ANGIOGENETIC AND HEREDITARY FACTORS IN ENDOMETRIAL DISEASE

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Angiogenetic and Hereditary factors in endometrial disease

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family and friends
ABSTRACT

The endometrium is a unique tissue in the adult human body because it is the only site where physiological renewal of vessels, angiogenesis, occurs. The regulation of this process under normal conditions is not fully understood. Heavy Menstrual Bleeding of endometrial origin (HMB-E) and Endometrial Cancer (EC) are two diseases which can neither occur nor be sustained without the process of angiogenesis. Clinically, both diseases are also associated with a strong hereditary component where female relatives of affected patients demonstrate a high incidence of the same condition. The aim of this thesis is to further elucidate the angiogenic and hereditary components of these diseases. The first part of this project (studies I and II) explores HMB-E and its relationship to angiogenesis, while the second part (studies III and IV) examines the association between incidence of EC in relation to previously known and unknown hereditary factors.

Study I explored the relationship between expression of the pro-angiogenic factor SDF-1 and the number of circulating epithelial progenitor cells (EPCs) in peripheral blood in 10 women with a confirmed diagnosis of HMB-E, and compared these results with those of 10 healthy controls using flow cytometry and cell culture. The results showed a significant decrease of SDF-1 throughout the entire menstrual cycle, a 16% decrease overall, with the most substantial decline noted during the proliferative phase of the menstrual cycle in women with HMB-E, compared with controls. The number of EPCs in peripheral blood was also significantly reduced in HMB-E patients, showing a significant positive correlation between number of EPCs and SDF-1 levels. These findings are consistent with the literature, which has shown that SDF-1 is essential for recruitment of EPCs from bone marrow into the bloodstream, and further suggests that this signaling axis is important for physiological angiogenesis in the endometrium.

Study II addressed microvascular morphology within the endometrium among 17 women with HMB-E and compared the results with 10 controls, using immunohistochemistry and electron microscopy. We found a significant decrease in pericyte coverage of microvessels during the mid-proliferative phase, as well as an increase in vessel perimeter among women with HMB-E. We also found a negative correlation between vascular expression of the known pro-angiogenic factor VEGF-A and pericyte coverage. These findings indicate that HMB-E may be caused by altered microvessel maturation and that the overexpression of VEGF-A seen in HMB-E might be the underlying cause.

Study III examined the clinical challenge of reducing EC risk in women with Lynch Syndrome (LS), who collectively have a 42-54% lifetime risk of developing this disease. Sweden has no national guidelines regarding surveillance of these women for gynecological cancers. Therefore, we took a retrospective look at the modalities and screening intervals that are currently used in Sweden for known LS carriers. In all, 86 women had a known LS mutation and participated in various screening programs.
Of the 41 women who decided to have prophylactic hysterectomy while under screening, EC/complex hyperplasia was found in the uterus of 4 of them postoperatively. The remaining 45 women opted for annual surveillance without prophylactic surgery. In this group, 9 women developed EC, 2 complex endometrial hyperplasia and 2 ovarian cancers, both of which were found at an early stage by ultrasound. Among these 9 EC cases, 5 were detected through endometrial biopsy during regular surveillance at an asymptomatic stage, as were the two hyperplasias. Ultrasonography failed to detect any of these cases. The remaining 4 EC cases were associated with occult bleeding between screening visits. No difference in tumor stage was noted between the ECs found because they were symptomatic and those found through routine surveillance. No mortality resulted from the gynecological cancers found in the study group as a whole. The results did not reveal any clear benefit from conducting annual gynecological screening for women with LS if the endpoint is to reduce mortality. The single most important factor may be knowledge and awareness among doctors and patients of LS and the associated increased risk of cancer, which should trigger prompt investigation if symptoms develop. However, when screening is undertaken, endometrial biopsy seems to be the diagnostic modality of choice to detect asymptomatic ECs or hyperplasia.

Study IV investigated additional genetic risk factors that help to explain the twofold increase in relative risk of developing EC among women with a first or second degree relative who have the disease, even when known single gene mutations are excluded. After genotype studies and quality control, a total of 332,906 SNPs among genotypes derived from 1116 EC cases and 5021 controls were compared. The results showed five haplotypes located on chromosomes no. 2, 10, 13, 15 and 20 that were significantly more common among EC patients than controls. The frequency of these haplotypes in the EC population ranged between 1.58-3.69% and the odds ratio for EC ranged between 1.58-3.05 for the five haplotypes. The five haplotypes were found in regions of the genome with no previously known link to EC development and without close proximity to any genes known to be involved with this disease. These findings add to our knowledge about the genetic risk factors associated with EC and may offer one explanation for why the incidence and clinical course of this disease differs among people of different ethnic backgrounds. Nevertheless, other risk factors must be taken into account when interpreting these results, including known environmental risk factors for EC and those that could potentially be inherited, such as obesity.

In conclusion, this thesis provides new information concerning the angiogenic and hereditary factors involved in development of both HMB-E and EC. The papers add to our knowledge about how the SDF-1 axis is involved in endometrial vascular regeneration during physiological endometrial angiogenesis and how vascular maturation appears to be inadequate in HMB-E. The thesis also confirms the knowledge about the role of known inherited cancer syndromes in development of EC, and finds that gynecological surveillance per se does not seem to reduce mortality from gynecological cancer; instead, the most important protective factor appears to be knowledge and awareness of the cancer syndrome among both carriers of the syndrome and the care providers.
Additionally, this thesis found five haploptypes that are overrepresented in a Swedish cohort of EC cases, which may explain some of the risk of inheriting EC. The future challenge will be to examine the potential link between HMB-E and EC from the perspective of both clinical and basic research.
LIST OF SCIENTIFIC PAPERS

I. E.Elsheikh, E.ANDERSSON, C.Sylvén, B-G.Ericzon, J.Palmblad, M.Mints
   Plasma levels of stromal cell-derived factor-1 (CXCL12) and circulating
   endothelial progenitor cells in women with idiopathic heavy menstrual
   bleeding
   Human Reprod, Volume 29, Issue 1, 2014, Pages 49–56

II. E.ANDERSSON, E.Zetterberg, I.Vedin, K.Hultenby, J.Palmblad, M.Mints
    Low pericyte coverage of endometrial microvessels in heavy menstrual
    bleeding correlates with the microvessel expression of VEGF-A

    The gynecological surveillance of women with Lynch Syndrome in
    Sweden
    Gynecol Oncol, Volume 138, Issue 3, 2015, Pages 717-722

IV. E.ANDERSSON, W.Liu, E.Tham, A.Lindblom, K.Broliden, M.Mints
    Five haplotypes associated with endometrial cancer risk found in a
    Swedish population
    In manuscript, to be submitted
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1 INTRODUCTION

1.1 Angiogenetic and Hereditary physiology

1.1.1 Endometrial anatomy and physiology

The human uterine wall consists of two anatomically distinct layers. The innermost layer, known as the myometrium, consists of smooth muscle and is thicker than the superficial layer, known as the endometrium, which surrounds the uterine cavity. The endometrium can then be further subdivided into the basal and functional layers. The basal layer, adjacent to the myometrium, does not shed during menstruation, which distinguishes it from the adjacent functional layer that develops in conjunction with the menstrual cycle and sheds during menstruation. The functional layer consists of a single-cell layer of columnar epithelium adjacent to the uterine cavity, with underlying vascular dense glandular stroma.

The sex steroid hormones produced in the ovaries, mainly estrogen and progesterone, control growth and shedding of the functional layer in the endometrium during the menstrual cycle. Shedding usually occurs over a period of 4-5 days. Estrogen levels then increase to a point where they begin to stimulate the cells in the basal layer to regenerate a new functional layer. This is referred to as the proliferative phase of the menstrual cycle (1).

When ovulation later occurs around day 14 in the cycle, hormonal production in the ovaries shifts toward progesterone produced by the corpus luteum. This process halts mitotic activity in the endometrial cells, while causing maturation of vessels and early decidualization changes in the stroma. This process describes the secretory phase of the menstrual cycle.
If embryo implantation fails to occur during the remaining days of the cycle, the corpus luteum will eventually start to deteriorate, thereby causing progesterone levels to drop, which in turn leads to the breakdown and shedding of the functional endometrial layer. Exactly how progesterone withdrawal leads to this event on a cellular level is not completely understood (2), but we do know that such withdrawal leads to vasoconstriction of the spiral arterioles, causing hypoxia, which in turn triggers an inflammatory response and shedding of the necrotic material. The necrotic tissue sheds over a period of 1-2 days, after which endometrial healing begins over the remaining days of the bleeding period, a process where inflammatory, coagulatory, angiogenic and cellular factors all play crucial roles for tissue stabilization, culminating in hemostasis and healing (1).

Observations within the uterine cavity during menstrual bleeding have shown that endometrial shedding is not an orderly process. Instead, patchy loss of the old layer occurs with islands of bleeding and shedding, while tissue regeneration simultaneously occurs in other areas of the uterine cavity, which implies that the process is under local control and involves a delicate balance of various molecular factors within the endometrium (2).
Molecular regulation of endometrial shedding, repair and regeneration is believed to entail a multistep process. The process begins with the previously described withdrawal of progesterone at the end of the menstrual cycle. As progesterone levels drop, the functional layer is already in a proinflammatory state, which is necessary for embryonic implantation to occur (3). Progesterone withdrawal leads to five observable changes: an increase in local expression of the prostaglandin synthesizing enzymes COX 1 and 2 (i); a subsequent increase in prostaglandin production with expression of chemokines and chemotactic proteins that stimulate tissue leukocyte infiltration (ii); production of local vasoconstrictors, including endothelin (ET-1) and PGF2, leading to vasoconstriction (iii); subsequent development of local areas of hypoxia in the inner portions of the functional layer secondary to vasoconstriction, causing an increase in inflammatory reactivity (iv), as well as an upregulation of local matrix metalloproteinases (MMPs) that break down the extracellular matrix (ECM) (v). These changes achieve a controlled inflammatory response with a controlled amount of bleeding, while enabling regeneration of the tissue without scarring (4).

In order for the endometrium to degenerate and regenerate itself, the blood supply must be controlled, including growth and proliferation of blood vessels. This process is called angiogenesis, and the endometrium is the only known adult human tissue in which angiogenesis occurs as a physiologic process (4).

1.1.2 Endometrial angiogenesis

The endometrial vasculature in the human body has been extensively studied due to its unique characteristics. The uterine artery, which is the main blood vessel supplying the uterus, branches and gives rise to the arcuate arteries of the myometrium. The radial arteries branch out from the arcuate arteries and give rise to two types of small arteries, the basal and spiral arteries of the myometrial-endometrial interface. The basal arteries supply the basal region of the endometrium, while the spiral arteries penetrate and vascularize the more superficial areas of the functional endometrial layer (5).

The spiral arteries elongate, branch, and coil as the endometrium grows during the menstrual cycle. As the menstrual cycle progresses, they form a dense network of capillaries, known as the subcapillary plexus, located just below the epithelium. During the secretory phase of the cycle, coiling of the spiral arteries becomes more extensive.
Furthermore, the venous system develops concurrently with the arteries, and several smaller veins coalesce over the course of the menstrual cycle to form sinuses, which converge to form larger veins as they pass from the superficial into the deeper regions of the endometrium and ultimately the myometrium, where they drain into the uterine veins (6).

Angiogenesis in the human endometrium occurs during all three phases of the menstrual cycle, and at different anatomical locations therein. During the bleeding phase, angiogenesis mainly occurs in the basal layer, during the proliferative phase in the functional layer, and in the secretory phase in the subepithelial capillary plexus. In addition, mediation of angiogenesis differs between these phases (7). During the bleeding phase, angiogenesis in the endometrium occurs independently of sex hormone expression. Evidence for this comes from mouse models where rodents who underwent oophorectomy and subsequently had one menstrual cycle exogenously induced still managed to regenerate a new endometrial lining afterwards despite the lack of sex hormones. This suggests that angiogenesis that takes place during the bleeding phase is mainly under the control of locally produced endometrial factors (8).

