Role of angiogenesis in adenomyosis-associated abnormal uterine bleeding and subfertility: a systematic review

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BACKGROUND: Adenomyosis commonly occurs with abnormal uterine bleeding (AUB) and is associated with subfertility and a higher miscarriage rate. Recent evidence showed abnormal vascularization in the endometrium in patients with adenomyosis, suggesting a role of angiogenesis in the pathophysiology of AUB and subfertility in adenomyosis and providing a possible treatment target.

OBJECTIVE AND RATIONALE: We hypothesized that the level of abnormal vascularization and expression of angiogenic markers is increased in the ectopic and eutopic endometrium of adenomyosis patients in comparison with the endometrium of control patients. This was investigated through a search of the literature.

SEARCH METHODS: A systematic search was performed in PubMed and Embase until February 2019. Combinations of terms for angiogenesis and adenomyosis were applied as well as AUB, subfertility or anti-angiogenic therapy. The main search was limited to clinical studies carried out on premenopausal women. Original research articles focusing on markers of angiogenesis in the endometrium of patients with adenomyosis were included. Studies in which no comparison was made to control patients or which were not published in a peer-reviewed journal were excluded. A second search was performed to explore the therapeutic potential of targeting angiogenesis in adenomyosis. This search also included preclinical studies.

OUTCOMES: A total of 20 articles out of 1669 hits met our selection criteria. The mean vascular density (MVD) was studied by quantification of CD31, CD34, von Willebrand Factor (vWF) or factor-VIII-antibody-stained microvessels in seven studies. All these studies reported a significantly increased MVD in ectopic endometrium, and out of the six articles that took it into account, four studies reported a significantly increased MVD in eutopic endometrium compared with control endometrium. Five articles showed a significantly higher vascular endothelial growth factor expression in ectopic endometrium and three articles in eutopic endometrium compared with control endometrium. The vascular and pro-angiogenic markers α-smooth muscle actin, endoglin, S100A13, vimentin, matrix metalloproteinases (MMPs), nuclear factor (NF)-κB, tissue factor (TF), DJ-1, phosphorylated mammalian target of rapamycin, activin A, foll- and myostatin, CD41, SLIT, roundabout 1 (ROBO1), cyclooxygenase-2, lysophosphatidic acid (LPA) 1,4-5, phospho signal transducer and activator of transcription 3 (pSTAT3), interleukin (IL)-6, IL-22 and transforming growth factor-β1 were increased in ectopic endometrium, and the markers S100A13, MMP-2 and -9, TF, follistatin, myostatin, ROBO1, LPA1 and 4-5, pSTAT3, IL-6 and IL-22 were increased in eutopic endometrium, compared with control endometrium. The anti-angiogenic markers E-cadherin, eukaryotic translation initiation factor 3 subunit and gene associated with retinoic-interferon-induced mortality 19 were decreased in ectopic endometrium and IL-10 in eutopic endometrium, compared with control endometrium. The staining level of vWF and two pro-angiogenic markers (NF-κB nuclear p65 and TF) correlated with AUB in patients with adenomyosis. We found no studies that investigated the possible relationship between markers of angiogenesis and subfertility in adenomyosis patients. Nine articles reported on direct or indirect targeting of angiogenesis in adenomyosis—either by testing hormonal therapy or herbal compounds in clinical studies or by testing angiogenesis inhibitors in preclinical studies. However, there are no clinical studies on the effectiveness of such therapy for adenomyosis-related AUB or subfertility.

WIDER IMPLICATIONS: The results are in agreement with our hypothesis that increased angiogenesis is present in the endometrium of patients with adenomyosis compared with the endometrium of control patients. It is likely that increased angiogenesis leads to fragile and more permeable vessels resulting in adenomyosis-related AUB and possibly subfertility. While this association has not sufficiently been studied yet, our results encourage future studies to investigate the exact role of angiogenesis in the etiology of adenomyosis and related AUB or subfertility in women with adenomyosis in order to design curative or preventive therapeutic strategies.

Key words: adenomyosis / angiogenesis / abnormal uterine bleeding / subfertility / anti-angiogenic therapy / endometriosis

Introduction

Adenomyosis is a common gynecological disorder, predominantly occurring in premenopausal women (Bergeron, 2006; Cohen et al., 1995). Adenomyosis may cause abnormal uterine bleeding (AUB) and dysmenorrhea and is associated with a 28% lower clinical pregnancy rate as well as a more than doubled risk of miscarriage in women undergoing IVF with autologous oocytes (Tremellen and Russell, 2011; Martinez-Conejero et al., 2011; Vercellini et al., 2014; Cohen et al., 1995). The main histologic feature of adenomyosis is the presence of endometrial glands and stroma within the myometrium (Bird et al., 1972). The ectopic endometrium is generally associated with smooth muscle hyperplasia (Abbott, 2017). Recently, innovations in ultrasound skill and reporting systems have proven to be of essential value in diagnosing adenomyosis without the need for prior hysterecetomy (Van den Bosch et al., 2018; Stoelinga et al., 2018). Therapy options are still scarce and limited to hormonal suppression, hysterectomy, embolization or MRI-guided high-intensity focused ultrasound in experimental settings (de Bruijn et al., 2017; de Bruijn et al., 2017; de Bruijn et al., 2018; Ponts et al., 2016; Dueholm, 2017; Vannuccini et al., 2018; Fan et al., 2012). The pathology of adenomyosis explaining the symptoms of AUB and implantation failure remains inconclusive (Abbott, 2017; Vannuccini et al., 2017). Hypotheses include various biological mechanisms such as alterations in endometrial immunological environment, the increased local estrogen production due to P450 overexpression, alterations in apoptosis and/or increased angiogenesis in the endometrium (Tremellen and Russell, 2012; Brosens et al., 2004; Vannuccini et al., 2017).

