaps (P=0.0002). The number of VEGFR1 positive vessels (not VEGFR2) and the relative size of the gaps were also significantly correlated (r=0.37; P=0.03). Thus, an increase in the amount of VEGF-A or VEGFR1 yields an increase in the relative size of the gaps.

Similarly, VEGF-A, VEGFR1 and –2 correlated significantly with the perimeters of the vessels (r=0.51, P=0.0012; r=0.41, P=0.018, and r=0.35, P=0.04, respectively). Moreover, the more the vessels expressed VEGF-A as well as receptors 1 and 2, the larger were the vessel perimeters.

Discussion

The studies of surgical therapy

One in 20 women aged 30 to 49 consults a gynecologist due to excessive menstrual bleeding. Most medical treatments are only moderately effective and approximately 20% of women will have a hysterectomy before the age of 55[175]. Thus, menorrhagia is considered a major social and medical issue for women, their families and the health services.

Historically, many women with heavy menstrual bleeding were advised to undergo hysterectomy, which was the only way of enduring a "cure". Hysterectomy is an effective treatment of menorrhagia, but it is associated with substantial postoperative morbidity and convalescence.

In the early 1990s, endometrial resection or ablation became a well-established outpatient alternative for the surgical treatment of menorrhagia.

In Papers I and II, we evaluated the short- and long-term results of 104 women after TCRE.

In the following sections, I discuss TCRE as a surgical option for the treatment of patients with menorrhagia.

Selection of patients

Endometrial resection is not indicated for all types of menorrhagia and therefore women must undergo adequate evaluation before it is considered.

A benign cause of menorrhagia and lack of desire for future fertility are two important requirements.

In studies I and II, the diagnosis of menorrhagia was based on an accurate history of menstruation and a gynecological evaluation including ultrasound and diagnostic hysteroscopy. Moreover, the majority of patients in both of these studies used pictorial blood loss assessment charts (PBAC), described in the Introduction. The women included in studies I and II represented a clinical entity, which is generally understood by practicing gynecologists.

The presence of fibroids

In our study, we treated 37% of the women with fibroids up to 3 cm. According to our observations, there was no significant difference in the rate of complication between the women with fibroids compared to those with none.

Endometrial resection is most commonly used in the treatment of idiopathic menorrhagia. Small submucosal fibroids or polyps can also be treated using the resection technique. A crucial prerequisite for TCRE is an upper limit of the uterine size corresponding to 12 weeks of pregnancy and fibroids up to five cm [176]. However, the treatment of larger (more than 3 cm) non-peduncle fibroids has been shown to be associated with a higher incidence of complications, such as properative hemorrhage. Therefore, these women should be treated by a well-trained and experienced hysteroscopist[177].

Age of patients

We did not observe any significant differences in age between women who were amenorrheic after TCRE compared to those who had a slight menstrual bleeding.

In fact, this finding is in accordance with other publications. The randomized studies showed that women over 40 years of age had a higher satisfaction rate (88%) compared to younger women (79%)[178].

Amenorrhea induced by menopause should by itself improve clinical results in older women. Another factor is enhanced ovarian function and regenerative potential of residual endometrium in younger women.

The discrepancy with our results regarding age may be explained by the fact that the material from randomized studies was substantially larger. Moreover, 26% of all the women at follow-up used HRT, which may also influence our results.

Surgical technique and operative complications

The general clinical outcome in the present studies compares favorably with those of others. In our material, the peroperative rate of complication was as follows: fluid overloads in 4%, perforation in 1% and major bleeding in 1%.

Fluid overload

Standard electrosurgical operative hysteroscopy requires the use of electrolyte-free, low–viscosity solutions for distention of the endometrial cavity. Absorption of the irrigating solution occurs mainly into the vessels, opened during the endometrial resection. In 1992, Garry *et al.* showed that intrauterine pressures that exceed the mean arterial pressure are associated with increased fluid absorption; whenever the intrauterine pressure exceeded 100 mm Hg, there was significant fluid absorption[179].