During the proliferative phase, angiogenesis is estrogen-driven since no new functional layer will grow in its absence and thereby no new vessels either. Evidence for this finding comes from studies on monkeys that underwent oophorectomy followed by estrogen and progesterone implants to mimic a normal menstrual cycle. Endometrium sampling showed a peak in endothelial cell proliferation markers during the mid-proliferative phase when the estradiol concentration was at its highest. Vascular generation was absent in monkeys without an estradiol implant (7).

During the secretory phase, blood vessel growth decrease; instead, maturation with more coiling of spiral arteries and incorporation of mesenchymal cells into the vascular walls occurs. Some theories suggest that most of the maturation process is actually controlled by endometrial natural killer (NK) cells that modify growth of spiral arteries and mediate incorporation of mesenchymal cells into the vascular walls (9). Some studies using in vivo models have shown that a paucity of NK cells in the endometrium leads to increased mesenchymal cell recruitment into the vessels (10, 11).
The mechanisms by which sex steroid hormones exert control over growth of endometrial vessels on the cellular level is not understood, since endothelial cells lack both estrogen and progesterone receptors. The main hypothesis is that estradiol and progesterone exert their effect on endometrial angiogenesis through stimulation of other cells in the endometrial stroma, which in turn produce local factors that promote vessel growth and migration (12).

Regulation of angiogenesis on the molecular level is a tightly controlled process where the balance between promoters and inhibitors will tip the vessels into either a pro- or anti-angiogenic state. The paradigm that control of angiogenesis relies on a balance between promoters and inhibitors was first described by Judah Folkman et al., who discovered that primary tumor cells produce factors capable of promoting angiogenesis in their local vascular bed, while angiogenesis was inhibited in the vascular bed of metastases in the same patient, a process that may be explained by local differences in the balance between promoting and inhibiting factors. For angiogenesis to occur, an angiogenic switch must be in place where signals from promoters exceed those of inhibitors (13).

Angiogenesis is often initiated by vasodilation of existing vessels with an increase in vascular permeability. The best known promoter of permeability and vessel growth is vascular endothelial growth factor A (VEGF-A). Induction of this factor has been demonstrated in areas of local hypoxia through stimulation by hypoxia-inducible factor-a (HIFa), but VEGF-A has also been found around mature and stable vessels where angiogenesis does not occur, implying that VEGF-A is both an angiogenic promoter of and survival factor for vascular endothelial cells (14). VEGF-A promotes vessel growth by binding to the VEGF 1, 2 and 3 receptor sites found on the endothelial cell surface, where they mediate an increase in vascular permeability through redistribution of cellular adhesion molecules such as PECAM-1 and vascular endothelial cadherin. This increased permeability leads to leakage and extravasation of macromolecules and proteins, while also promoting endothelial cell migration, mitosis and division and preventing apoptosis (15). Following extravasation of macromolecules and fluid, degradation of the ECM in the stroma takes place, which enables migration of new endothelial cells. Degradation of the ECM also liberates growth factors previously bound therein, such as bFGF, TGF-b and IGF-1, and subsequently stimulates endothelial cell migration into the tissue (16).

Angiogenesis occurs through four different processes: sprouting (i), elongation (ii), intussusception (iii) and incorporation of circulating endothelial progenitor cells (iv). The new vessel is then stabilized through creation of a new basement membrane and recruitment of mesenchymal cells.
In smaller vessels and capillaries the recruited mesenchymal cells are usually pericytes, and in larger vessels smooth muscle cells, which enable the vessel to regulate contractility, blood flow and permeability (17). Regulation of vessel growth and the switch to vessel maturation is mediated by angiopoietin-1 (Ang1) and its binding to the tie-2 receptor (18). It is not known which of the four angiogenic processes is dominant in the endometrium. Markers for tip cells, a special cell involved in vessel sprouting, have not been found in isolated endometrial tissues, which may indicate that the endometrium instead uses the other three pathways to a higher extent during angiogenesis (19). To stabilize the newly formed vessel tubes, the endothelial cells produce PDGF-B and TGF-b to attract supporting mesenchymal cells. These cells migrate into the vicinity of the new basement membrane, where they help to build up the new basal membrane and support the endothelial cells. Vessels possessing a basement membrane and recruited mesenchymal cells are considered to be mature (20).

![Picture 3: Angiogenesis in the human endometrium (21) ©](image)

Immunohistochemical studies have shown that endometrial VEGF-A is found in the luminal and glandular epithelium, with increased expression during the secretory phase compared with the proliferative phase. The principal receptor, VEGF-R2, is mainly expressed on endothelial cells in the endometrium during the proliferative phase with a second peak in the mid-secretory phase. These findings indicate that VEGF-A plays a crucial role for angiogenesis of the human endometrium (22).
The effect that VEGF-A has on vascular permeability also increases expression of prostaglandins and nitric oxide in the endometrium, both of which are known to be vasodilators and chemotaxins for immune cells (21).

1.1.3 Cancer inheritance

The first description of biological inheritance of traits can be traced back to Moravian monk Gregor Mendel, who discovered that the color of his flowers could be predicted by imagining that the two parent flowers each passed on two copies of the same trait, which could be either dominant or recessive. After the discovery of DNA and subsequent whole genome sequencing, we now know that what Gregor Mendel described was two gene variants, alleles, of the same gene that coded for pigment in his flowers. In the case of a dominant allele, only that gene is used for mRNA transcription and protein synthesis, while the recessive allele remains dormant, unless both copies are of the recessive allele, which would then result in the color of the recessive phenotype in that flower. To better describe this phenomenon, biologists use the term autosomal dominant inheritance for phenotypes in which one dominant allele copy found on one of the non-sex chromosomes is sufficient to produce that phenotype, whereas autosomal recessive inheritance require two copies to do so.

In the case of known cancer syndromes, where a mutation in one specific gene increases cancer risk, the rule is that there is only one mutated allele from one parent, and one normally functioning allele from the other. This differs from Mendelian inheritance since the healthy allele initially functions as the dominant allele, and results in a normal phenotype for that individual during the first decades of life. However, should the healthy allele acquire a somatic mutation later in life from environmental damage to the DNA or by coincidence, there would be no normal backup and the cell would become susceptible to developing cancer. This is called the two-hit hypothesis (23), and explains how inherited cancer syndromes can have an autosomal dominant pattern of inheritance clinically, but requires two non functioning alleles on the cellular level in order for the cancer phenotype to be displayed (24). Many of the known cancer syndromes have mutations in genes that code for the proteins that are responsible for repairing and protecting the DNA strand during and after DNA replication.
1.1.4 Proofreading of DNA

DNA proofreading is a three-step process, occurring at different times during replication. The first proofreading step is carried out by DNA polymerase. Since attachment to the correct base pair is energetically more favorable, the affinity of polymerase to incorporate a correct new base is higher. Once the nucleotide bases have become attached, the polymerase must tighten its grip around the chain to facilitate covalent bonding of nucleotides to the skeleton of the chain, after which the polymerase can proceed to the next base pair. The entire process is also more efficient when correct base pairs become attached (24, 25).

Exonuclease is responsible for the second proofreading. The mode of action is dependent on the fact that DNA polymerase will not add another base to the daughter chain if the most recently attached base does not display a 3′-OH tail onto which the next base can attach. For DNA replication to continue, the exonuclease will remove the latest inserted base on the daughter strand until a 3′-OH tail is displayed on the active site of the polymerase, which can then continue to add a new base (26).

![Diagram](nature_reviews_immunology.png)  
Picture 5: The MMR system and its recognition of mismatch errors in the DNA-strand (27) ©

The Mismatch-repair (MMR) system carries out the third and final proofreading once DNA strand replication is complete. The process by which this occurs was discovered in mutant bacteria that lacked functional exonuclease, but surprisingly had a lower than expected mutation rate. The system recognizes sites of potential strand distortion that occur around incorrectly matched base pairs where they form a nick in the DNA strand. The system must also distinguish between the daughter strand and the parent strand, since it would otherwise correct the wrong strand in 50% of cases, thereby creating mutations at the same rate.
Eukaryotic cells contain natural *single strand breaks* in the daughter strand that are subsequently sealed by DNA ligase. MMR probably relies on these strand breaks as a recognition marker for the daughter strand. However, this theory only explains recognition of the lagging daughter strand, thereby implying that single strand breaks must be created in the leading daughter strand since it does not acquire them naturally in the replication process according to current knowledge, but the underlying mechanism is unknown (28).

1.2 Clinical diagnosis

1.2.1 Heavy menstrual bleeding (HMB)

About 20-30% of women of reproductive age experience excessive menstrual bleeding, known as heavy menstrual bleeding (HMB) (29). HMB has traditionally been defined as menstrual bleeding in excess of 80 ml per menstrual cycle (30). HMB is the fourth most common cause for gynecological referral in the developed world (31). A national cost estimate for diagnostics, treatment and supportive care for HMB in the UK in 2000 was approximately GBP 65 million.

The etiology of HMB can be subdivided according to the PALM-COEIN classification system, an acronym that stands for polyp, adenomyosis, leiomyoma, malignancy, coagulopathy, ovulatory, endometrial, iatrogenic, and not yet classified (32). The PALM diagnoses can be made based on imaging studies or tissue biopsies, while the COIEN diagnoses are attributable to causes that cannot be imaged or diagnosed based on histopathology (32). HMB may present at any time during a woman’s reproductive years and during any phase, both ovulatory and anovulatory, of the menstrual cycle, depending on its etiology (31). HMB prevalence increases with age because of the higher incidence of anovulatory cycles as women approach menopause. An estimated one of three women suffers from HMB during perimenopause (33). Women who develop HMB later in their reproductive life have secondary HMB, while women suffering from HMB from the time of menarche have primary HMB (31).

The clinical challenge when assessing HMB, aside from identifying the underlying cause, is to measure actual blood loss during the menstrual cycle. The gold standard for this determination is the alkaline haematin method in which all soiled sanitary products are collected and incubated, after which conversion of hemoglobin to haematin is measured with a spectrophotometer to provide an estimate of blood loss (34). For obvious reasons this method is difficult to use in everyday clinical practice.
Instead, the Pictorial Blood Assessment Chart (PBAC) is used, where patients themselves fill in the number of sanitary products and degree of blood soiling, based on which an estimate of blood loss can be calculated. A score of >100 on the PBAC has a sensitivity of >80% for HMB (35). Although not strictly objective, the simplicity and affordability of the PBAC have made it the most commonly used tool to measure blood loss in HMB.

![PBAC Chart]

Picture 4: The PBAC. A score >100 during a menstrual period is considered to be diagnostic for HMB (36).

Clinical management of women with HMB depends on the underlying cause, but some pharmacological and surgical tools can be used for all HMB to reduce blood loss regardless of etiology. Pharmacological treatment can be further subdivided into hormonal or hemostatic.

Hormonal treatment includes progestin-releasing intrauterine systems, combined oral contraceptives, cyclical oral progestogens or progestin-only contraceptives. The main mechanism by which these treatments function is to increase progesterone activity on the endometrium, which stabilizes the growth of tissue as previously described. Many studies have shown that these treatments are generally effective at reducing blood loss, especially in intrauterine systems, where a 70% reduction in PBAC score is seen in the first three months of use, with a sustained effect after four years in a large randomized control study (37).
Intrauterine devices have been proven to be more efficacious than oral contraceptives in decreasing blood loss and are usually well-tolerated by patients (38, 39), although hormonal side effects may occur, including irregular spotting, breast tenderness and pain (36). However, this approach is not an option for women who wish to become pregnant. The most commonly used hemostatic treatment for HMB is tranexamic acid, which prevents fibrinolysis of blood clots. A large meta-analysis of tranexamic acid treatment used for 5 days showed a reduction in blood loss during the menstrual period ranging from 34-56% (37).