Angiogenesis is the process of the outgrowth of new capillary blood vessels from existing blood vessels, which occurs in both physiological and pathological processes (Griffioen and Molema, 2000; Folkman, 1995). It occurs during the proliferative phase of the menstrual cycle when the endometrium is regenerated and is essential for successful embryonic implantation (Burton et al., 2009; Moller et al., 2001). The notion that the ectopic endometrium is surrounded by increased vascularization suggests that the ectopic endometrium releases factors that signal a quiescent vasculature to initiate capillary sprouting. This mechanism was first described in the cancer arena. Tumor cells mutate to start the production of angiogenic factors, which is the initiating event of the formation of new vasculature. This event is referred to as the angiogenic switch (Hanahan and Folkman, 1996) and has motivated the search for both angiogenic factors and angiogenesis inhibitors (Ramijawan et al., 2017). Estrogen, which is considered a crucial factor in the etiology of adenomyosis, causes mobilization and microvessel...
incorporation of endothelial progenitor cells resulting in angiogenesis (Chen et al., 2010; Rudzitis-Auth et al., 2016). Abnormal angiogenesis may occur due to modulation of the expression of angiogenesis-regulating genes, such as genes that regulate vascular endothelial growth factor (VEGF). Overexpression of VEGF leads to enhanced vascular sprouting (Hillen and Griffioen, 2007), decreased pericyte coverage and augmented vascular permeability and leakage. Such vasculature is expected to bleed more easily (Weis and Cheresh, 2005).

The role of angiogenesis is well established in endometriosis (Rudzitis-Auth et al., 2016) and has been recognized as a potential treatment target (Laschke and Menger, 2012). Adenomyosis has a strong relationship with endometriosis and can be found in one-third of patients with endometriosis (Larsen et al., 2011). Both endometriosis and adenomyosis are invasive diseases in which the endometrial cells have acquired invasive properties that require angiogenesis to establish an ectopic site (Kang et al., 2010; Yen et al., 2017). Accordingly, polymorphisms in the angiogenic factor fibroblast growth factor 1 and 2 gene have been found to be associated with the risk of developing both endometriosis and adenomyosis (Kang et al., 2010). In line with endometriosis, where it is known that angiogenesis plays a role in the establishment of ectopic foci outside the uterus, this is also expected to play a role concerning ectopic foci inside the uterus (Groothuis et al., 2005). If angiogenesis exists in the eutopic endometrium, it may be additionally relevant if it is associated with AUB or subfertility. In sub-fertile patients undergoing surgery for deep endometriosis, pregnancy rates were 68% reduced when patients had coexistent adenomyosis (Vercellini et al., 2014). Although this suggests that adenomyosis negatively impacts the eutopic endometrium, the finding should be interpreted with caution since it was reported in a systematic review including not more than five observational studies. Prior studies have noted the potential role of angiogenesis in the pathophysiology of adenomyosis (Ota and Tanaka, 2003; Ota, 1992; Benagiano et al., 2012).

With the knowledge that adenomyosis is found to be a common cause of AUB in women of the perimenopausal age group (Rizvi et al., 2013) and is associated with a higher risk of miscarriage and lower pregnancy rates in women treated with IVF, it is suggested that aberrant angiogenesis plays a role in the pathophysiology of adenomyosis-related AUB and subfertility.

However, very little is known about the role of angiogenesis in adenomyosis. To improve our knowledge of angiogenesis in the endometrium of women with adenomyosis, study its possible relation with alterations in vascular characteristics that may cause AUB and/or subfertility and explore the therapeutic potential of targeting angiogenesis, we performed a literature review to summarize the current knowledge of possible markers related to the angiogenesis in the endometrium of women with adenomyosis. The main objective was to test the hypothesis that abnormal vascularization and the level of angiogenic markers are increased in the ectopic and eutopic endometrium of adenomyosis patients in comparison with the endometrium of control patients. In addition, we review the biology of angiogenesis in adenomyosis and discuss possible therapeutic opportunities.

**Methods**

This systematic review is conducted in accordance to the Prisma guidelines (Liberati et al., 2009). The methods, inclusion/exclusion criteria, type of studies, study selection, data collection, risk of bias assessment, type of interventions and type of outcome measures were specified in advance and registered in the PROSPERO International prospective register of systematic reviews (CRD42017069832).

**Literature search**

Two databases, PubMed and Embase, were consulted until February 2019. Combinations of terms for angiogenesis, angiogenic markers and adenomyosis were applied as well as AUB, infertility or anti-angiogenic therapy. References of the studies and related articles were checked for inclusion if not already included in the primary search (see Supplementary Data for full search strategy).

**Eligibility criteria**

The first search (Search I: angiogenic-related outcome in adenomyosis patients) was limited to laboratory studies carried out on the endometrium of premenopausal women. Original research articles, such as cohort or case control studies, and prospective clinical trials, which investigated the presence of angiogenesis in the endometrium of adenomyotic patients and were published as full papers in peer-reviewed journals, were included. The research needed to focus on markers that indicate the presence of angiogenesis or are known to (among other functions) induce angiogenesis. Papers had to be written in English. Case reports, studies on cells cultured in vitro not taken from women with adenomyosis or studies that did not compare the endometrium of adenomyosis with the endometrium of control patients without adenomyosis or uterine fibroids were excluded. A second search (Search II: anti-angiogenic therapy for adenomyosis) was carried out to evaluate the current status of research into therapeutic options targeting angiogenesis in adenomyosis. For this search, all original research articles, including preclinical studies that evaluated angiogenesis inhibitors in experimental settings, were included.

**Data extraction**

Data from the included studies were extracted according to a predefined standardized format. The following information was extracted from each of the included articles: first author, year of publication, study design, number of patients included, patient and control characteristics, use of hormones, phase in menstrual cycle, study method, angiogenic-related outcome, type of cells and method of evaluation. All the angiogenic-related markers studied in the articles are summarized by category: vascular outcomes (including mean vascular density: MVD), pro-angiogenic markers and anti-angiogenic markers. We present findings on the differences in tissue expression of the angiogenic-related markers by endometrial type: ectopic (the displaced endometrium to a location within the myometrium, also noted as endometrium with abnormal location (Goteri et al., 2009), adenomyosis (Schindl et al., 2001), adenomyotic lesions (Huang et al., 2014), adenomyotic nodules (Carrarelli et al., 2015), adenomyotic tissue (Li et al., 2013) or adenomyotic foci (Liu et al., 2016) in the articles) or eutopic (the endometrium lining the uterine cavity in adenomyotic patients also noted as endometrium with normal location (Goteri et al., 2009)). Also, we report on the type of cells (endothelial, epithelial or stromal cells) where the angiogenic markers were found and on the tissue expression of angiogenic-related markers during the menstrual phase.
Quality assessment of the included studies

The quality assessment of the included studies was performed by two independent reviewers (MH and CW). The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) checklist was used to assess reporting quality of the included studies (Vandenbroucke et al., 2014), and the Newcastle Ottawa Scale was used to assess methodological quality of all included studies (Stang, 2010). Although the use of quality assessment scales is less common in the field of preclinical medicine, we considered the use of the Gold Standard Publication Checklist (GSPC) to Improve the Quality of Animal Studies, a valid method to enable quality evaluation of the preclinical studies (Hooijmans et al., 2010). The scales will be used as a guide to critically evaluate the quality of the studies, but will not be used to rate articles (da Costa et al., 2011; Sanderson et al., 2007).