Pulmonary and metabolic complications, as well as cerebral edema, may occur due to fluid absorption. Fluid overloads resulted in

hypervolemia, hyponatremia and hypo-osmolarity.

Istre *et al.* showed a linear correlation between a peroperative decrease in serum sodium and a deficiency of glycine. Moreover, these authors observed that patients who absorbed 500 ml of glycine or more experienced nausea, whereas those who absorbed 1000 ml of glycine or more had cerebral edema, detected on a CT scan[147].

Careful monitoring of fluid absorption during the entire surgical procedure with the use of an electronic fluid pump and a "working-pressure" below 100 mm Hg may be the reasons for the low rate of fluid overload in our study.

In an attempt to overcome the problem with electrolyte-free, low-viscosity solutions, a new technique for endometrial resection, using bipolar electrodes (GyneCare, USA) with a saline solution as a distension media has been developed[180].

Perforation of the uterine wall

Perforation of the uterine wall is usually recognized, as the uterine walls fall together and visibility is poor. The intrauterine pressure falls as the distension medium flows into the cavity. If perforation occurs with electrosurgical instruments, there is a risk of injury to structures outside the uterus, such as major blood vessels, the ureter, bladder or bowel. In that event a laparoscopy should be performed immediately and the uterus and surrounding structures, carefully examined. The damaged structures should be subjected to careful scrutiny and appropriate repair[160].

Hemorrhage

Hemorrhage can occur during TCRE if the depth of resection is over 3 mm of the myometrium or if perforation has occurred where a major vessel is involved. The preoperative endometrial thickness reduces vascularity and bleeding.

Bleeding that occurs after TCRE can be controlled by electrocoagulation of surface vessels and introduction of a Foley's catheter with distension of the bulb for four to six hours[181].

Preoperative endometrial thickness

Endometrial resection may prove safer and more effective with preoperative endometrial preparation.

The use of endometrial thinning in the majority of the women (81%) in our study may have contributed to the low rates of complication.

The Cochrane review reports a decrease in fluid absorption when endometrial thinning with either danazol or GnRh analogues is used. Moreover, there was a higher incidence of amenorrhea; GnRh analogues appear to result in better rates of amenorrhea than danazol. However, the use of progestogens did not demonstrate any improvement in the outcome[177].

Outcomes after TCRE

During a span of five years following TCRE, 21% of the women became ammenorrhoic and 51% had hypomenorrhea. Within two years after the resection, the rate of hysterectomy in our study was 13%, indicated mainly due to persistent menorrhagia or pain. The histopathology after hysterectomy showed signs of adenomyosis and fibroids.

Our results are in agreement with those of previous studies. However, the rate of hysterectomy was lower than in other published studies.

Ray *et al.* reported a rate of amenorrhea between 26 and 40%, with an 85% improvement in those continuing to menstruate. The rate of satisfaction was reported to be 84%[182].

Success or failure of endometrial ablation is multifactorial. A prediction of the outcome is still unreliable for individual cases. Based on randomized trials, women with objectively verified menorrhagia have a better outcome than those with periods perceived to be heavy (a 9% rate of failure compared to 18%). Furthermore, women over 40 years of age have a higher rate of satisfaction (88%) compared to younger women (79%) [183].

Davis *et al.* reported that failure of ablation was associated with the finding of fibroids in 30% of the patients and adenomyosis in 27%[161].

Most studies seem to yield similar results at a follow–up after five years; about 15% of women will have undergone a second ablation and 20%, a hysterectomy.

That is to say about 80% of women, treated with TCRE, have the possibility of avoiding hysterectomy.

Is dysmenorrhoea a contraindication for TCRE?

We observed that 11% of the women, eight of whom had amenorrhea, suffered recurrent episodes of pain. Of 40 women with dysmenorrhea before treatment, 17 still had cyclic pain afterwards, and in three women, dysmenorrhea developed post-operatively. Moreover, the persistent dysmenorrhea was often the main reason for performing a hysterectomy.

Menstrual pain may develop on the basis of several pathogenic mechanisms. Endometrial release of prostaglandins represents an important pathogenic mechanism in primary dysmenorrhea[184].