Surgical treatment of HMB is usually reserved for women who do not achieve an adequate response to the pharmaceutical options described. Surgical intervention for HMB is quite common; for example, the rate of surgical treatment for HMB was 14.3 out of 10 000 women in the UK in 2009 (40). Alternative surgical treatments include endometrial ablation or hysterectomy. Ablation technique has improved over the years, with fewer complications and less need for secondary ablations later on (41). It should be noted, however, that a comparison between ablation and intrauterine contraceptive devices has shown no significant benefit in long-term outcome for treatment of HMB (42). Hysterectomy is the definitive treatment when all other options have failed. In recent years, the number of hysterectomies for HMB has dropped due to improved first-line treatments. For example, in the UK the number of hysterectomies for HMB decreased from 20 000 in 2000 to 7000 in 2005, which is in line with the current rate for this procedure (43).

1.2.2 Endometrial cancer (EC)

Endometrial cancer (EC) is the most common cancer of the female reproductive tract in the developed world with an age-standardized incidence rate of 13.6 per 100 000 women in 2012 in Europe (44). EC usually occurs in postmenopausal women; more than 90% of cases are diagnosed after age 50 with a median age of 63 years, although 4% of cases occur prior to age 40 (45). Because most cases are diagnosed in response to occult postmenopausal bleeding, the majority of patients are diagnosed at FIGO stage I, with an excellent five-year survival rate (>95%), when surgery is the only treatment necessary. However, survival rates decrease when there is regional spread or metastatic disease, associated with five-year survival rates of 68% and 17%, respectively (44). Worldwide, there are approximately 74 000 deaths per year from this disease (46).
The old classification system divided EC into two different subtypes based on histopathological appearance; type I was known as endometroid EC, while type II was based on histology of non-endometroid origin such as serous, clear cell, and undifferentiated tumors. Recent advances in tumor biology now enable classification of EC tumors into four different groups based on biological tumor characteristics; 1) POLE (ultramutated) tumors, 2) MSI (microsatellite instability) tumors, 3) TP53 mutated tumors, and 4) remaining tumors. The new classification system correlates better with tumor behavior and long-term prognosis (47).

Many known environmental risk factors have been linked to EC, all of which are associated with higher exposure of endometrial tissue to estrogen. The best known risk factor is obesity, with a relative risk of 2.21 in obese women compared with controls, found by a large meta-analysis (48). Additional risk factors associated with higher estrogen exposure and thereby higher risk of EC include nulliparity, early menarche, late menopause, estrogen hormone treatment and polycystic ovary syndrome (49).

EC due to genetic predisposition is a well-established phenomenon linked to germline mutations in the MMR genes, the most common of which is Lynch Syndrome (LS). Other germline mutations that increase risk of EC include mutations in the BRCA1/BRCA2, PTEN, ATM and CHEK2 genes (50). Based on data obtained from unselected cases of EC, it is believed that approximately 10% of all ECs result from one of these germline mutations, with LS being responsible for approximately 5% of all EC cases. What clinically sets LS apart from other germline mutations is earlier age at diagnosis, with half of all LS-associated EC tumors detected before age 50, and location within the lower uterine segment (LUS) to a higher extent (50).

1.3 Angiogenetic factors in endometrial disease

1.3.1 Known angiogenetic factors in HMB-E

A study by Kooy et al. observed an increased proliferation of endometrial cells in HMB-E compared with controls, suggesting an imbalance in angiogenic factors within the endometrium (51). Other studies have shown a lower number of mesenchymal cells surrounding microvessels in HMB-E. Moreover, a previous study by Mints et al. showed that small endometrial vessels demonstrate abnormal morphology in HMB-E, with an increase in number and size of endothelial cell gaps lining the vascular lumen (52).
This lack of maturation has been hypothesized as a possible explanation for HMB-E since a lack of contractile cells surrounding the vessels would lead to an inability to restrict blood flow when needed and might prevent the distal hypoxia necessary for the shedding process (53). Abnormal activation of MMPs and their tissue inhibitors of metalloproteinases (TIMPs), which could impair the controlled breakdown of endometrial tissue during menstruation, has been observed in women with HMB-E (54). Other studies of women with HMB-E have also shown down-regulation of Ang-1, which could affect maturation of vessels (55).

Yet other studies have shown that the ratio between vasoconstrictors and vasodilators is altered in HMB-E, with an increase in the vasodilators PGE and VEGF-A, while there is also a lack of vasoconstrictors such as PGF and ET-1 in the endometrium of women with HMB-E (56). It is not yet clear whether all of these changes occur in all cases of HMB-E, or whether a single imbalance is sufficient to alter control of menstrual bleeding, nor has the actual underlying etiology for HMB-E been determined.

1.3.2 Known angiogenetic factors in EC

In order for a tumor to grow to a size greater than 1-2 mm it must develop new blood vessels. These vessels are important for metastasis of the tumor at a later stage. The most extensively studied angiogenic factor in cancer in general and in EC in particular is VEGF-A. VEGF-A is overexpressed in about 2 out of 3 EC tumors, and a higher level of expression correlates with greater likelihood of metastasis and poor clinical outcome (57-59). Because it is known that VEGF-A is overexpressed, especially in advanced disease, VEGF inhibitors have been studied in trials and used in the clinical setting for patients with advanced EC, but unfortunately a high number of tumors become resistant to this treatment (60). Some studies also recommend VEGF-A as a serum marker in advanced EC to monitor the effects of chemotherapy (61). A new approach in recent trials is to inhibit VEGF-A and certain additional axes in endometrial angiogenesis, such as the mTOR intracellular protein, which is known to be a key regulator for activation of HIF-a (62).

Other angiogenic factors that have been studied and correlated to tumor stage in EC include MMPs 2 and 9, which are also involved in the physiological breakdown of the endometrium during menstruation. Both MMPs are independently involved in angiogenesis and metastasis in EC, but which of them plays the more significant role remains controversial (61). It is known that both MMPs are usually overexpressed in EC tumors in general, and in type I tumors in particular (63), although the significance of this overexpression is unclear.
Some studies have reported one or the other of them to be poor diagnostic markers of histologically poorly differentiated tumors and aggressive tumor invasion respectively (64, 65) while others have found them to correlate with clinical outcome but not with tumor differentiation or invasion (66). Recruitment of epithelial progenitor cells (EPCs) from the bone marrow and bloodstream has also been shown to be essential for growth of tumor microvessels (67).

1.3.3 SDF-1 and angiogenesis

Stromal Cell-derived factor-1 (SDF-1) (or CXCL12) is a chemokine that binds to the CXCR4 receptor on target cells. SDF-1 is most abundant in the ECM in liver, spleen, pancreas and heart, but is found in all human tissues (68). The SDF-1/CXCR4 signaling axis is known to be of central importance in preserving the hematopoietic stem cell niche (69) and to act as a homing beacon for blood cells to the bone marrow (70). In peripheral tissues CXCR4 has proven to be the most expressed chemokine receptor on endothelial cells, and the interaction with SDF-1 is fundamental to angiogenesis since knocking out one or both factors in mice results in a lack of vessels, especially in the gastrointestinal tract (71). Studies have also shown that expression of CXCR4 can be upregulated by other angiogenic factors such as VEGF-A, TNF-a and bFGF, ultimately making the cells more sensitive to SDF-1 (72). The observed effect on endothelial cells after SDF-1 exposure is to increase production and release of angiogenic factors (VEGF-A and bFGF), which ultimately leads to a positive feedback loop with a further increase of production of CXCR4 and SDF-1 in the endothelium (73). It has been suggested that this positive feedback loop is important for maintaining endothelial structure and survival during angiogenesis. A growing body of evidence has found that SDF-1 plays an essential role in vascular injury and in recruitment of progenitor cells from the bloodstream to injured tissue. This is done through upregulation of cellular adhesion molecules (CAMs) on the endothelial cells and selectins present on the vascular endothelium of the injured tissue (74).

The properties associated with the SDF-1/CXCR4 axis have been shown to be vulnerable to hijacking by pathological processes, especially cancer where SDF-1 overexpression is usually a negative prognostic factor (75). First of all, the feedback loop between VEGF-A and SDF-1 signaling creates a strong angiogenic signal in areas of local hypoxia, resulting in more efficient recruitment of new vessels to these parts of the tumor, while the ability of SDF-1 to attract and keep ECs in the surrounding tissue helps to preserve the structure of the vessels as the tumor expands (76).
1.3.4 Endothelial progenitor cells (EPCs)

EPCs are phenotypically defined as a cell population expressing CD133, CD34 and VEGFR2 surface markers, although this definition is currently under debate (77). Previously, it was believed that this cell population originated exclusively from the bone marrow, but recent evidence shows that these cells can also originate from nonhematopoietic tissues such as liver, spleen and adipose tissue (78). When a vascular injury is signaled, these cells are released into the bloodstream and incorporated into vascular walls, where they mature into endothelial cells (79). Recruitment of EPCs to sites of vascular injury has been demonstrated to result from signals of local hypoxia, which induce increased production of SDF-1 in the injured tissue. SDF-1 then travels through the bloodstream to the bone marrow, where it enables release of EPCs into the bloodstream (80). Therefore, binding of SDF-1 to the CXCR4 receptor on the EPCs is essential for attracting EPCs to the injured tissue (81).

During the maturation process of EPCs into ECs with incorporation into the vascular wall, they secrete proangiogenic factors that ultimately result in vessel growth with resolution of local hypoxia. This ability of SDF-1 and EPCs to respond to local hypoxia have made them a promising target for medical treatment of cardiovascular disease, but unfortunately without long-term clinical effect, mainly because of an inability to mimic physiologic recruitment of EPCs into the hypoxic tissue (82). Exactly how the EPCs are recruited to the endometrium is not completely clear, although some studies have shown that estrogen and progesterone have a stimulating effect on EPCs. A study by Matsubara et al. measured the number of EPCs cultured in vitro from peripheral blood taken during the bleeding, proliferative and secretory phases, which showed a significantly higher number of cultured EPCs in bleeding phase blood stimulated with estrogen and progesterone (83). Furthermore, additional studies have demonstrated that estrogen and progesterone stimulate EPC recruitment and survival, an effect which also seems to be mediated by the SDF-1/CXCR4 axis (84-86).

1.3.5 The pericyte

The pericyte was first discovered in 1873 as a cell that was wrapped around capillary vessel walls (87). Pericytes are indeed present in smaller arterioles and capillaries, but are mostly found in post-capillary venules. Pericytes display filaments with contractile properties such as alpha-smooth muscle actin (αSMA), a similarity shared with smooth muscle cells, although some characteristics set them apart, such as pericyte attachment along the longitudinal axis of the vessel with multiple extensions into the cytoplasm of the basement membranes or directly into the endothelial cells (88).
Pericyte coverage varies greatly among different tissues. In the capillaries of the central nervous system, there is a 1:1 ratio between endothelial cells and pericytes, while the ratio is approximately 1:100 in peripheral tissues, such as smooth muscle, indicating a role in vessel barrier function (89). The lifetime of endothelial cells in any given tissue correlates with pericyte coverage; higher coverage is associated with longer lifetime, indicating the importance of the support role to endothelial cells (90). A difference in pericyte coverage can also be found between the lower and upper body, with higher coverage in the lower body, indicating a regulatory function on blood pressure at the capillary level (89).