Results

Study characteristics

The search using the abovementioned databases resulted in 1669 hits. After removal of duplicates and screening of both the titles and abstracts, 73 articles were retrieved for full-text screening. A total of 20 articles met our selection criteria and were included in our review (Fig. 1a).

The second search yielded 120 articles, out of which 17 full-text articles were assessed for eligibility and nine were included in this review (Fig. 1b).

The study characteristics of the included articles are shown in Tables I and II. The factors that were identified in the search are not all solely angiogenic factors. Some are usually categorized in the group of inflammatory cytokines, with angiogenesis regulatory functions (Table III). Since the factors were included in this study for their (anti-)angiogenic capacity, they will be referred to as (anti-)angiogenic factors for the remainder of this paper. All articles used immunohistochemical staining to study endometrial samples from premenopausal women. Some articles also used other techniques, such as western blotting, real-time PCR and ELISA. The terms ectopic and eutopic were used by most studies to describe the type of endometrium studied.

Assessment of risk of bias

The methodological quality was assessed using the STROBE checklist for the quality of reporting (Table IV), the Newcastle Ottawa Scale for possible sources of bias (Table V) and the GSPC for the preclinical studies (Table VI) to assess the reporting as well as the methodological quality of the included studies. Only 12 studies reported specifications of the location and inclusion dates of the conducted study. The lack of information poses risk of selection bias of study participants. Only a limited number of studies controlled for possible confounders, such as comorbidity, menstrual phase, age or parity (Liu et al., 2016; Yang et al., 2017; Cai et al., 2018; Liu et al., 2018). The preclinical animal studies on anti-angiogenic therapy often lacked clear reasoning why a specific animal model was chosen, a clear description of how the disease was defined and full disclosure on the type of anesthesia/euthanasia, description of general well-being and relevant physiological parameters of the animals. The discussion of the findings and clinical relevance, on the other hand, was accurate in all included studies.

Microvascular density and vascular characteristics

In nine articles, vascular characteristics or the MVD in endometrium of patients with adenomyosis was studied by quantification of microvessels through either CD31, CD34, von Willebrand Factor (vWF) or factor VIII (FVIII) antibody staining (Liu et al., 2016; Wang et al., 2016; Nie et al., 2011; Tokyol et al., 2009; Huang et al., 2014; Li et al., 2006; Schindl et al., 2001; Nie and Liu, 2016; Ota et al., 1998). Staining of these markers exclusively occurs in endothelial cells of blood vessels (Table III). In all articles that assessed the ectopic endometrium, the MVD was significantly increased compared with the control endometrium. Six studies found a significantly increased MVD in the eutopic endometrium from adenomyosis patients compared with the control endometrium (Liu et al., 2016; Huang et al., 2014; Wang et al., 2016; Nie et al., 2011; Ota et al., 1998; Nie and Liu, 2016), while two studies reported no significant difference (Schindl et al., 2001; Tokyol et al., 2009), and one did not study the difference between eutopic endometrium and control endometrium (Liu et al., 2016).

One article assessed the characteristics of capillaries in the endometrium through staining vWF (Ota et al., 1998). Compared with the control endometrium, the eutopic endometrium displayed an increase in mean and total surface area of capillaries, irrespective of the menstrual cycle phase. The ectopic endometrium was not investigated in this study. The highest increase was reported for the total surface area of capillaries; this was 1.6 times higher in the eutopic endometrium in adenomyosis patients than in the endometrium of controls during the proliferative phase. One group studied endoglin (Eng), a transmembrane homodimer glycoprotein that is known as an angiogenic marker because it is overexpressed in activated endothelial cells (Hayrabedyan et al., 2005). Eng staining was not observed in the endothelial cells of microvessels of the control endometrium, while it was expressed in all microvessels of the eutopic and ectopic endometrium, with stronger expression in eutopic than in ectopic endometrium.

Pro-angiogenic and vascular markers

A total of 22 markers were studied for their angiogenic characteristics in the 20 articles retrieved by our search (Table III). Five articles compared VEGF expression in the endometrium of adenomyosis patients with controls (Li et al., 2006; Wang et al., 2016; Huang et al., 2014; Liu et al., 2016; Li et al., 2013). All studies reported a significantly higher VEGF expression in the ectopic endometrium compared with control endometrium. In three articles, the eutopic endometrium was compared with the control endometrium (Wang et al., 2016; Huang et al., 2014; Li et al., 2006), where VEGF expression was reported to be increased in the eutopic endometrium compared with control endometrium. Immunoreactivity of cyclo-oxygenase 2 (COX-2) was studied in three studies (Tokyol et al., 2009; Ota et al., 2001; Li et al., 2013). One study reported significantly increased protein expression levels of COX-2 in adenomyotic tissue compared with control tissue (Li et al., 2013). Both other studies reported no significant difference in COX-2 expression between the groups.