However, secondary dysmenorrrhea is thought to be due to organic changes, such as adenomyosis or endometriosis. In our study, histopathology of the removed uteri from women after hysterectomy due to dysmenorrhea frequently showed signs of adenomyosis. Whether this finding represents true adenomyosis or only scattered islands of residual endometrial glands in the myometrium, is a matter of debate[185]. The healing process following TCRE may stimulate regeneration of residual endometrium in the myometrium.

Some evidence suggests that women with both dysmenorrhea and menorrhagia were less satisfied after TCRE than those with menorrhagia alone[160]. However, in 1991, Magos *et al.* reported that satisfaction of the patient was independent of whether or not menorrhagia was associated with dysmenorrhea[186]. These data may indicate that only a deep invasion of adenomyosis is connected with symptoms not alleviated by resection of the endometrium and the superficial myometrium.

Furthermore, cyclic pain after TCRE may also develop as a result of hematometra or hematosalpinx, located in the proximal and intramural parts of the Fallopian tube. This may occur if the tissue around the tubal ostiae is insufficiently removed during the surgical procedure. On the other hand, in some women, resection in the isthmus area may have been too deep, inducing the formation of intrauterine or cervical synechiae, which may obliterate the passage to the uterine cavity. This may result in hematometra.

Uterine morphology after TCRE

Second-look hysteroscopy was performed in 60 women (Paper II). In agreement with previous studies[187], we showed that the uterine cavity in most women was reduced to a narrow, fibrotic sac with synechiae. Intrauterine synechiae were present in all of the assessed uteri.

The fibrotic changes or synechiae hamper access to the cavity. However, with the exception of one uterus, we were able to assess all of the cases with use of hysteroscopy.

Hysteroscopy revealed the presence of residual endometrium in 85% of the women, but according to a histological examination, residual endometrium was found in only 67%. This discrepancy can mainly be attributed to difficulties in obtaining representative material due to fibrosis and synechiae.

In short, endometrial tissue was present in all but five women after TCRE. This indicates that not all of the endometrium may be removed and that a regeneration of the endometrium can occur after TCRE.

Contraception after TCRE

We observed one pregnancy that ended in a spontaneous abortion. Both intrauterine and ectopic pregnancies have been reported following endometrial ablation; Istre *et al.* reported that tubal potency was found in 13% of women after TCRE[188]. Therefore, patients following TCRE should be advised to continue taking contraceptives. It is likely that in the future, TCRE will be combined with hysteroscopic sterilization.

Choice of hormone replacement therapy after TCRE

At follow-up, islands of endometrial tissue were present in all of the women except five. Therefore, when hormone replacement therapy is indicated, a combination of estrogen and gestagen should be recommended.

Diagnostic management of women after TCRE

TVS was performed in all of the women except for two. However, hydrosonography could not be employed in the majority of women, since their uterine cavities were stenotic, rigid and stiff. Hysteroscopy was

the only method possible for evaluation of both the uterine cavity and its lining.

Despite the fact that TCRE has been performed, the potential risk of developing endometrial cancer still remains. If early signs of bleeding are concealed due to closed cavities, such a development may proceed without detection[189,154].

If women that have undergone TCRE develop symptoms, such as bleeding and/or severe pain, *both* vaginal ultrasound and hysteroscopy with directed biopsies should be recommended to exclude the presence of endometrial atypia.

"One –stop" clinic

Recent developments have given rise to "one-stop" clinics. The combination of investigation and outpatient hysteroscopy, including endometrial ablation, renders these clinics a very attractive option for women with menstrual disorders. The provision of information, continuity of care, reduced waiting time and organization suits their needs.

Women with menorrhagia should be more involved in the decision-making process regarding different treatment options. A "minor" surgical technique, such as endometrial resection/ablation, is effective and has a high success rate with few complications in the hands of an experienced surgeon. Furthermore, it is an outpatient procedure, which for most women would be preferable to hysterectomy.