Pericytes have been shown to play an active role in the process of angiogenesis. First of all, these cells have the ability to produce and secrete MMPs that help to degrade the basement membrane and surrounding tissue, thereby enabling new vessels to sprout (91). When new EC tubes penetrate new tissues, they are unstable with low barrier integrity. The new ECs then secrete PDGF-b, which attracts pericytes to the new vessel, thereby initiating construction of a new basement membrane and ultimately stabilization of the vessel (92, 93). Meanwhile, the newly recruited pericytes begin to secrete inhibitory angiogenic factors such as TGF-b and Ang-1, which stops angiogenesis once vessel maturation has been achieved (35). In vivo studies on diabetic retinopathy have shown that one of earliest signs of dysfunctional vascular integrity, even before formation of pathological vessels, is a significant loss of pericytes (94). Just how local hyperglycemia leads to pericyte loss is not fully understood, but it is known that levels of PDGF-b in the retina decrease, while Ang-2 levels increase (95, 96), both of which act as a proangiogenic signal, while also inhibiting pericyte recruitment and vessel maturation. In conclusion, all evidence points to the pericyte as a key player in angiogenesis and even more importantly in the next step of the process where vessel maturation needs to occur.

1.4 Hereditary factors in endometrial disease

1.4.1 Hereditary HMB-E

Some past studies have looked for a genetic predisposition for developing HMB-E. The first study by Sneider et al. found that the rate of hysterectomies for the indication of HMB-E was higher among first degree relatives of women who also underwent surgery for HMB-E (97). A subsequent study found high clinical overlap between the diagnosis of HMB-E and family history of HMB-E among first degree relatives (98).
The reason for these findings has not yet been studied. Results from studies on excessive angiogenesis in diabetic retinopathy have shown a link between certain polymorphisms in the VEGF-A gene and risk of developing retinopathy given similar blood sugar control, which indicates that inherited differences in the VEGF-A gene may play a crucial role in whether angiogenesis is physiological or pathological (99, 100).

1.4.2 Lynch Syndrome (LS)

LS, also called HNPCC, was first described in 1966 by Henry Lynch when he detected a hereditary pattern for cancer in two large families with strikingly similar carcinomas that developed at an unusually early age for cancer and demonstrated a clear autosomal dominant pattern of inheritance. Carcinomas of the colon and the endometrium were the most commonly found diseases in these two families (101). Today it is known that patients with LS are at increased risk of malignancy in various tissues, including colon, endometrium, stomach, small bowel, biliary tract, pancreas, urinary tract, bladder, ovary, brain, prostate and breast (102).

Genetically, four different mutated genes comprise the clinical syndrome, in which one mutation singlehandedly causes the Lynch phenotype with varying degrees of susceptibility for different cancers. The four genes are MLH1, MSH2, MSH6 and PMS2. These genes code for the proteins involved in single-nucleotide repair of DNA during cell replication known as the MMR-system which function has been previously described. The DNA in affected cells will eventually display MSI due to an inability to correct the single nucleotide errors that are constantly acquired during DNA strand replication, thereby leading to an accumulation of additional single nucleotide base pairs at certain loci in the genome which are referred to as microsatellites (103). Individuals who inherit the trait for this cancer syndrome usually have only one mutated gene copy (allele) from one parent, and one functioning allele from the other. However, if the healthy allele develops an acquired somatic mutation the cell lineage will display the hazardous phenotype, following the rule of the two-hit paradigm, which has also been previously described (23).

1.4.3 Clinical and laboratory screening methods for LS

Clinical screening for LS is based on the Amsterdam II (AmII) and revised Bethesda Criteria. These criteria have been updated since previously they did not take cancers other than Colorectal Cancers (CRC) into consideration, and therefore lacked sensitivity to other cancers associated with the syndrome.
All AmII criteria must be met in order for the patient to be considered positive and a candidate for germline gene testing of MMRs to determine LS status. Concerning the Bethesda criteria, fulfilling one criterion is sufficient to pursue further laboratory studies to confirm an MSI tumor as a next step, and if positive, to continue with further testing of germline DNA.

**Amsterdam II (Am II):**

1. Three or more relatives with an *LS associated cancer* (CRC, EC, small intestine, ureter or renal pelvis etc)
2. Two or more successive generations affected
3. One or more relatives diagnosed before the age of 50 years
4. One should be a first-degree relative of the other two
5. FAP should be excluded in cases of colorectal carcinoma
6. Tumors should be verified by pathologic examination (104)

**Revised Bethesda criterias**

1. Colorectal or uterine cancer diagnosed <50 years of age
2. Presence of synchronous or metachronous LS associated cancers regardless of age
3. CRC with MSI-histology in a patient <60 years of age
4. CRC diagnosed in one or more first-degree relatives in a patient with a LS-associated tumor, with one of the cancers being diagnosed before 50 years of age.
5. CRC diagnosed in two or more first or second degree relatives in a patient with a LS-associated tumor regardless of age (105).

However, 25% of LS-carriers presenting with their first tumor will not meet the Am II criteria at the time of diagnosis, which makes these patients easy to miss (106), and only 54% of LS-carriers with EC meet the Am II criteria. Patients presenting with EC as their first cancer (sentinel cancer) are especially easy to miss (107). The sensitivity of the Bethesda criteria is higher at approximately 90%, but their positive predictive value is only 3-5% because of the many false positives (108). Because of this limitation in clinical screening, many recent studies have attempted to implement universal laboratory screening for EC in order to improve the detection rate. The primary pathological screening method currently in use is immunohistochemistry (IHC) on MMR proteins in the tumor. When MLH1, MSH2, MSH6 and PMS2 antibodies are used simultaneously, IHC has 91% sensitivity and 83% specificity for detection of MMR protein loss (109, 110). Confirmed MMR protein loss or MSI-H tumor together with one positive Bethesda criteria have a positive predictive value of 25-30% for finding an LS mutation in the germline DNA (108).
The second option is determination of MSI by PCR analysis, which strongly correlates with IHC testing. Depending on the number of microsatellites the PCR detects, the answer will be either microsatellite stable (MSS) which means no instability at all, or microsatellite low (MSI-L) meaning that there are some but few microsatellites, or microsatellite high (MSI-H) which means that the tumor displays a MSI phenotype.

1.4.4 LS associated EC

Henry Lynch described the high susceptibility for EC in female family members of LS patients in the first paper in which he described LS syndrome. Lifetime risk for EC in the general female population is approximately 2.6%, while EC risk in LS ranges from 42% to 54%. EC associated with LS accounts for 2.5-5% of all EC tumors. The incidence of EC in the general population rises with age until age 70, after which it steadily declines. The ages in the Lynch group are different; mean age at diagnosis is 48 years and over 50% of all patients are diagnosed before age 50 (111).

Studies of the various genes involved in LS show clear differences in EC risk associated with different genes, as well as different mean ages at diagnosis. The lifetime risk of EC associated with the MSH6 mutation is 64-71%; MSH6 mutation carriers are most prone to develop this malignancy. The lifetime risk for EC in MLH1/MSH2 varies between 40-50%. Mean age at time of diagnosis of EC also varies between 48-51 years for MLH1/MSH2 and 56-62 years for MSH6 (109).

Histological classification of EC tumors is the same in the LS group as in sporadic cases of EC; 80% are endometroid and 20% non-endometroid. However, the histopathological features of ECs related to LS are usually more diverse than those seen in sporadic tumors and may include both endometroid and non-endometroid malignant histology within the same tumor. MSI-H status correlates with an increase in: mucinous features, signet ring cells, number of tumor-infiltrating lymphocytes and inflammatory activity at the tumor periphery (112). Moreover, LS tumors are more likely to involve the LUS (111) and 34% of LUS ECs demonstrated MSI-H, while 29% of these MSI-H tumors were associated with a LS mutation, as reported in a study of EC involving the LUS linked primarily to MSH2 mutations (50).
Currently, the National Comprehensive Cancer Network (NCCN) in the US recommends that LS testing be considered in all patients under the age of 55 diagnosed with EC, regardless of clinical criteria fulfilment. Many centers already screen for LS on all CRC tumors newly diagnosed before age 60 using IHC for MMR protein loss without undertaking further clinical triage of patients, but not on ECs routinely (113).

1.4.5 Additional hereditary factors in EC

LS is the best-known hereditary cancer syndrome linked to a high incidence of EC. As previously mentioned, there are other cancer syndromes in which the incidence of EC is higher than in the normal population. Once these known mutation carriers are excluded from the EC population, researchers still observe a twofold increase in risk of developing the disease among women with a family history of EC in a first or second-degree relative compared with the remaining female population without a family history of EC (114).

This over-representation cannot fully be explained solely by environmental factors such as obesity and estrogen exposure, which would probably have a more equal effect on the entire female population. Nor is there a linear relationship between incidence of EC and incidence of obesity, as would be the case if the increased incidence of EC depended solely on increased obesity (115). These observations suggest that other hereditary factors are involved in development of EC, not just single gene mutations or environmental risk factors. Certain single nucleotide polymorphisms (SNPs) in the genes involved in estrogen production, signaling and degradation have already been found to be associated with EC risk, such as in the genes ER-a, CYP17A1 and CYP 19A1 (116-119). Recent Genome Wide Association Studies (GWAS) (120-122) analyzing SNPs have already identified several EC risk loci in the human genome. These taken together explain <5% of relative family risk for EC development, but it should be noted that most of these studies were conducted in heterogeneous populations, which makes it more difficult to draw conclusions on how much overrepresentation of single SNPs contribute to cancer risk in the next generation of a homogenous population. Therefore, many current GWAS are based on analysis of haplotype instead.

Haplotypes can be described as a specific consecutive combination of SNPs found in close proximity to each other on a chromosome; they are preserved through many generations because they are not usually affected by large relocations of the genome during homologous recombination cycles. This is why haplotypes are often used as markers for ethnicity (123).
Haplotypes in close proximity to genes that are known to be involved in EC development have already been found, which helps explain the differences in EC risk between different ethnicities (118, 124, 125). Other GWAS have also found certain variations in gene loci within the HNF1B and TERT-CLPTML1 genes associated with an overall increased risk of cancer, including for EC (126, 127).
1.3 Aims

1.3.1 General aims of the thesis

The overall aim of this thesis is to contribute knowledge about the hereditary and angiogenic endometrial factors involved in both HMB-E and EC.

This has been done by investigating the cellular and molecular processes of endometrial angiogenesis that are involved in HMB-E, as part of a search for further evidence in support of HMB-E as a disorder of angiogenesis. The hereditary aspects of EC were investigated by examining a known high-risk group of women (LS carriers) and also by assessing cases from the EC population at large with a family history of EC, but without hereditary cancer syndrome, all of which could help to reveal new genetic risk markers for the disease.

1.3.2 Study I: aims

The aim was to investigate whether levels of stromal cell derived factor-1 (CXCL12), and numbers of circulating EPCs, EPC colony-forming units (EPC-CFU) and mature endothelial cells differ between women with HMB-E and controls, and whether there is any correlation between these and plasma levels of other known angiogenic growth factors. The results could explain the importance, if any, of EPCs to angiogenesis in the endometrium.

1.3.3 Study II: aims

The objective was to conduct a prospective clinical study to investigate whether endometrial capillaries and microvessels in patients with HMB-E have less vascular pericyte coverage and thereby increased vascular fragility, and whether there is any correlation between pericyte coverage and expression of VEGF-A in endometrial vessels. The results may elucidate whether or not alterations in vessel maturation cause HMB-E.

1.3.4 Study III: aims

The purpose of this study was to assess the diagnostic modalities for gynecological screening of LS patients used in Sweden today and the clinical outcome achieved thus far using a retrospective study approach. The study could show whether screening visits are important for prevention of higher stage cancer and what diagnostic tools to recommend.
1.3.5 Study IV: aims

The aim of this study was to investigate a Swedish population group to determine whether there is an overrepresentation of certain haplotypes within the genome of patients with EC who have a family history of EC but not hereditary cancer syndrome. The results could help to explain the higher incidence of EC in certain populations and to more effectively predict and prevent EC in the future.
2. METHODS

2.1 Study I

2.1.1 Study design, population and data collection

Patients and controls were enrolled consecutively to avoid selection bias. The diagnosis of HMB-E was based on an accurate bleeding history and the pictorial blood loss assessment chart, where a score of >100 ml was considered to be HMB-E. Women in the control group were selected based on the pictorial blood loss assessment chart, where a score of <80 ml was defined as normal menstruation. To exclude intrauterine pathology, all participants were required to have a normal transvaginal ultrasound (TVUS) and gynecological examination. Blood samples from the HMB-E patients were tested for platelet count, APTT, INR and bleeding time to exclude coagulation defects.