α-Smooth muscle actin (SMA), S100A13, vimentin, matrix metalloproteinases (MMPs) 2 and 9, nuclear factor (NF)-κB (subunits p50/p65), tissue factor (TF), DJ-1, phosphorylated mammalian
Figure 1: Prisma flow diagrams for the two searches carried out for the systematic review. (a) PRISMA 2009 Flow Diagram Search I: angiogenic-related outcome in adenomyosis patients. The search was limited to laboratory studies carried out on the endometrium of premenopausal women. (b) PRISMA 2009 Flow Diagram Search II: anti-angiogenic therapy for adenomyosis. The search was for all original research articles, including preclinical studies that evaluated angiogenesis inhibitors in experimental settings.
| Study, year       | Study type         | N participants | Patient group | Control group | Matching cycle phase | Time of no hormone use | Methods                  | Angiogenic parameters | Outcome | Clinical symptom |
|------------------|--------------------|----------------|---------------|---------------|---------------------|------------------------|-------------------------|-----------------------|---------|------------------|
| Cai et al., 2018 | Case–control       | 80             | 40¹            | 40 NA         | Proliferative and secretory | 6 months               | IHC                     | eIF3e                 | ↓       | -                |
|                  |                    |                |               |               |                     |                        |                         | TGF-β                 | ↑       | -                |
|                  |                    |                |               |               |                     |                        |                         | E-cadherin            | ↓       | -                |
|                  |                    |                |               |               |                     |                        |                         | Vimentin              | ↑       | -                |
|                  |                    |                |               |               |                     |                        |                         | Snail                 | ↑       | -                |
|                  |                    |                |               |               |                     |                        |                         | PCNA                  | ↑       | -                |
| Yang et al., 2017 | Case–control       | 60             | 10            | 10 Age        | Proliferative       | 6 months               | IHC and ELISA           | LPA1¹                 | ↑↑      | -                |
|                  |                    |                |               |               |                     |                        |                         | LPA2¹                 | ↓↓      | -                |
|                  |                    |                |               |               |                     |                        |                         | LPA3¹                 | ↓↓      | -                |
|                  |                    |                |               |               |                     |                        |                         | LPA4¹                 | ↑↑      | -                |
|                  |                    |                |               |               |                     |                        |                         | LPA5¹                 | ↑↑      | -                |
|                  |                    |                |               |               |                     |                        |                         | LPA6¹                 | =       | -                |
| Nie et al., 2016 | Case–control       | 68             | 50²            | 18 NR         | Proliferative and secretory | 6 months               | IHC                     | vWF                   | ↑       | ↑ Menorrhaga |
|                  |                    |                |               |               |                     |                        |                         | CD68                  | ↑       | ↑ Menorrhaga |
| Liu et al., 2016 | Cross-sectional case control | 81             | 34¹            | 20² Age, parity, menstrual phase | Proliferative and secretory | 6 months               | IHC, IF and western blot | TGF-β¹                | ↑↑↑↓↑↑ |
|                  |                    |                |               |               |                     |                        |                         | CD41¹                 | ↑↑↑↓↑↑ |
|                  |                    |                |               |               |                     |                        |                         | Vimentin²             | ↑↑↑↓↑↑ |
|                  |                    |                |               |               |                     |                        |                         | E-cadherin            | ↑↑↑↓↑↑ |
|                  |                    |                |               |               |                     |                        |                         | VEGFa                 | ↑↑↑↓↑↑ |
|                  |                    |                |               |               |                     |                        |                         | CD31¹                 | ↑↑↑↓↑↑ |
|                  |                    |                |               |               |                     |                        |                         | α-SMAa                | ↑↑↑↓↑↑ |
| Zhihong et al., 2016 | Case–control       | 42             | 18            | 24 NA         | Secretory           | 1 year¹                | IHC, quantitative RT-PCR | IL-6a                 | ↑       | -                |
|                  |                    |                |               |               |                     |                        |                         | IL-10a                | ↓       | -                |
| Wang et al., 2016 | Case–control       | 40             | 30²            | 10 NR         | Proliferative and secretory | 3 months               | IHC, in situ TUNEL, western blot | GRIM-19b              | ↓↓↓↓↓↓ |
|                  |                    |                |               |               |                     |                        |                         | VEGFb                 | ↑↑↑↑↑↑ |
|                  |                    |                |               |               |                     |                        |                         | CD34¹                 | ↑↑↑↑↑↑ |
| Guo et al., 2015 | Prospective case–control | 47             | 30            | 17 NR         | Proliferative and secretory | 3 months               | IHC and western blot    | Dj-1¹                 | ↑↑      | -                |
|                  |                    |                |               |               |                     |                        |                         | p-mTOR¹               | ↑↑      | ↑↑               |

(Continued)
Table I (Continued)

| Study, year   | Study type          | N participants | Patient group | Control group | Matching Menstrual cycle phase | Time of no hormone use | Methods | Angiogenic parameters | Outcome | Eutopic | Ectopic | Dysmenorrhea | Association clinical symptom |
|---------------|---------------------|----------------|---------------|---------------|--------------------------------|------------------------|---------|-----------------------|---------|---------|---------|--------------|-----------------------------|
| Carrarelli et al., 2015 | Prospective case–control | 20 | 8 | 12 | NR | Proliferative | 1 month | IHC, RT-PCR | Activin A<sup>a</sup> | = | ↑ | ↑↑↑ | - | Assistend clinical symptom |
| Huang et al., 2014 | Prospective case–control | 130 | 100 | 30 | NR | Proliferative, secretory | 3 months | IHC, ELISA, western blot | VEGF<sup>b</sup> | ↑↑↑↑↑↑ | ↑↑↑↑ | ↑↑↑↑↑↑↑↑ | - |
| Wang et al., 2014 | Case–control | 20 | 10 | 10 | NA | NR | NR | IHC | IL-22, IL-22R1, IL-10R2 | ↑ | ↑ | - | |
| Li et al., 2013 | Case–control | 31 | 23 | 8 | None | NR<sup>b</sup> | 3 months | Electrophoretic shift assay, gene, protein expression | NF-κB (p50, p65) | - | ↑↑↑↑ | - | Dysmenorrhea |
| Nie et al., 2011 | Case–control | 68 | 50<sup>c</sup> | 18 | NR | Proliferative and secretory | 6 months | IHC | SLIT<sup>b</sup> | = | ↑ | - | Dysmenorrhea (SLIT) |
| Liu et al., 2011 | Case–control | 68 | 50<sup>c</sup> | 18 | NR | Proliferative and secretory | 6 months | IHC | TF<sup>c</sup> | ↑↑↑ | ↑ | AUB dysmenorrhea |
| Nie et al., 2009 | Retrospective case–control | 68 | 50 | 18 | None | Proliferative and secretory | 6 months | IHC | PR-B<sup>c</sup> | ↓↓↓ | ↓ | AUB dysmenorrhea |
| Tokyol et al., 2009 | Retrospective case–control | 45 | 25 | 20 | NR | Proliferative and secretory | NR | IHC | MMP-2<sup>c</sup> | =/= | ↑/↑ | - | |
| Li et al., 2006 | Prospective case–control | 88 | 68 | 20 | NR | Proliferative and secretory | 3 months | IHC | MMP-2<sup>c</sup> | ↑/↑ | ↑/↑ | - | |
| Hayrabedyan et al., 2005 | Case–control | 50 | 20 | 5 | NR | Secretory | NR | IHC | Endoglin<sup>c</sup> | ↑ | ↑↑ | - | |