The studies of angiogenesis

Why does the endometrium regenerate and what regulates this process? Alterations in the structure and function of the human uterine vasculature are known to occur in normal and diseased states through changes in vascular integrity that may influence patterns of vaginal bleeding.

In this thesis, we have investigated the expression and distribution of VEGF, its receptors and the morphology of vessels in the endometrium in healthy women and those with menorrhagia in order to determine if these variables are aberrant in menorrhagia. The methods employed and results will be discussed here.

Selection of patients

Validity of the diagnosis of *menorrhagia and selection biases.* In order to avoid any bias in patient selection, we enrolled patients consecutively. The diagnosis of menorrhagia is based on an accurate history of menstruation and pictorial blood loss assessment charts (PBAC), described in the Introduction. This method has previously been evaluated and found to be semi-objective, with more than 80% sensitivity and specificity[98]. Normal findings at hysteroscopy were a diagnostic requirement for patients with idiopathic menorrhagia.

However, since patients were referred to a specialized surgeon, they probably represented a cohort of severe cases. That is obviously an advantage when mechanisms for menorrhagia are explored. Furthermore, from a therapeutic point of view, an evaluation of surgical procedures has shown that the number of women satisfied with objective menorrhagia is higher than in women with periods perceived to be less profuse.

Exclusion criteria. A number of defined conditions made patients ineligible for participation in the study. It is well known that smoking of cigarettes has an anti-estrogenic effect and that sex hormones influence endometrial regrowth after menstruation. Furthermore, contraceptive pills and anticoagulantia can cause abnormal menstrual bleeding. Hence, smokers and patients on contraceptives were excluded from the study. Similary, a large majority of the patients with blood clotting disorders (evaluated by a series of blood tests (papers I-V), could be eliminated. Based on these measures, we believe that the patients who were examined are representative of idiopathic menorrhagia.

Laboratory techniques

The computer-assisted stereological analysis of immunoassayed blood vessels. Most of the previous studies on VEGF and corresponding receptors focused on the expression of VEGF in stroma and glands. However, the human endometrium is a complex tissue comprised of different types of cells, including epithelial, stromal, inflammatory, perivascular and blood vessels cells. Therefore, with respect to expression of the various proteins, our investigation was specific in terms of classification of involved vessels (arterioles, venules and capillaries).

When the study was intiated, we had evidence that VEGF existed in stroma and glands, yet we proceeded to investigate in which stromal structures VEGF was expressed. Specifically, we determined if proteins were expressed in vessels and endothelial cells. However, we were unable to use ELISA or PCR methods, which are quantitative methods for detection of mRNA or protein in whole samples. *In situ* hybridization assays are qualitative methods and require further analyses to assess cell type and if the corresponding protein is expressed. Therefore, immunohistochemistry, involving the use of single and double stainings and confocal microscopy, was the optimal choice.

As reported in the Introduction, there are contradictory results regarding the expression of VEGF in the endometrium. These discrepancies may be explained by inherent problems with assessment of the intensity of immunohistochemical staining. This semi-quantitative assessment is the subject of a variety of methodological pitfalls and thus better assays are warranted. In our study, we introduce an analysis of the number of vessels that express the variable of choice. It represents a more objective assessment and may be further refined if it can also calculate the fraction of vessels, related to all vessels, expressing this molecule.

The assessment of blood vessel morphology. In paper V, we used the immunohistochemical expression of CD31, CD34 and VWF to determine the identity of endometrial blood vessels. Endothelial cells are phenotypically heterogeneous[190] and they are also heterogeneous in their immunoreactivity[119], which justifies the choice of a panel of markers to assess endometrial vascularization. Moreover, in order to investigate if abnormalities of the vessels, if any, were due to deficient development with the Fast-red stain, we decorated the secondary antibody with the fluorescent dye Alexa 488 and created double stainings with CD34 and VWF, etc. Since our results were congruent, we feel confident that gaps are not the result of uneven stainings.

Finally, we completed the investigation of the endometrial vessels with 3D-computer-aided image analysis. With the use of 35 µm thick sections, it was possible to visualize the architecture and abluminal appearance of the vessels.