Ten healthy control women and ten HMB-E patients, all of whom had regular menstrual cycles (25-32 days) and no hormonal contraception within three months prior to study start, provided four blood samples during a single menstrual cycle: two in the proliferative phase, one at ovulation and one in the secretory phase. The phases of the menstrual cycle were determined by using the first day of menstruation as the starting point, and by measuring the estradiol and progesterone levels in the blood samples obtained. The day of ovulation was determined by a urine test for luteinizing hormone (LH). The first blood sample was collected on day 1-2, the second on day 5, the third on the day of ovulation (day 14) and the fourth on day 22-24. First, the blood samples were used to isolate peripheral blood mononuclear cells (PBMCs) and to culture EPCs, after which the remaining blood was centrifuged (1000g) for 15 minutes and the plasma was then frozen at -70 C before conducting an analysis.

Plasma from all four samples was analyzed to measure levels of SDF-1, VEGF-A and bFGF, as well as granulocyte and granulocyte-macrophage colony stimulating factor (GM-CSF) using ELISA (R&D systems, Minneapolis, USA), all according to manufacturer instructions. A kinetic microplate reader, with an intra-assay coefficient ranging between 3-9%, measured the growth factor level on a single plate for each growth factor tested.
2.1.2 Flow cytometry and cell culture

To determine PBMC phenotype for selection of EPCs, the flow cytometry test used an array of antibodies targeting specific markers for EPCs (VEGFR-2, VEGFR-1, Tie-2, CD133, CD146 and CD34), as well as markers for mature circulating ECs (CD141, CD144 and CD31). Homeostatic regulating molecules, including vascular adhesion protein-1 (VAP-1), CD142, heparin sulfate proteoglycan (HSP) and plasminogen activator inhibitor-1 (PAI-1), were also targeted in the flow cytometry test. The following antibodies used were for the test: anti-VEGFR-2, anti-CD141, anti-PAI-1, HSP (Biogenesis Ltd, Poole, UK), (Reliatech, Mascheroder, Germany), anti-VAP-1 (Serotec, Dusseldorf, Germany), anti-VEGFR-1 (R&D Systems), anti-CD133 (Miltenyi Biotec, GmbH, Bergisch, Germany), Tie-2, CD34, CD144, CD31 and CD142 (Becton Dickinson, San Jose, CA, USA). To determine non-specific binding of monoclonal antibodies, corresponding control isotypes were used. The test was conducted using a fluorescence-activated flow cytometer (FACSorter, Becton Dickinson).

The PBMCs found in the flow cytometry test were then cultured on fibronectin-coated (20 mg/ml) tissue culture plates in an endothelial-selective medium (EndoCult, Stem Cell Technologies, Vancouver, BC, Canada). The incubator had the following settings: 78.09% nitrogen, 20.95% oxygen, 0.93% argon, 0.039% CO2, 37°C, and 95% humidity. On day 2 of culturing, the non-adherent cells were collected from the initial plates and placed in a second set of fibronectin-coated plates. The final CFU count on both sets of plates was done on day 5.

2.1.3 Statistics

For statistical analysis the non-parametric Wilcoxon matched pairs test was used to compare growth factor levels and number of CFUs between plates and at different time points. The Friedman non-parametric test was used to test differences between repeated estimates. Analysis was performed using SPSS software version 18.0, with P<0.05 considered to be significant.
2.2 Study II

2.2.1 Study design, population and data collection

A total of 17 women with a normal menstrual cycle and a history of HMB for <5 years, and 10 healthy women with a normal menstrual cycle who did not use hormonal contraception during the last three months were consecutively recruited from an outpatient clinic for women with HMB. Women with a pictorial blood loss score >100 ml were considered to have a diagnosis of HMB, while women with a score <80 ml served as controls. All study subjects underwent TVUS, as well as blood tests for platelets, INR, vWF and APTT to exclude uterine pathology and coagulopathy, after which a diagnosis of HMB-E could be made. The HMB-E patients in the study were also examined by hysteroscopy to rule out abnormalities.

Blood samples and endometrial biopsies were obtained from each patient at the same time, along with a history concerning the number of days since the last menstruation. Blood samples were analyzed for estradiol and progesterone to help determine the phase of the menstrual cycle. Study subjects provided a urine dipstick LH test to determine the day of ovulation.

This showed that 8 patients and 5 controls were in the proliferative phase, and 9 patients and 5 controls were in the secretory phase on the day of biopsy.

2.2.2 Immunohistochemistry and pericyte quantification

The functional endometrial layer in the biopsy specimen was stained. Endothelial tissues embedded in 5 µm of paraffin were stained with antibodies to CD34 (Cat. no. ABIN343723; QBEnd/10; BioGenex, San Ramon, CA, USA) and a-SMA (SMAα; M0874; with a FITC conjugated IgG2a; Dako, Glostrup, Denmark) to identify endothelial cells and pericytes, respectively. We used Alexa 488 (I36933) or Alexa 568 (Z-25006; Molecular Probes, Eugene, OR, USA) as secondary antibodies, respectively. The Leica Q550IW image analysis system, a Leica DM RXA color video camera, and software based on Leica QWin Image Analysis were used to measure and quantify the endometrial microvessels after SMAα staining. First, microvascular density (MVD), the number of microvessels per high-power field (HPF), was determined. HPFs with a minimum of ten visible vessel lumens per HPF were randomly selected at ten individual spots on each endometrial slide. Thereafter, the number of microvessels covered with pericytes in each HPF was counted and finally summed for the entire endometrial slide.
A microvessel with >50% pericyte coverage of the perimeter was defined as a positive microvessel. Next, ten randomly selected CD34-stained microvessels were imaged with a camera on each slide using an x63 oil immersion lens.

### 2.2.3 Transmission electron microscopy

Smaller sections of the endometrial biopsies were fixed for 30 min at room temperature, and then for 24 h at 4°C using 2% glutaraldehyde + 0.5% paraformaldehyde in a 0.1 M sodium cacodylate buffer containing 0.1 M sucrose and 3 mM CaCl₂, pH 7.4. The specimens were then rinsed in 0.15 M cacodylate buffer and postfixed by incubating for 2 h in 2% osmium tetroxide in 0.07 M cacodylate buffer containing 3 mM CaCl₂, after which they were dehydrated in an ascending series of alcohol and acetone solutions and finally embedded in LX-112 epoxy resin (Ladd Research Industries, Burlington, VT). The embedded tissue was then cut into ultrathin sections and given contrast by staining with uranyl acetate followed by lead citrate, after which they were examined under a Tecnai 10 (FEI, Eindhoven, Netherlands) transmission electron microscope at 80 kV, where thickness and endothelial volume density were measured. Total microvascular area was defined by the area occupied within the basal membrane, and luminal area as the area surrounded by endothelial cells. A ratio could then be calculated by dividing the luminal area by the total vascular area to obtain an estimate of endothelial volume density.

Two individual observers independently performed the vascular quantifications and measurements in the endometrial slides after reaching agreement on defining HPFs, vascular lumen and positive pericyte vessels by observing sample slides together. Each observer then analyzed each slide at least two times.

### 2.2.4 Statistics

Data in this study are presented as median values using 95% confidence intervals (CIs). The Kruskal-Wallis, Mann-Whitney, and Spearman statistical tests were performed in this study using the Statistica software package to obtain the results. A P-value of <0.05 was considered to be statistically significant.
2.3 Study III

2.3.1 Study design, population and data collection
The study included all female patients with a genetically confirmed germline LS mutation identified between 1994 and 2013 at the department of genetics at the universities in Stockholm, Uppsala, Linkoping, Lund and Gothenburg. Of the 260 women who were identified and asked to participate, 170 agreed. Clinical data from these 170 women with molecularly confirmed LS were collected. Data including gynecological LS screening history, biopsy results (if any), genetic records, number of screening visits, results from screening including TVUS, endometrial biopsy (EB) and blood test for tumor marker cancer antigen (CA) 125, as well as prophylactic surgery including age at procedure, and the setting from which the screening data derived were all obtained from the medical records. All diagnoses in the study are based on the original pathology reports in the medical records. All TVUSs included assessment of endometrial thickness, uterus size, and examination of the ovaries (size, position, cysts, free fluid, or other sonographic abnormalities). After the data were collected, 160 women remained with sufficient clinical data for inclusion in the study.

2.3.2 Statistics
The Statistica® software package was used to perform statistical analyses in this study. A p-value < 0.05 was considered to be significant. All p-values are reported for all statistical tests in the study because of the small total patient number. The Pearson's chi-squared test, Fisher's exact test and Kaplan–Meier estimator were used to calculate proportional differences between two independent subsets of patients in the study. The Kruskal–Wallis test was used when it was necessary to test more than two independent samples.
2.4 Study IV

2.4.1 Study design, population and data collection

The cases in this study came from two EC cohorts. The first cohort is the Registry of Endometrial Cancer in Sweden (RENDOCAS) which is a hospital-based case-control study cohort. The patients in this cohort (n=520) underwent surgery for pathology-verified EC at Karolinska University Hospital in Solna, Sweden, between 2008 and 2011, regardless of age at diagnosis. For each patient, the following data were previously obtained: basic blood tests with whole genome genotyping, histological reports on tumor samples, detailed family history with individual pedigree based on all substantiated family data collected from the medical records of family members. Patients fulfilling the Amsterdam or Bethesda criteria had previously been screened for LS and the mutation carriers are known. Data on the cohort were also available for relevant environmental factors for EC (BMI, hormonal treatment, parity etc.), collected at the time of diagnosis. All patients included in the RENDOCAS cohort with no known cancer syndrome genotype were included in this study as cases. The remaining cases in the study derive from a cohort collected from ECs diagnosed throughout Sweden in postmenopausal women only, with consecutive enrollment beginning in 1994 (n=2184) (128). The same data as in the RENDOCAS cohort regarding family history, genotyping and exclusion of cancer syndromes were collected.

The controls were collected from the KARMA cohort described in greater detail in previous papers (129). In Sweden, women aged 40–74 are invited every 18–24 months to participate in the national breast cancer screening program. Women who attended mammographic screening at four hospitals in Sweden with negative screening results and no other cancer diagnosis were invited to be included in the KARMA control cohort between January 2011 and March 2013. All patients in the KARMA control cohort were eligible for inclusion in the control group in this study.

2.4.2 Genotyping and quality control

Genotyping was done on DNA extracted from peripheral blood samples of both cases and controls using standard procedure. The genotyping data were merged, taking TOP strand format into account. A total of 9080 individuals (2704 cases and 6376 controls) were processed for Quality Control (QC) analysis. In the first QC round (QC1), heterozygous haploid genotypes were excluded, as were samples with gender inconsistency and same position variants.
After QC1 474,706 SNPs and 9080 individuals (2704 cases and 6376 controls) remained in the data set. A second QC round (QC 2) was then performed, where SNPs with <98% call rate, <1% minor allele frequency (MAF) and SNPs inconsistent with the Hardy–Weinberg Equilibrium (HWE 0.001) in the controls were excluded, ultimately leaving 332,906 SNPs after QC 2. In the third and final QC round (QC 3), a multidimensional scaling (MDS) analysis was conducted on all remaining SNP markers to stratify the population and to identify ethnic outliers. These outliers were excluded from the dataset, while the remaining SNPs were plotted using an MDS plot in an effort to achieve maximum homogeneity between cases and controls. After QC 3 there were 332,906 SNPs and 9,062 individuals (2700 cases, 6362 controls) remaining in the dataset on which GWAS analysis could potentially be performed. For simplicity, we used only 1116 of the endometrial cases and all 5021 Karma controls to calculate the results, since this arrangement provided sufficient statistical power.