(Continued)
### Table I (Continued)

| Study, year   | Study type                  | N participants | Patient group | Control group | Matching | Menstrual cycle phase | Time of no hormone use | Methods  | Angiogenic parameters | Outcome | Association clinical symptom |
|---------------|-----------------------------|----------------|---------------|---------------|----------|------------------------|-------------------------|----------|------------------------|---------|-------------------------|
| Schindl et al., 2001 | Retrospective case-control  | 70             | 53            | 17            | NR       | Proliferative and secretory | 4 months              | IHC      | CD34                   | Eutopic | Ectopic                |
|               |                             |                |               |               |          |                        |                         |          |                        | =       | ↑↑↑/↑↑↑                |
| Ota et al., 2001 | Case–control                | 83             | 331          | 50            | NR       | Proliferative and secretory | None                  | IHC      | COX-2                  | =/=     | =/=                    |
| Ota et al., 1998 | Case–control                | 71             | 422          | 29            | NR       | Proliferative and secretory | None                  | IHC      | vWF                    | ↑/↑     | -                      |

Superscripts: 1focal and diffuse, 2all diffuse, 3all focal adenomyosis, 4group includes nine patients with ovarian cyst, 5includes nine patients with leiomyoma less than one-third of uteri corpus uteri, 6includes patients with carcinoma in situ, 7no comparison performed between the phases, 8no significant differences observed between the phases, 9no P-value known for difference between the phases

Symbols: - not included in the study, = no difference observed compared with control endometrium, ↑ increase, ↓ decrease, ↑↑ or ↓↓ significant difference compared with control endometrium with P < 0.05; ↑↑↑ or ↓↓↓ significant difference compared with control endometrium with P < 0.001

/ difference between the menstrual phases indicating: proliferative phase / secretory phase

NR, not reported; HIC, immunohistochemistry; IF, immunohistochemistry; VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; p-mTOR, phosphorylated mammalian target of rapamycin; COX-2, cyclooxygenase-2; LPA, Lysophosphatidic acid 1,4-5, phospho signal transducer and activator of transcription 3; IL-, interleukin-; TF, tissue factor; ROBO 1, roundabout 1; AUB, abnormal uterine bleeding

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### Antiangiogenic markers

Four articles reported on markers with antiangiogenic capacity that were identified (Tables III and IV). Two articles reported decreased expression of VEGF (Table IV). The immunohistochemical staining and protein expression levels of all markers were significantly increased in the ectopic endometrium compared with the endometrium of controls. The immunohistochemical staining and protein expression levels of all markers were significantly increased in the ectopic endometrium compared with the endometrium of controls. The immunohistochemical staining and protein expression levels of all markers were significantly increased in the ectopic endometrium compared with the endometrium of controls.
| Substance group | Mechanism | Anti-angiogenic agent | Studies | Effect on endo- or myometrium | Effect on clinical symptoms |
|-----------------|-----------|-----------------------|---------|-----------------------------|-----------------------------|
| GnRH agonist    | Reduction of estrogen and progesterone concentrations | Leuprolide acetate | (Khan et al., 2010) | Decreased staining of vWF (MVD) and angiogenesis in endo- and myometrium. No difference in MVD in adenomyotic lesions | - |
| Progestogens    | Reduction of estrogen concentration and progesterone receptors | Levonorgestrel (LNG-IUS) | (Laoag-Fernandez et al., 2003) | Decrease in VEGF expression in endometrial epithelium and stroma | No correlation between number of bleeding days and expression levels of VEGF |
| Growth factor inhibitors | Alleviating expression of IL-6, IL-8, TGF-β, EGF, VEGF, MMP-2 | Berberine | (Liu et al., 2018) (Liu et al., 2017) | Inhibit proliferation and viability of eutopic and ectopic endometrial stromal cells | - |
| NF-κB inhibitor | Andrographolide (Andrographis paniculata) | | (Li et al., 2013) | Decrease in mRNA and protein levels of COX-2, VEGF and TF | - |
| Predclinical studies | Mechanism | Anti-angiogenic agent | Studies | Model | Effect on endo- or myometrium | Effect on clinical symptoms |
| Anti-platelet therapy | Thromboxane A2 (TXA2) synthesis inhibitor or platelet deplletion | Ozagrel; rat anti-mouse GP Ibα polyclonal IgG antibody | (Zhu et al., 2016) | ICR mice with neonatal dosing of tamoxifen | Both ozagrel and platelet depletion reduced the depth of myometrial invasion, platelet aggregation and staining level of COX-2 and NF-κB p65 | Diminished hyperalgesia (improvement in hot plate latency) |
| Growth factor inhibitors | Anti-VEGF antibody | Bevacizumab | (Huang et al., 2014) | Xenotransplantation of human adenomyosis in NODSCID mice | Reduce blood vessel formation | - |
| | Annexin A2 inhibition (ANXA2) | ANXA2 knockout via siRNA | (Zhou et al., 2012) | Nude mouse model | Inhibition of endometrial tissue growth | Diminished hyperalgesia (improvement in hot plate latency) |
| | Fumagillin analogues | Reduction mean surface area blood vessels | TNP-470 | (Zhou et al., 2003) | Virgin female SHN mice implanted with pituitary gland | Inhibition of endothelial cell migration and proliferation | - |

MVD, mean vascular density; LNG-IUS, levonorgestrel-releasing intrauterine system; EGF, epidermal growth factor; NODSCID, non-obese diabetic severe combined immunodeficient.
Table III  Outcome of vascular characteristics and angiogenic markers.

| Location in uterus | Eutopic endometrium | Ectopic endometrium | Studies |
|--------------------|---------------------|---------------------|---------|
|                    | Endothelium | (Glandular) epithelium | Stroma | Endothelium | (Glandular) epithelium | Stroma |       |
| MVD and vascular characteristics |          |                      |        |          |                      |        |       |
| MVD by CD31        | ↑         | ↑↑↑                  |        | (Huang et al., 2014; Liu et al., 2016) |
| MVD by CD34        | ↑/=       | ↑                    |        | (Schindl et al., 2001; Tokyol et al., 2009; Nie et al., 2011; Wang et al., 2016) |
| MVD by FVIII-Rag   | =         | ↑↑↑                  |        | (Li et al., 2006) |
| vWF                 | ↑         |                      |        | (Ota et al., 1998; Nie and Liu, 2016) |
| Endoglin           | ↑         | ↑                    |        | (Hayrabedyan et al., 2005) |