Endothelial gaps and fenestration have previously been observed in various organs, e.g. liver sinusoids, the bone marrow and endocrine glands. McDonald *et al.*[9] compared five methods, used for establishing the presence of endothelial gaps, and concluded that each of these methods had benefits and limitations. However, scanning with electron microscopy was determined to be the optimal method for providing detail on the morphology of the gaps. The method we used was not included in that assessment. However, here we have found that it represents a new means to visualize gaps that offers advantages but requires further evaluation in order to determine if the endometrial gaps are of the same nature as gaps in other organs.

In our study, immunohistochemistry combined with morphometry, is a valuable technique for estimatating the number and size of the gaps.

Since we observed gaps in endometrial blood vessels, by using light microscopy and 3D-computer analysis, our data supports the notion that idiopathic menorrhagia is associated with an endothelial abnormality in the anatomy of the vessel wall. Based on these results, we suggest that the use of electron microscopy may be impractical in this study since the distance between gaps is so large that thousands of sections have to be obtained in order to localize a single gap. However, other methods, e.g. staining with antibody caveolin-1 to reveal caveolae, may be worthwhile.

The expression of VEGF-family in healthy endometrium

In paper III, we demonstrated that endometrial arterioles, venules and capillaries expressed VEGF-A, -B, -C and their receptors (except forVEGF-C, which was not expressed in arterioles).

Our findings have two implications. Firstly, endometrial capillaries, composed by definition of endothelial cells only, express all the members of the VEGF-family. Our results do not indicate whether endothelial cells produce VEGF molecules or if VEGF is taken up by, for instance, by receptor-mediated endocytosis. Although previous results suggest that endothelial cells did not produce VEGF, recent data favor an endogenous production[191, 192]. Our data show co-localization of VEGF and CD34, suggesting that VEGF is in fact expressed in the endothelial cells, not only by PC or other cells adjacent to the capillaries.

Secondly, we have increased the range of knowledge about the role of VEGF-C in the endometrial vessels, where arterioles were completely negative, and unidentified vessels, probably lymphatics, stained positively. VEGF-C, similar to VEGF-A has also been reported to enhance the permeability of blood vessels and induces fenestrations in the endothelial cells both *in vivo* and *in vitro*[193.194]. VEGF-C is also a ligand for VEGFR2 and may be able to induce angiogenesis via this receptor.

Moreover, the novel observation in this study was the detection of VEGFR3. Previous experiments showed that inactivation of the VEGFR3 gene leads to abnormally organized vessels and cardiac failure. Furthermore, Alitalo *et al.* detected an increased expression of VEGFR3 on the fenestrated capillaries of several organs, including bone marrow, kidney glomeruli and endocrine glands[195]. This finding suggests that VEGFR3 plays a role in the transport functions of the more permeable endothelia in specific locations, as in the endocrine organs.

Therefore, in order to explain the cellular and molecular process of endometrial repair, these aspects may be of interest.

Endometrial vascular abnormalities in idiopathic menorrhagia

A major finding of this study is that vessels in patients with menorrhagia displayed an unusual morphology with focal regions, gaps, where no vessel wall CD31, CD34 or VWF but where abluminal Tie-2 immunoreactivity was detected. The relative size of the gaps was significantly higher for menorrhagia samples than for controls (P=0.000002).

Endothelial gaps have been described previously for other organs and situations. However, to our knowledge, this is the first study that demonstrates the presence of gaps in patients with menorrhagia and as part of a specific disease process.

Our data of enhanced VEGF-A and VEGF receptor expressions, with strong statistical interrelations as well as with the occurrence of gaps, point to the possible existence of a discrete signaling chain of molecules, involved in the pathogenesis of menorrhagia.

Why are endometrial vessels abnormal?

Is aberrant angiogenesis the reason?