2.4.3 Statistics
A logistic regression model was used to examine the association between a single SNP or haplotype and cancer risk. Corresponding OR, standard errors, 95% confidence intervals and P values were calculated using PLINK v1.07. An MDS plot that showed P values sorted by chromosomal position was generated to provide a visual illustration of top association findings across the genome. A P-value of <6.0×10^{-9} was considered to meet genome-wide statistical significance of SNP according to Bonferroni-adjusted P value criteria.

2.5 Ethical approvals
Participation in the four studies was voluntary and all participants were informed about the voluntary nature of the studies, as well as the study aims. Informed consent was obtained from all participants and all data were anonymized immediately after collection.

The Ethical Review Board of Karolinska Institutet South Huddinge, Sweden, granted permission for study I under registration no. 528/03. Study II was approved by the Ethical Review Board of Karolinska Institutet North under registration no. 99-299. The Stockholm Regional Ethics Committee (EPN) granted ethical approval for studies III and IV under registration no. 2010/1536-31/2.
3. Results

3.1.1 Study I

Figure 1: SDF-1 levels at different times of the menstrual cycle. Boxplots show medians and IQRs, as well as maximum and minimum values. The significant difference occurred during the mid-proliferative phase on day 5 between HMB-E patients and controls.

We found that the levels of SDF-1 were significantly lower overall in HMB-E patients by an average of 16% compared with the control group (P = 0.0001). When assessing levels during the different menstrual phases, a significant decrease (P = 0.013) was seen between the mid-proliferative phase and ovulation in HMB-E patients compared with controls. The greatest difference in SDF-1 levels occurred when comparing the mid-proliferative phase between patients and controls (see figure1).

VEGF-A showed a similar decreasing trend as SDF-1 in the HMB-E patients, but it was not statistically significant (P = 0.086), while VEGF-A levels among controls did not change during the different phases of the menstrual cycle (P = 0.473).
Figure 2A: Number of EPC-CFUs in HMB-E patients and controls. Boxplots show medians and IQRs, as well as maximum and minimum values.

Figure 2B: The relationship between SDF-1 levels in blood and number of EPC-CFUs. 

HMB-E patients also had a lower number of EPC-CFUs compared with controls (P = 0.014), showing a positive correlation between SDF-1 level in blood and EPC-CFUs (r = 0.428; P = 0.047); see figures 2A and 2B. We also found that the level of circulating endothelial cells in HMB-E patients was higher than in controls, although this result did not reach statistical significance. In contrast, the level of the EPC marker, CD34, in blood was significantly lower in HMB-E patients than among controls (P = 0.020).

We also investigated the levels of different mature EC markers in peripheral blood and found that the levels of CD144 (P= 0.014), CD141 (P=0.001) and CD31 (P=0.002) were significantly elevated in HMB-E patients compared with controls; see figures 3A and 3B. The EPC marker was significantly lower in HMB-E patients compared with controls (P=<0.0001); see figure 3C. We also looked at homeostatic circulating molecules in peripheral blood and of these, HSP was found to be significantly lower in HMB-E patients than in controls (P = 0.009). In contrast, PAI-1 was higher in HMB-E patients (P=0.004); see figure 3C.
In all, the results in study I show differences in SDF-1 levels between HMB-E and controls, with significant variations during the proliferative phase. SDF-1 levels also correlate with number of EPC-CFUs and number of circulating EPCs in the blood, which suggests a possible explanation for the altered angiogenesis seen in HMB-E.
3.1.2 Study II

Figure 1: Number of stained aSMA-covered microvessels in HMB-E patients compared with controls. The data are presented as means with 95% confidence interval.

Total microvascular density did not differ between HMB-E patients and controls, with a median of 19.5 microvessels/HPF (standard deviation (SD) 6.0; 95% CI) in HMB-E patients compared with 16.0 microvessels/HPF (SD 2.5; 95% CI) in controls, but the number of SMAα-positive microvessels in the proliferative phase was significantly (P=0.005) lower in HMB-E patients than in controls (27 (18-34) 95% CI for patients vs 42 (35-49) 95% CI for controls); see figure 1.

The number of microvessels that expressed VEGF-A was significantly higher in HMB-E patients over the entire menstrual cycle with a median of 17 (16-22) 95% CI for patients and 10 (9-15) 95% CI for controls. The measured luminal perimeter of the endometrial microvessels also showed a significant change during the secretory phase, with a median increase of 9.3 µm ((6.1-14.9) 95% CI) in HMB-E patients compared with 3.6 µm ((1.3-7.2 µm) 95% CI) in controls (p=0.0007).
When assessing pericyte coverage of aSMA-positive microvessels within the control group, a significant decrease in coverage was seen during the secretory phase (P=0.04) compared with the proliferative phase, while among HMB-E patients coverage did not differ between menstrual phases. When correlating coverage of aSMA microvessels with expression of VEGF-A in both controls and HMB-E patients, a significant negative correlation was observed between expression of VEGF-A and pericyte coverage (r=0.8; P=0.04); see figure 2.

The endothelial density as measured by electron microscopy revealed that endothelial cells accounted for a significantly higher percentage of total vessel area compared with controls (median 77.4 % (70.4-81.8) 95% CI vs 53.3 % (49.6-58.4) 95% CI, P<0.0001). No morphological defects were seen within the endothelial cells or the pericytes, nor was any defect in cell adhesion or cell contact evident.
3.1.3 Study III

Of the 170 women contacted for the study, 160 had sufficient clinical data for inclusion. Among these, 79 had MLH1, 51 MSH2, 25 MSH6, and 5 PMS2 mutations.

Further analysis showed that 43 women had undergone hysterectomy with or without bilateral salpingo-oophorectomy prior to a diagnosis of LS; consequently, these cases could not be used to assess the effectiveness of the screening program for gynecological cancer in LS carriers. Interestingly, the indication for surgery was EC in 35 of these cases (81%) and ovarian cancer (OC) in 3 cases (7%), while the remaining 5 cases were for benign indications (11%). Another subset of women (n=26) who were known LS carriers, but too young to have started the cancer screening program, could not be included to assess the effectiveness of the screening program. Additionally, 3 patients chose not to attend the screening program, and in two cases the physician opted not to screen at all. In all, of 160 patients, only 86 patients remained who were known LS carriers and had attended one or more gynecological screening visit.
In this group of 86 women, 41 (47.7%) eventually underwent prophylactic hysterectomy and/or bilateral salpingo-oophorectomy. Two patients from the prophylactic surgery group (4.9%) were postoperatively diagnosed with EC and two (4.9%) with atypical hyperplasia of the uterus. The remaining 45 (52.3%) women instead regularly attended gynecological surveillance with no decision to have prophylactic surgery. The total incidence of gynecological cancer in this group was 20% EC and 4% OC. Five patients had EC and two had complex hyperplasia with atypia, all asymptomatic and detected by EB during screening visits. Four additional cases were detected because of interval bleeding between visits. Two cases of OC were detected by TVUS in patients with ovarian cysts under surveillance. The youngest woman with EC was diagnosed at age 35, before she was aware of her diagnosis of LS.

Figure 2: Proportion of cancer-free women from time of LS diagnosis to time of gynecological cancer diagnosis. Red line = prophylactic surgery group. Blue line = surveillance group

The incidence of gynecological cancer showed a clear declining trend in the group of women who opted for prophylactic surgery, although this trend did not reach statistical significance, possibly because of the low total number of participants in the two groups; see figure 2.
3.1.4 Study IV

Using a 25 base-pair frame for the SNP analysis, we identified five distinct haplotypes in the EC group, none of which had been described earlier in the literature, and which were associated with an increased risk of endometrial cancer compared with controls. These haplotypes were found on chromosomes 2, 10, 13, 15 and 20, and contained between 7 and 17 SNPs, with lengths ranging from 1445 to 89,722 base pairs; see table 1 and figure 1.

<table>
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<th>Odds ratio EC (OR)</th>
<th>Frequency</th>
<th>Bp-length</th>
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</table>

Table 1: The five risk haplotypes found in the EC population and their p-values (significance= < 6x10^-9). OR = Odds ratio for EC compared to the control group. Frequency = Frequency of occurrence of the haplotype in the study group of EC patients as a whole. Bp-length = Basepair length

The haplotype on chromosome 2 is located in the ITGA6 (integrin subunit alpha 6) gene. All SNPs in the haplotype occur in intron sequences of the gene. The ITGA6 protein is highly expressed in normal endometrial tissue, especially in endometrial glandular cells. The subunit forms a heterodimer with a beta chain and resides in the cell membrane where it interacts with the ECM. ITGA6 expression is known to be a favorable prognostic marker in renal cancer (p<0.001) and an unfavorable marker in head and neck cancer (p<0.001). ITGA6 has not been shown to be of any prognostic value in cancers of the female reproductive tract, although *in vitro* studies of overexpression have shown a general link to an aggressive metastatic phenotype (130). Other *in vitro* studies have shown a link between ITGA6 expression and higher invasiveness in gallbladder cancer, and that blocking ITGA6 suppresses tumor growth and angiogenesis (74, 131).
Figure 1: The rs numbers and the SNP order in each of the five risk haplotypes

The haplotype found on chromosome 10 is located in the TTC40 gene. This gene codes for the CFAP46 protein, which is expressed in benign fallopian tube tissue, but found in EC tissue as well. Three SNPs in this haplotype (rs 2254419, rs4880433, rs10870341) are located in the coding sequence for the TTC40 gene, where the minor allele leads to missense mutation variants (rs 2254419 S/G, rs4880433 A/T, rs10870341 C/R), but with no known evident effect on protein function. Expression of CFAP46 has no evident prognostic value for any cancer. Interestingly, in a Japanese population the SNP rs2254419 (G) missense variant has been associated with obesity, a well-known major risk factor for developing EC (132).

The haplotype on chromosome 13 spans one gene (TUBA3C) and two pseudogenes (CENPIP1, SMPD4P2), including the intergenic regions. All the SNPs reside in the intron sequence of the genes. Normally, TUBA3C is found only in testicular tissue, but in cancer it may be expressed in several tissues, especially in the female genital tract and breast tissue. There is no evidence that the protein is associated with any prognostic value for any cancer.
The haplotype on chromosome 15 resides in a regulatory region with binding sites for several transcription factors. Of these, expression of the E2F3 transcription factor is known to be a favorable prognostic finding in OC. However, recent studies have shown that the Mir-152 microRNA, which usually acts as a tumor suppressor, is often methylated and silenced in ECs. One of the main targets of Mir-152 is E2F3, which may indicate that this transcription factor plays a role in EC that is not yet fully understood (133, 134). Another factor, PITX1, binds to the same region and is usually overexpressed in cervical cancer, but has no demonstrated prognostic value in any disease so far. However, PITX1 has been shown to act as a target for ERα in ER-positive breast cancer, with up-regulation of both PITX1 and its target genes, although other ER-dependent cancers in other tissues have not been studied in this regard thus far (135).

ZSCAN16 is another transcription factor that binds to the same region and is associated with a favorable prognosis in ovarian and cervical cancer. One study has shown a significant alteration in the epigenetics of the regulatory region for the ZSCAN16 gene on chromosome 6 in ER-positive breast cancer with subsequent overexpression of the protein in those cancers. ZSCAN16 expression in other ER-dependent tumors is not known (136). None of the transcription factors binding to the region where this haplotype is located are currently known to be altered or to have a prognostic value for EC.
4. Discussion

4.1.1 Study 1

This study demonstrated altered levels of SDF-1 in HMB-E patients compared with healthy women. The underlying mechanism is unknown, nor is it known whether the systemic circulation or the local circulation within the endometrium is the source of the SDF-1. Because SDF-1 levels vary cyclically with the menstrual cycle in healthy women, and the endometrium is known to be the site of angiogenesis during the proliferative phase, it suggests that the main source of peaking SDF-1 during the proliferative phase is the endometrial response to stimulation by estradiol and progesterone.