Pro-angiogenic and vascular markers

| VEGF                | ↑         | ↑↑↑                  |        | (Li et al., 2006; Li et al., 2013; Huang et al., 2014; Liu et al., 2016; Wang et al., 2016; Liu et al., 2016; Wang et al., 2016; Huang et al., 2014; Li et al., 2006; Li et al., 2013) |
| S100A13             | ↑         | ↑↑↑                  |        | (Hayrabedyan et al., 2005) |
| Vimentin            | =         | ↑↑↑                  |        | (Liu et al., 2016; Cai et al., 2018) |
| MMP-2, -9           | ↑         | ↑                    |        | (Li et al., 2006; Tokyol et al., 2009) |
| NF-kB (p50, p65)    | ↑         |                      |        | (Nie et al., 2009; Li et al., 2013) |
| Tissue factor       | ↑         | ↑                    |        | (Liu et al., 2011; Li et al., 2013; Liu et al., 2011; Li et al., 2013) |
| Dj-1                |         | ↑↑↑                  |        | (Guo et al., 2015) |
| p-mTOR              | ↑↑↑       | ↑                    |        | (Guo et al., 2015) |
| Activin A           | ↑         | ↑                    |        | (Carrarelli et al., 2015) |
| Follistatin         | ↑         | ↑                    |        | (Carrarelli et al., 2015) |
| Myostatin           | ↑         | ↑                    |        | (Carrarelli et al., 2015) |
| Platelet count by CD41 |       | ↑                    |        | (Liu et al., 2016; Cai et al., 2018) |
| SLT                 |           |                       |        | (Nie et al., 2011) |
| ROBO1               | ↑         | ↑                    |        | (Nie et al., 2011) |
| COX-2               | =         | =                    |        | (Ota et al., 2001; Tokyol et al., 2009; Li et al., 2013) |
Table III (Continued)

| Location in uterus | Eutopic endometrium | Ectopic endometrium | Studies |
|--------------------|---------------------|---------------------|---------|
|                    | Endothelium         | (Glandular) epithelium | Stroma | Endothelium | (Glandular) epithelium | Stroma |
| LPA1, 4-5          | ↑                   | ↑                   | ↑↑↑     | ↑↑↑         | (Yang et al., 2017) |
| pSTAT3             | ↑                   | ↑                   | ↑↑↑     | ↑↑↑         | (Wang et al., 2016) |
| IL-6**             | ↑                   | ↑                   | ↑↑↑     | ↑↑↑         | (Zhihong et al., 2016) |
| α-SMA              | ↑                   | ↑                   | ↑↑↑     | ↑↑↑         | (Liu et al., 2016) |
| IL-22**            | ↑                   | ↑                   | ↑↑↑     | ↑↑↑         | (Wang et al., 2014) |
| TGF-β1**           | ↑                   | ↑                   | ↑       | ↑           | (Liu et al., 2016; Cai et al., 2018; Liu et al., 2016; Cai et al., 2018) |

Anti-angiogenic markers

| Marker               | Changes | Studies |
|----------------------|---------|---------|
| GRIM-19              | ↓       | (Wang et al., 2016) |
| IL-10**              | ↓       | (Zhihong et al., 2016) |
| E-cadherin           | ↓       | (Liu et al., 2016; Cai et al., 2018) |
| eIF3e                | ↓       | (Cai et al., 2018) |

**No difference; ↑↓/↑↓ increase/decrease compared with control endometrium; ↑↑/↑↓↑↓ increase/decrease compared with control endometrium; ↑↑↑/↑↓↑↓ increase/decrease compared with control endometrium; ⋆Type of cell not reported, ⋆Anti-inflammatory cytokines with angiogenesis regulatory functions.

Type of cells expressing (anti-)angiogenic markers

All markers for MVD and vascular characteristics were specific for endothelial cells in order to quantify and characterize blood vessels in the studied samples. Immunoreactivity of S100A13 (Hayrabedian et al., 2005), MMP-2 and ROBO1, a receptor for SLIT, was also observed on the surface and within vascular endothelial cells (Nie et al., 2011; Tokyol et al., 2009). Glandular epithelial cells (Wang et al., 2016; Li et al., 2006; Goteri et al., 2009; Liu et al., 2016; Cai et al., 2006; Orazov et al., 2016), stromal cells (Li et al., 2006; Goteri et al., 2009; Li et al., 2013; Orazov et al., 2016) and endothelial cells (Goteri et al., 2009; Orazov et al., 2016) in the endometrium were stained positive for VEGF in women with adenomyosis whereas the staining in the control endometrium was either very low or not reported (Wang et al., 2016; Li et al., 2006; Goteri et al., 2009; Liu et al., 2016; Li et al., 2013). The expression of COX-2 was observed not only in the endothelial, glandular (Ota et al., 2001; Tokyol et al., 2009) and stromal (Li et al., 2013) cells but also in plasma cells (Ota et al., 2001). S100A13 (Hayrabedian et al., 2005) staining was present in glandular epithelial cells, stromal cells and vascular smooth muscle cells as well as in the perivascular space. For the markers activin A and its receptors ActRIIA and ActRIIB, the type of cell was not studied.

Immunoreactivity of α-SMA, TGF-β1 and vimentin (only ectopic) (Liu et al., 2016; Cai et al., 2018), MMP-2–9 (Li et al., 2006; Tokyol et al., 2009), TF (Liu et al., 2011; Li et al., 2013), DJ-1/p-mTOR (Guo et al., 2015), follistatin, myostatin (Carrarelli et al., 2015), LPA1, 4–5 (Yang et al., 2017), pSTAT3 (Wang et al., 2016), IL-6 and IL-10 (only eutopic) (Zhonghong et al., 2016) and IL-22 (Wang et al., 2014) was reported in both glandular epithelial cells and stromal cells. Tissue expression of E-cadherin, eIF3e (Liu et al., 2016; Cai et al., 2018), SLIT (Nie et al., 2011) and GRIM-19 (Wang et al., 2016) was only reported in epithelial cells, while NF-κB (p50, p65) (Li et al., 2013; Nie et al., 2009) and CD41 (Liu et al., 2016) expression was only reported in the stroma. ROBO1 (Nie et al., 2011) and MMP-2 were reported in both endothelial and epithelial cells.