Microvascular fragility has been proposed as a mechanism for breakthrough bleeding in patients who use progesterone[196]. Perivascular SMA is found in pericytes and smooth muscle cells and both of these types of cells are known to contribute to the structural integrity and strength of blood vessels[121]. Hickey *et al.* observed a reduction in perivascular SMA in patients who use HRT[197]. Microvascular pericytes are associated with vascular maturity and stability and also play an important role in angiogenesis. Ang-1 promotes vascular maturation via the tie-2 receptor, while Ang-2 is its natural antagonist, destabilizing vessels and initiating neovascularization in the presence of VEGF[124]. The recruitment of pericytes is regulated by Ang-1 as part of the vascular stabilization process following angiogenesis[198]. It is possible that the gaps, in which abluminal Tie-2 immunoreactivity was detected, indicate involvement of the angiopoetins system with a reduction of perivascular SMA in these patients. Double staining for CD34 and SMA might have provided further information on the relationship

between endometrial vessels and SMA in these subjects.

Is altered vascular perfusion the reason?

Hickey *et al.* showed that in patients who use Norplant, there was reduced blood perfusion. However, this aspect was not investigated in patients with idiopathic menorrhagia. In paper V, we observed that in the secretory phase, the perimeter and diameter of vessels in the patients were larger compared to controls. Vascular dilatation may be a reaction to reduced perfusion and may lead to endometrial hypoxia. Hypoxia is a potent vascular destabilisator which compromises with endothelial integrity, induces vascular breakdown and stimulates angiogenesis via several angiogenetic factors, including VEGF, PDGF and Ang2. One hypothesis may be that there is a common reason for our observations and others, *viz*. that unknown factors trigger regional hypoxia in the endometrium, resulting in up-regulation of hypoxia-sensitive mechanisms (e.g. HIF1-a) that lead to enhanced generation of VEGF, vascular growth, vessel dilation and the formation of gaps.

Is aberrant action of ovarian steroids the reason?

Ovarian steroids modulate the uterine vascular tone and perfusion and may thus contribute indirectly to disturbances in menstruation. Despite normal levels of circulating ovarian steroids, the actions of estrogen and progesterone at an endometrial level may be altered in women with menorrhagia and be of significance for vascular abnormalities.

Is an altered balance between activators and inhibitors of angiogenesis the reason?

In Paper IV we showed, that the expression of VEGF and its receptors is increased in the endometrium in association with menorrhagia. Subsequently, in Paper V, we found that the number of VEGF-A, as well as VEGFR1 positive vessels and gaps, were positively correlated.

Previous studies have shown that VEGF not only enhances proliferation of endothelial cells, but also augments vascular permeability and induces fenestrations in the endothelium of small capillaries and venules[8]. VEGF can also lead to dilatation of the vessels. Hypoxia is a major stimulant of VEGF up-regulation. Changes in endometrial oxygenation are likely to accompany changes in vascular perfusion. Oxygen tension in superficial endometrial vessels or the surrounding tissues has hitherto not been assessed. These effects may also be influenced by the local release of vasoconstrictor substances, such as endothelins, and lead to future alterations in local flow and dilatation.

Moreover, as stated in the Introduction, in the presence of VEGF-A, Ang-2 promotes a rapid increase in the diameter of blood vessels, leading to hypoxia.

Recently, Qi *et al.* showed that TIMP3 blocks the binding of VEGF to VEGFR-2 and inhibits downstream signaling and angiogenesis[199]. The fenestration of vessels in menorrhagia patients may also be explained by changes in the endometrial balance between some MMPs and expression of their tissue inhibitors (TIMPs).

In conclusion, our data indicate that there is an up-regulation of the agonist-receptor pathway of VEGF in idiopathic menorrhagia. Furthermore, deregulation of the VEGF signaling system leads to anatomical differences in blood vessels, manifested, inter alia, as gaps.

Changes in vessel morphology per se cannot account for bleeding, but may be associated with altered vascular control and function in menorrhagia. Without a doubt, stimulation of angiogenesis involves a wide spectrum of agents, with potential roles in specific aspects of the formation of the vessels.

However, our novel observations may be significant in order to understand some of the underlying mechanisms that contribute to idiopathic menorrhagia.

Future perspectives: why do some women bleed more?