A previous but similar study undertaken on endometrium and blood from healthy women also showed cyclical variation of SDF-1 levels with the menstrual cycle, which correlated negatively with the number of EPC-CFUs that could be formed in vitro (137). The study concluded that during the proliferative phase, healthy endometrium appears to produce SDF-1 to recruit EPCs to the growing vessels, while incorporation of EPCs into the vessels results in a negative feedback loop on SDF-1 production. This conclusion is consistent with the findings of this study as well, and may serve as a possible explanation for the high blood loss in HMB-E patients should such recruitment malfunction, thereby rendering leaky vessels because of fewer ECs.

However, studies conducted using different methodology showed contradictory results. A study by Laird et al. that measured mRNA expression in endometrial biopsies from healthy fertile women during different phases of the menstrual cycle did not detect any significant changes in SDF-1 expression, but did find a significant change in CXCR4 mRNA expression with substantially higher levels of expression localized to the vascular walls during the early proliferative phase compared with all other phases in the cycle (138). Although no change in SDF-1 was seen, the CXCR4 mRNA expression in the proliferative phase seen in this study is actually consistent with our own study, which found that SDF-1 signaling is likely to be most active during this phase. The lack of a SDF-1 mRNA peak in the biopsies may be due to different study methodology since Laird’s group extracted mRNA from solid tissue, while our study extracted it from blood; this may indicate that most of the SDF-1 in the endometrium is in soluble form. However, these findings beg the question of whether SDF-1 actually originates in the endometrium or from peripheral tissue.
This study also showed a significant decrease in the number of EPC-CFUs among HMB-E patients with a positive correlation between SDF-1 levels and number of EPC-CFUs; moreover, the number of circulating EPCs was significantly lower in patients than in controls. These findings are also in line with the previous study (78), which suggested that SDF-1 is important for recruitment of EPCs from the bloodstream to endometrial vessel walls. The question is how lack of recruitment could affect angiogenesis and the integrity of the endometrial vessels, since other known angiogenic pathways may possibly provide sufficient compensation.

In a mouse model study using DNA marked cells originating from hematopoietic tissues to create granulation tissue, the number of endothelial cells of hematopoietic origin in the granulation tissue was compared with other mature tissues and significant differences were found. In the granulation tissue, endothelial cells of hematopoietic origin accounted for 8.3-11.2%, while the percentage was much lower in other tissues, 0.2-1.4% (139). This indicates that incorporation of circulating EPCs is an important mechanism for acquiring endothelial cells in adult tissues, where de novo angiogenesis from existing mature vessels takes place. This may be even more important in the endometrium since vessel sprouting does not seem to be the main mechanism of new vessel formation (19). EPCs have also been shown to express both estrogen receptors alpha and beta (140), making them sensitive to estrogen stimulation, which in vitro has been shown to increase EPC proliferation (141). This could also explain why recruitment of EPCs may be an extremely important source of endothelial cells, especially in the endometrium.

Several studies have reported that the ability to recruit EPCs during angiogenesis and vascular injury is beneficial for vascular integrity in patients with or at risk of cardiovascular disease (142-144), including evidence that recruitment protects the vascular wall from further injury. The important role of EPCs in neovascularization of ischemic tissues has also been shown by several studies (145), further suggesting the importance of the endometrium to neovascularization since local hypoxia is critical to the shedding process. A recent study has described the mechanism by which SDF-1 release into the bloodstream is activated by HIF-a in hypoxic tissue, and how SDF-1 both recruits EPCs from the bone marrow and attracts these cells to ischemic tissue through simultaneous upregulation of E-selectin on existing ECs (74). These findings provide a key as to how hypoxia, SDF-1 and EPCs interrelate in the neovascularization process; our study findings also fit this paradigm.
4.1.2 Study II

The main finding of this study was the significantly lower pericyte coverage of microvessels during the proliferative phase in HMB-E patients compared with controls; moreover, pericyte coverage decreased during the secretory phase among controls, while coverage remained stable throughout the phases of the menstrual cycle in HMB-E patients. This finding supports the hypothesis that HMB-E primarily results from dysregulated angiogenesis.

As previously mentioned, the pericyte is important for EC function and stability. This study does not answer the question of whether dysregulation stems from the pericyte or from inability of ECs to recruit them properly. One finding that suggests the latter explanation is the negative correlation between VEGF-A levels and pericyte coverage, which might indicate that the strong VEGF-A signal from endothelial cells primarily inhibits pericyte adherence during neovascularization in the proliferative phase, ultimately leaving behind immature vessels that are prone to leakage. VEGF-A and its binding to the VEGF-R2 receptor on mesenchymal cells have been shown to inhibit the PDGFR-B receptor on the same cells, thereby preventing proliferation and recruitment of these cells into newly formed EC tubes (146). The same study also tested pericyte coverage of microvessels in mice treated with PDGF or VEGF-A alone or in combination. Monotreatment with PDGF clearly led to robust recruitment of pericytes and formation of new vessels, while the combination of PDGF and VEGF-A resulted in inhibition of vascular formation, suggesting an antagonistic relationship between the two factors. Both this study and prior studies have shown significant overexpression of VEGF-A throughout the menstrual cycle in HMB-E patients (52, 147, 148). This finding, along with the inhibitory effect VEGF-A has on pericytes, may explain the disruption of vascular morphology among HMB-E patients in this study.

Similar to the findings in the first study on SDF-1, pericyte coverage was cyclical in healthy controls, with highest coverage seen during the proliferative phase and a decrease during the secretory phase, while coverage in HMB-E patients remained unchanged throughout the menstrual cycle. The reason why the endometrium of HMB-E patients lacks such a response in the proliferative phase is unclear. Endometrial growth is controlled by estrogen and progesterone, where estrogen levels are highest during the proliferative phase; however, studies have shown that estrogen may be both pro or anti-angiogenic under different circumstances (12).
What is known from ovariectomized mouse models is that single treatment with estradiol leads to angiogenesis in the endometrium through mediation of VEGF-A, since blocking of VEGF-A results in complete absence of new vessels with estradiol treatment (149); however, these vessels failed to show recruitment of pericytes. In contrast, treatment with progesterone had a strong positive effect on pericyte proliferation and coverage of vessels, which remained unaltered regardless of whether or not estrogen was used for pre-treatment (150). Logically, this would suggest that the highest coverage would be expected during the secretory phase rather than the proliferative phase, as in the case of our study. Abberton et al. studied coverage of aSMA cells around spiral arterioles and showed higher coverage during the secretory phase (151, 152). The same group conducted a comparative study between HMB-E patients and controls regarding aSMA expression in spiral arterioles and found no significant differences (153). It should be noted that these studies examined large vessel maturation, rather than microvasculature, as in our study. It may be possible that the need for pericyte coverage in the microvasculature changes as the larger vessels mature and become contractile later in the cycle, when they can start to regulate peripheral blood flow more efficiently, ultimately rendering pericyte coverage in the capillaries unnecessary during the secretory phase. Further studies of HMB-E patients and healthy controls comparing aSMA/pericyte coverage between arterioles and microvessels would be of interest.

Our study also identified morphological changes in microvessel lumens among HMB-E patients that were not present in controls, including an increase in endothelial cell area of vessels and an increase in vessel perimeters. As previously mentioned, earlier studies have shown that HMB-E vessels lack essential vasoconstrictors such as ET-1 and PGF, which would affect vascular tone and vessel perimeter. Lack of aSMA could also contribute to these morphological changes since involved vessels lose the ability to contract. This inability to contract results in a larger vessel perimeter, which could also help explain increased leakage in vessels.
4.1.3 Study III

To our knowledge, this is the first study that attempted to determine the effectiveness of gynecological surveillance in Sweden. We found that 15% of women in the surveillance group developed cancer over the course of the surveillance period. This may appear to be an unexpectedly low percentage given that the lifetime risk of EC in LS carriers is approximately 50%. The explanation may lie in the relatively short follow-up period for each case and the high frequency of prophylactic hysterectomies (48%) in the cohort.

The overall question is how screening for gynecological cancers among LS carriers affects morbidity and mortality in the group since it is known that EC usually gives rise to early symptoms like occult vaginal bleeding and is therefore diagnosed at an early stage with an excellent cure rate by surgery alone (154). It should be noted, however, that this is true for FIGO stage I EC that accounts for approximately 80% of all cases with a five-year survival rate of 95%. For FIGO II-V, which account for the remaining 20%, the long-term prognosis is much worse with a five-year survival rate between 25-75% (https://www.cancercentrum.se/globalassets/cancerdiagnoser/gynekologi/kvalitetsregister/nationell-kvalitetsrapport-gynekologisk-cancer-2017.pdf), for which reason screening could be especially beneficial for such LS patients.

Our study identified four cases of FIGO stage I-II EC because of bleeding between checkups, which was not different in stage from the other EC cases detected during checkups. This could indicate that early detection is not significantly improved by the checkups themselves but rather that awareness of increased EC risk in this subset of patients who bleed may be the most important factor for prevention of patient and/or doctor delay for finding cancer at an earlier stage. A large national study by De Jong et al. that compared mortality rates for different cancers in 140 Lynch families in the Netherlands before and after introduction of the national screening program in 1987 did not detect any significant decrease in EC mortality (155). This could be explained by the relatively low mortality rate associated with EC overall, but could also indicate that the gynecological screening methods were not effective. A study in Finland that followed 175 known female mutation carriers showed promising results for the effectiveness of gynecological surveillance by detecting 14 cases of EC and 14 cases of premalignant complex hyperplasia. All of the women detected through the surveillance program survived their EC, compared with a group of 83 women with symptomatic EC, 6 of whom died from the disease, although this difference does not reach statistical significance.
More importantly, this study shows that screening methodology is crucial in order for screening checkups to detect EC or hyperplasia, since in the relevant study EB detected all cases of hyperplasia and 8 ECs, while ultrasound found only 4 ECs and missed all cases of hyperplasia (156).

These findings are consistent with our own results since in our study the 5 cases of EC and complex hyperplasia found in asymptomatic women were diagnosed through EB, while TVUS missed all of these cases. Of the four cases that presented with symptoms between visits, EB detected EC in all cases while TVUS only saw thickening of the endometrium in two cases. This indicates that in order for EC surveillance to be effective, EB must be used. Other studies have reported a paucity of findings from TVUS screening for EC detection, while yet others have concluded that annual screening is sufficient, and that EB is only indicated when symptoms of occult bleeding occur (157).

The most effective intervention to prevent gynecological cancer in LS patients is prophylactic hysterectomy with or without bilateral oophorectomy, which reduces risk of both EC and OC. Our study uncovered four additional cases of EC in patients who had already undergone prophylactic surgery. The optimal time to carry out this procedure is still debatable; to date, no study has shown a decrease in long-term mortality among prophylactically operated patients. The current recommendation is to opt for surgery when having children is no longer an issue, although this also gives rise to other questions concerning surgical risk and early menopause, which may have negative long-term effects on health and quality of life.