Tissue expression of angiogenic markers during the menstrual cycle

Eighteen studies mentioned the phase of the menstrual cycle in which the samples were collected (Table I). Two of the six articles on MVD in which the difference between the menstrual phases was taken into account reported an increased MVD in the secretory phase compared with the early proliferative phase (Huang et al., 2014; Tokyol et al., 2009), whereas the other studies found no difference in MVD between the secretory and proliferative phases (Nie et al., 2011; Li et al., 2006; Schindl et al., 2001; Wang et al., 2016; Nie and Liu, 2016). All Eng samples were taken in the secretory phase (Hayrabedian et al., 2005). The levels of staining for vWF were increased in all adenomyotic samples, with no difference between the menstrual phases (Ota et al., 1998). One study reported that the staining of VEGF had a greater density in the secretory phase compared with the proliferative phase (Huang et al., 2014), while two studies found no difference between the phases (Li et al., 2006; Wang et al., 2016) and two others did not compare menstrual phases (Li et al., 2013; Liu et al., 2016).
### Table IV  STROBE: Strengthening the Reporting of Observational Studies in Epidemiology.

| Item  | Cai et al., 2018 | Liu, 2018 | Liu, 2017 | Yang et al., 2017 | Liu, 2016 | Zhihong, 2016 | Wang, 2016 | Guo, 2015 | Carrarelli, 2015 | Huang, 2014 | Wang, 2014 | Li et al., 2013 | Nie, 2011 | Liu, 2011 | Goyer, 2009 | Khan, 2010 | Nie, 2009 | Tokyo, 2009 | Li, 2006 | Hayrabedyan, 2005 | Laoag-Fernandez, 2003 | Schindl, 2001 | Otta, 2001 | Otta, 1998 |
|-------|------------------|-----------|-----------|------------------|-----------|--------------|-----------|----------|------------------|-----------|----------|------------------|---------|---------|-------------|----------|---------|--------------|--------|------------------|------------------------|-----------|----------|-----------|
| 5     | 1                | 0         | 1         | 0                | 1         | 0            | 0         | 0        | 1                | 1         | 1        | 1                | 1       | 1       | 1            | 1        | 1       | 1            | 1       | 1       | 1            |
| 6     | 1                | 0         | 0         | 1                | 1         | 1            | 1         | 1        | 1                | 1         | 0        | 0                | 0       | 0       | 0            | 0        | 0       | 0            | 0       | 0       | 0            |
| 6b    | 0                | NA        | NA        | 1                | 1         | 1            | 1         | 1        | 0                | 1         | 1        | 1                | 1       | 1       | 1            | 1        | 1       | 1            | 1       | 1       | 1            |
| 7     | 1                | 0         | 0         | 1                | 1         | 1            | 1         | 1        | 0                | 1         | 1        | 1                | 1       | 1       | 1            | 1        | 1       | 1            | 1       | 1       | 1            |
| 8     | 1                | 1         | 1         | 1                | 1         | 1            | 1         | 1        | 0                | 1         | 1        | 1                | 1       | 1       | 1            | 1        | 1       | 1            | 1       | 1       | 1            |
| 9     | 1                | 0         | 0         | 0                | 0         | 0            | 0         | 0        | 0                | 0         | 0        | 0                | 0       | 0       | 0            | 0        | 0       | 0            | 0       | 0       | 0            |
| 10    | 0                | 0         | 0         | 0                | 0         | 1            | 0         | 0        | 0                | 0         | 0        | 0                | 0       | 0       | 0            | 0        | 0       | 0            | 0       | 0       | 0            |
| 12    | 1                | 0         | 0         | 1                | 1         | 1            | 1         | 1        | 1                | 1         | 1        | 1                | 1       | 1       | 1            | 1        | 1       | 1            | 1       | 1       | 1            |
| 13    | NA               | NA        | NA        | NA               | NA        | NA           | NA        | NA       | NA               | NA        | NA       | NA               | NA      | NA      | NA           | NA       | NA      | NA           | NA      | NA      | NA           |
| 14    | 1                | 0         | 0         | 1                | 1         | 1            | 1         | 0        | 0                | 0         | 0        | 0                | 0       | 0       | 0            | 0        | 0       | 0            | 0       | 0       | 0            |
| 16    | NA               | 1         | 1         | NA               | 0         | NA           | 0         | 0        | 0                | 0         | 0        | 0                | 0       | 0       | 0            | NA       | 1       | NA           | NA      | 0       | NA           | NA      | NA      | NA           |

Assessment of risk of bias according to the STROBE checklist for observational studies: each item is classified as adequate (1), inadequate (0) or not applicable ‘NA’ in the evaluation of a paper.
### Table V

Newcastle Ottawa Scale for case control studies.

| Study Authors and Year | Selection | Comparability | Exposure |
|------------------------|-----------|---------------|----------|
| Cai et al., 2018       | 1         | 1             | 1        |
| Liu, 2018              | 0         | 0             | 0        |
| Liu, 2017              | 1         | 1             | 0        |
| Yang et al., 2017      | 1         | 1             | 0        |
| Nie, 2016              | 1         | 1             | 1        |
| Liu et al., 2016       | 1         | 1             | 1        |
| Nie, 2015              | 1         | 1             | 1        |
| Li et al., 2013        | 1         | 1             | 1        |
| Wang, 2014             | 1         | 1             | 1        |
| Huang, 2014            | 1         | 1             | 1        |
| Carré, 2013            | 1         | 1             | 1        |
| Guo, 2015              | 1         | 1             | 1        |
| Wang, 2016             | 1         | 1             | 1        |
| Zhao, 2016             | 1         | 1             | 1        |
| Liu, 2016              | 1         | 1             | 1        |
| Nie, 2015              | 1         | 1             | 1        |
| Yang et al., 2017      | 1         | 1             | 1        |
| Liu, 2017              | 1         | 1             | 1        |
| Liu, 2018              | 1         | 1             | 1        |
| Cai et al., 2018       | 1         | 1             | 1        |

Assessment of risk of bias according to the Newcastle Ottawa Scale for case control studies: each item is classified as adequate (1), inadequate (0) or not applicable 'NA'.
In the secretory phase, there was an increased expression reported of the pro-angiogenic parameters COX-2, MMP-2 and MMP-9 (only for eutopic endometrium) (Tokyol et al., 2009; Li et al., 2006). However, Ota et al. (2001) reported no difference in COX-2 expression between the phases. No difference in expression between the phases was reported for SLIT and ROBO1 (Nie et al., 2011), TF (Liu et al., 2014) and GRIM-19 or pSTAT3 (Wang et al., 2016). Dj-1 and p-mTOR were the only parameters with a higher expression reported in the proliferative phase than in the secretory phase (Guo et al., 2015).