Endothelial progenitor cells in the endometrium

Until recently, it was thought that the formation of blood vessels in postnatal life was mediated by sprouting of endothelial cells from existing vessels. However, recent studies have suggested that endothelial stem cells may persist in adulthood, where they contribute to the formation of new blood vessels. This, in turn, suggests that neoangiogenesis in the adult may depend at least in part on the process of vasculogenesis similar to what is seen during early development in life. Precursors of endothelial cells (EPCs) have been isolated from bone marrow and peripheral blood.

Asahara *et al.* identified EPCs in endometrial neovasculature in transgenic mice[200]. These findings indicate that EPCs contribute to physiological neovascularization associated with the postnatal regenerative process.

Moreover, growth factors in the endometrium, among them VEGF, may contribute to enhanced homing of circulating EPCs to the endometrium, where they may further differentiate and/ or become incorporated into foci of neovascularization[201].

It is of interest that Hattori *et al.* pointed out PlGF binding to VEGFR-1, as an important mediator of stem cell recruitment and mobilization[202].

Bone marrow-derived EPCs were also observed within tissue stroma at sites of pathological neovascularization.

But how does this occur in the human endometrium? Does angiogenesis in the endometrium develop from the existing endothelial cells or are the EPCs derived from the bone marrow?

Do the increasing levels of VEGF enhance homing of these cells to the endometrium?

Endoglin and the fragility of vessels

Endoglin, also known as CD105, is a receptor for transforming growth factor (TGF-ß1 and- ß3) in vascular endothelial cells and is a proliferation-associated antigen[203,204]. CD105 has been located in human vascular endothelial cells in both large and small vessels, whereas vSMC, fibroblasts, macrophages, leukemic cells and erytroid precursors express CD 105 to a lesser extent[204].

CD105 is required for the formation of mature blood vessels. Based on studies on CD105 knockout mice, which had multiple vascular and cardiac defects, the absence of CD105 resulted in vessel fragility and internal bleeding.

Furthermore, hereditary hemorrhagic telangiectasia is the result of the CD105 gene mutation. Telangiectases, arteriovenous malformations of skin, mucosa and viscera, are characteristics of this disease[205].

Thus, staining for CD105 may have provided further information about the relationship between gaps and CD105.

Endoglin is also a marker for mesenchymal stem cells. Despite the possibility that ECPs may be imported from the bone marrow, it is also conceivable that there are mesenchymal stem cells in the endometrium that cause the formation of glands, stroma and smooth muscle cells. This aspect can be investigated using endoglin as marker for these cells.

Idiopathic menorrhagia as a vascular malformation

Vascular malformations are localized errors in angiogenic development[206]. Most of them are cutaneus and are called vascular "birthmarks". However, vascular malformations also occur in visceral organs, such us the respiratory and gastrointestinal tract, but are more prevalent in the brain. They include capillary, venous, arteriovenous, lymphatic and combined malformations. Genetic studies of families with such malformations have resulted in the identification of mutated genes, which suggests their important role in the regulation of angiogenesis. Case-control studies have shown an association between polymorphisms of the VEGF gene and diabetic retinopathy[207]. Moreover, it was demonstrated that a mutation in the Tie-2 gene caused venous malformations, present as bluish-purple lesions on the skin and mucosa[208].

The relation of these vascular malformations to endometrial gaps and menorrhagia remains to be elucidated.

Summary and general conclusions

- TCRE provides a minimally invasive technique for treatment of menorrhagia with good clinical results at a five-year follow up: about 80% have the possibility of avoiding hysterectomy.
- Endometrial resection/ablation should be offered as a surgical option to all women with idiopathic menorrhagia who have completed their family.
- Residual endometrium was found in most of the patients after TCRE. In case of hormone replacement therapy, a combination of estrogen and gestagen should be recommended.
- Normal endometrial angiogenesis is perturbed in idiopathic menorrhagia, with an up-regulation of the agonist-receptor pathway of VEGF-A.
- The endometria in women with menorrhagia display fenestrations in the vessel walls.
- These findings provide novel opportunities for therapeutic intervention in the future.

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