Our study also investigated whether there were any screening procedure differences between university, county and private clinics. No significant differences were found, although regarding age distribution, private clinics demonstrated a trend to continue screening into older age groups before referral for prophylactic surgery, compared with university and county hospitals, which tended to refer patients for surgery earlier. This may reflect either a patient or care provider bias. It may be in the interest of private care providers to keep patients under surveillance as long as possible for economic reasons, but alternatively the explanation may be that patients who do not want to undergo surgery actively avoid surveillance at hospital clinics.
One limitation of this study is its retrospective design, which may lead to selection bias. In this regard, selection bias may be detrimental to the conclusions of the study since awareness about LS and the risk of developing cancer may be the most important single factor for reducing mortality in this group, so if this subgroup of LS-aware carriers is more likely to participate in this study there could be substantial confounding of results. We cannot check for this since we do not have access to the medical records of non-participants. The strength of the study design, however, is that it provides a good representation of the effectiveness of the surveillance program in Sweden without the risk for recall or ascertainment bias, which would be the case if the study had a prospective design.
4.1.4 Study IV

Our results demonstrate five haplotypes that are significantly more abundant in a Swedish cohort of EC patients compared with controls. Nevertheless, the frequency of these haplotypes is fairly low among all cancer patients; the most frequent haplotype located on chromosome 20 is present in 3.69% of all ECs. The odds ratio for all chromosomes is also relatively low; the haplotype on chromosome 10 is associated with the highest OR of 3.05. Overall, this indicates that the haplotypes that were discovered are insufficient to account for development of cancer in individual patients, but may explain the increased occurrence of EC in a larger homogeneous group of people. This idea is also consistent with the location of the haplotypes in the genome, since no individual SNP in these haplotypes leads to deleterious mutation of any protein, as might be expected if the SNP haplotype itself were the direct cause of disease. Instead, as expected these SNPs are either located in noncoding regions or intergenic regions of the genome. These SNPs are mostly found in intron sequences or lead to missense variants, with no apparent effect on protein function.

Interestingly, these genes and the transcription factor binding regions in which the five described haplotypes are found have been associated with estrogen-dependent cancers, especially breast cancer. Endometrioid EC is also estrogen-dependent, and the environmental factors associated with this disease are strongly linked to estrogen excess (158-160), which could explain the increased susceptibility for EC among these haplotypes. Neither potential estrogen excess nor other environmental risk factors for EC have been investigated among the patients and controls in this particular study, which is a weakness of the study design.

These five haplotypes may also affect the epigenetic structure of the genome. Studies involving other types of cancer have shown that different alleles affect methylation of DNA differently, and thereby also transcription of genes. One striking example is methylation of the MLH1 promoter in CRC (161), where different MLH1 alleles render the DNA more or less susceptible to methylation. Other studies have shown a similar pattern in other loci associated with CRC within non-coding regions of the genome (162, 163). Another possible explanation for increased susceptibility to EC for these haplotype carriers could be linkage disequilibrium with adjacent genes on the chromosome that may be more closely related to EC pathology.
The haplotype on chromosome 20 is located between the PLCB1 and HAO1 genes. Neither of them is known to be linked to cancer pathogenesis. The haplotype on chromosome 15 is located between GERM1 on one side and ARHGAP11A and SCG5 on the other. GERM1 is upregulated in endometriosis and has been linked to EC (164, 165). Meanwhile, SCG5 polymorphism has been linked to an increased risk of CRC, but no studies of EC have been conducted in relation to this locus (166).

The chromosome 13 haplotype has no other transcript genes in close proximity. The haplotype on chromosome 10 lies close to the INPP5A gene, the presence of which is an unfavorable prognostic factor in breast cancer. This gene is usually overexpressed in all cancers of epithelial origin, including cancers of the female reproductive tract. INPP5A overexpression has been shown in vitro to inhibit cell proliferation and to increase apoptosis in cervical cancer cells, suggesting a tumor-suppressing effect in some tissues (167).

Finally, the haplotype on chromosome 2 is located near the PDK1 gene. The presence of this gene has an unfavorable prognosis in cervical cancer. PDK1 is involved in the akt-mTOR pathway in the cell, which stimulates cell survival, and one study showed that altered phosphorylation of PDK1 in EC cell lines influenced cancer cell viability in vitro (168).

The strategy of using haplotype analyses is based on the assumption that Sweden has a fairly homogeneous population. A prior study using a similar approach identified seven novel risk loci for cancer in a Swedish population, although those risk loci were not associated with any specific type of cancer (169). It has long been known that EC differs among various ethnicities regarding clinical outcome; it has been suggested that this is due to a combination of both environmental and genetic factors (170, 171). A recent study compared the protein profiles of ECs among people of different ethnicities using the human genome atlas and found clear differences in molecular profile between African American, Caucasian, and Asian women (172). Although this study cannot answer the question of whether tumors are due to environmentally induced mutations or genomic susceptibility, it does indicate that tumor development varies based on inherited genomic differences, where inherited haplotypes may be one of many important factors.
5. General conclusions

Endometrial angiogenesis

- Study I confirmed a cyclical difference in SDF-1 levels between the proliferative and secretory phases of the menstrual cycle in non-HMB-E subjects.
- Study II study showed a cyclical difference in pericyte coverage of microvessels in the endometrium between the proliferative and secretory phases in non-HMB-E subjects.
- A cyclical pattern of SDF-1 levels and pericyte coverage was absent in HMB-E patients in whom both were significantly lower during the proliferative phase.
- The number of EPC-CFUs was lower in HMB-E patients than in controls, suggesting that EPC incorporation during physiological angiogenesis of endometrial vessels plays an important role.
- The number of EPC-CFUs correlated positively with SDF-1 levels, which indicates that the SDF-1/CXCR4 axis is responsible for recruitment of EPC-CFUs into the bloodstream, which is in line with previous literature.
- Pericyte coverage of microvessels correlated negatively with VEGF-A levels in the endometrial vessels, indicating that overexpression of VEGF-A has a negative effect on microvascular maturation.

Hereditary factors in the endometrium

- Study III showed a high percentage of prophylactic hysterectomy (52.3%) after childbearing age among women who were known LS carriers.
- A total of 5 ECs and 2 endometrial hyperplasias were found during surveillance visits. All these cases were diagnosed through endometrial biopsy; 4 ECs were found between visits due to symptoms.
- All ECs found in the study were in FIGO stage I-II. Whether this favorable outcome is due to the surveillance program or the mere knowledge of the increased risk of EC is unknown.
- Study III could not determine whether gynecological surveillance itself reduces morbidity or mortality in female LS carriers.
- Study IV found five haplotypes that were significantly overexpressed in women with a family history of EC, but who had no cancer syndrome themselves.
- All five haplotypes were found in genes or chromosomal regions with no apparent effect on risk of EC; the reason for overexpression is not yet known.
6. Future perspectives

6.1 Novel HMB-E treatments

Current HMB-E treatments effectively decrease blood loss, but unfortunately, they all have a contraceptive effect, which makes them unsuitable for women with HMB-E who wish to become pregnant. Moreover, all current HMB-E treatments address the symptoms, without affecting the underlying pathogenesis. This research suggests that HMB-E patients have both altered EPC recruitment to endometrial vessels during physiological angiogenesis and a change in endometrial vascular maturation, as indicated by low pericyte coverage.

Based on this research, a future goal may be to search for novel drugs that could either stimulate EPC recruitment to the endometrium or pericyte recruitment to immature vessels. Further studies are needed to elucidate whether HMB-E is primarily due to defective angiogenesis or failed vessel maturation, or possibly both simultaneously. A topic of interest for future study is to elucidate whether administration of a PDGF-b agonist or a VEGF-A inhibitor to HMB-E patients \textit{in vivo} shortly before or during menstruation could enhance vascular maturation and reverse the symptoms. The difficulty with such a study would relate to mode of administration. Preferably, the substance would be administered locally into the endometrium, but such an approach would require hospital resources. On the other hand, oral administration would be associated with a risk of systemic side effects.

A third approach is to increase incorporation of EPCs into the endothelium of vascular walls and thereby possibly improve the angiogenic process, but first other issues must be addressed. Our study investigated systemic SDF-1 levels in the bloodstream, a substance that purportedly exerts its main effect by causing EPC detachment from the bone marrow. Local expression of is also needed to attract EPCs into the growing endometrial vessels. SDF-1 production could be stimulated by inducing local hypoxemia, but this approach would increase VEGF-A levels and thereby have a negative impact on vascular maturation. A different strategy could involve \textit{in vitro} cultivation of EPCs which could be readministered to the patient during menstruation in an effort to reduce bleeding. However, although this strategy would hardly be considered cost-effective as clinical treatment, it could reveal the potential importance of EPCs in the pathogenesis of HMB-E.
6.2 Improved LS surveillance programs

As genetic analysis improves, a greater number of patients will be diagnosed with cancer syndrome requiring accurate and cost-effective medical surveillance. Our study found no obvious benefit regarding tumor stage or long-term survival among women with known LS who were diagnosed with EC as a result of surveillance or symptoms between visits. Instead, the most important factor appears to be awareness of LS, but that may be reinforced by the surveillance program. A possible future study focused on tumor stage and long-term outcome could compare one group of LS patients who attend annual gynecological screening with a similar group that only receives yearly EC risk reminders from their clinical geneticist.

Another study, previously discussed in the literature, suggests combining annual colonoscopies with simultaneous endometrial biopsies. Such an approach could make surveillance more efficient by reducing the need for separate procedures, thereby saving both time and resources.

The time at which to recommend gynecological prophylactic surgery is still under debate since the risk of cancer must be weighed against surgical risks and the effects of early menopause induced by bilateral oophorectomy. Because penetrance of EC varies depending on which of the four genes is affected, a general surveillance program must be designed to take this into account and the recommended time of prophylactic surgery should be based on this information. The recommendation for MLH1 and MSH 2 mutation carriers should probably be to have prophylactic surgery between age 40-50. MSH6 carriers could remain under surveillance without risk for a longer time and could possibly refrain from prophylactic surgery before menopause since EC would not be expected until late in life, provided that they do not have a family history of early EC.

6.3 Improved EC-risk assessment

New genetic variants are constantly being identified and an increasing number are linked to a higher risk of certain diseases, including EC. Usually such genetic risk factors interact with environmental risk factors, which makes certain populations more likely to develop certain diseases. Regarding EC, for which we have already identified several environmental risk factors, information concerning the genetic make-up of individual patients could help to better predict their risk of developing disease, given knowledge of environmental hazards.
However, more studies are needed to explore the relationship between known polymorphisms and environmental risk factors to address the possible risk of confounding that has not previously been elucidated. For example, should it be the case that the increase in relative risk for EC in certain populations can be more or less fully explained through inheritance of BMI, then additional genetic profiling would not provide useful information to the clinician.

### 6.4 The relationship between HMB-E and EC

The studies in this thesis have focused on angiogenic and hereditary risk factors for HMB-E and EC separately. As the thesis explains, known angiogenic factors are involved in development of EC and there are indications that hereditary factors are involved in HMB-E. Surprisingly, to date no studies have addressed HMB-E as a possible independent risk factor for EC, given that normal menstrual physiology is so tightly intertwined with the physiology of angiogenesis. It would not be difficult to imagine that the dysregulation of angiogenesis seen in HMB-E could act as a breeding ground for mutations and thereby for uncontrolled cell proliferation at a future stage. Such studies would be even more revealing in populations with a high incidence of EC among premenopausal women, for example in LS carriers. Even if the LS mutation explains the increase in cancer risk, could that risk be reduced through control of endometrial angiogenesis? Furthermore, would it be possible to predict and control HMB-E and angiogenesis if the underlying hereditary factors are known?

The first future task would be to design a clinical case-control study to compare the incidence of EC in women with confirmed HMB-E with a control group, and to check for confounding risk factors during selection. A second study could be to look at VEGF-A expression, as well as SDF-1 and EPC levels in peripheral blood in HMB-E patients and attempt to correlate those levels with risk of developing EC later in life. Conversely, it would be interesting to compare EC cases that have a known hereditary component with ECs of other origin regarding menstrual history, to assess the rate of HMB-E in these EC subsets, which would also provide greater insight into the etiology of HMB-E.

In conclusion, HMB-E and EC are two separate diagnoses that both share some risks and pathophysiological abnormalities. The future could possibly reveal that in certain cases, HMB-E should be regarded as pre-stage EC, and thereby highlight the importance of accurate diagnosis and appropriate treatment of HMB-E.
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