Differences between the two menstrual phases were not studied for α-SMA, TGF-β1, platelet aggregation (CD41), vimentin, E-cadherin, elf3e (Liu et al., 2016; Cai et al., 2018), NF-κB (Nie et al., 2009; Li et al., 2013) and IL-22 (Wang et al., 2014). The samples for LPA (Yang et al., 2017), activin A, follistatin and myostatin (Carrarelli et al., 2015) were all collected in the proliferative phase, while the samples for IL-6 and IL-10 (Zhihong et al., 2016) and Eng were only taken in the secretory phase (Hayrabedyan et al., 2005).

**Anti-angiogenic therapy for adenomyosis**

The search on anti-angiogenic therapy in adenomyosis identified nine articles reporting on targeting angiogenesis in (pre)clinical studies (Table II). GnRH agonists (GnRHa) may act indirectly, through estrogen or estrogen’s effect on the expression of VEGF, on angiogenesis in the endometrium (Khan et al., 2010). As GnRHa induces a hypo-estrogenic state, they lower VEGF and COX-2, which in turn may result in a downregulation of the abnormal endometrial vascularity (Khan et al., 2010). The vWF-positive MVD in biopsy specimens obtained after reduction surgery and hysterectomy for adenomyosis in women with GnRHa therapy for 3–6 months was significantly decreased compared with the non-treated group (Khan et al., 2010). The levonorgestrel-releasing intra-uterine system (LNg-IUS) is a common treatment modality for menorrhagia, and the first line of treatment for adenomyosis (Vannucini et al., 2018). One study demonstrated that after 3 months of LNg-IUS use, the level of expression of VEGF was decreased in the eutopic endometrium of patients with adenomyosis, while the effect on the ectopic endometrium was not studied (Laag-Fernandez et al., 2003). On the contrary, the level of VEGF expression was not correlated to the number of bleeding days. A number of herbal compounds have been studied for their anti-angiogenic potential. Andrographolide has been reported to disrupt NF-κB activation (Li et al., 2013). Treating adenomyosis patients with andrographolide resulted in reduced expression levels of COX-2, VEGF and TF (Li et al., 2013; Zheng et al., 2018). Another study investigated the growth inhibitory effect of berberine and

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**Table VI  Gold standard publication checklist.**

|                          | Zhu, 2016 | Huang, 2014 | Zhou, 2012 | Zhou, 2003 |
|--------------------------|-----------|-------------|------------|------------|
| **Introduction**         |           |             |            |            |
| Background information (3p) | 3         | 2           | 2          | 2          |
| Research question or hypothesis (2p) | 1         | 0           | 1          | 1          |
| Clinical relevance (2p)  | 1         | 0           | 1          | 2          |
| **Methods**             |           |             |            |            |
| Experimental design (1p) | 1         | 1           | 1          | 1          |
| Experimental groups and controls (19p) | 14        | 6           | 13         | 13         |
| Regulations and ethics (2p) | 2         | 1           | 1          | 1          |
| The intervention (12p)   | 12        | 4           | 7          | 11         |
| Outcome (3p)             | 2         | 1           | 2          | 2          |
| **Results (7p)**         | 6         | 4           | 3          | 3          |
| **Discussion (3p)**      | 3         | 3           | 3          | 3          |
| **Total score out of 54** | 45        | 22          | 34         | 39         |

Assessment of risk of bias: each topic contains a list of items which ought to be addressed in every research article on animal experiments (Hooijmans et al., 2010). The total of items per topic (1 point per item) is stated behind each topic.
reported a decrease in the expression levels of IL-6, IL-8, TGF-β, EGF, VEGF and MMP-2, thus inhibiting the growth of ectopic endometrial stromal cells (Liu et al., 2017; Liu et al., 2018). This effect was also partly exerted through the inhibition of nuclear NF-κB/p65 translocation.

Four studies were identified that targeted angiogenesis in a preclinical study (Table II). In ovariectomized mice xenografted with human adenomyosis lesions and treated with bevacizumab (anti-VEGF monoclonal antibody), the MVD decreased and the expression of VEGF was reduced (Huang et al., 2014). Another study targeted angiogenesis in mice that developed adenomyosis after pituitary gland implantation using TNP-470, a potent inhibitor of angiogenesis known from arthritis research (Zhou et al., 2003). The mean surface area of blood vessels in the endometrium of the TNP-470-treated group reduced to 60.5% of that in the control group, and the TNP-470-treated group did not develop signs of uterine adenomyosis as opposed to 80% in the

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**Figure 2 Overview of interactions between the (anti-)angiogenic markers reported in this review.** Red: association with abnormal uterine bleeding; green: association with subfertility; ↑ increase, = no change, ↓ decrease. TNF-α, tumor necrosis factor alpha; NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells; HIF-1α, hypoxia-inducible factor 1 alpha; eIF3e, eukaryotic translation initiation factor 3 subunit; DJ-1; p-mTOR, phosphorylated mammalian target of rapamycin; TGF-β, transforming growth factor beta; VEGF, vascular endothelial growth factor; IL-, interleukin-; LPA, lysophosphatidic acid; MMP, matrix metalloproteinase; COX-2, cyclooxygenase-2; pSTAT3, phospho signal transducer and activator of transcription 3; GRIM-19, gene associated with retinoic-interferon-induced mortality 19; SLIT; ROBO1, roundabout 1. Note: adjusted figure based on information described in the literature. However, these interactions do not necessarily represent the actual or complete biological pathways. Diagram made with Microsoft Visio 2010.
control group. Zhou et al. (2012) identified the angiogenic potential of overexpressed annexin A2 (ANXA2), an estrogen-responsive protein, through hypoxia inducible factor (HIF)-1α/VEGF-A activation, in the ectopic endometrium compared with the eutopic endometrium of adenomyosis patients. Expression of ANXA2 was associated with the epithelial-to-mesenchymal transition (EMT), as well as to the severity of dysmenorrhea in adenomyosis patients. In an in vivo nude mouse model, they demonstrated that ANXA2 knockdown reduced angiogenesis, suggesting its potential as a therapeutic target.

Zhou et al., 2016 investigated the effect of high- and low-dose anti-platelet therapy using a thromboxane A2 synthesis inhibitor (ozagrel) or platelet depletion therapy using the rat anti-mouse GPIbα polyclonal IgG antibody in an adenomyosis mouse model. Platelet activation is related to angiogenesis since its co-occurrence with TGF-β1 release leads to EMT and fibroblast-to-myofibroblast transdifferentiation. Both ozagrel and platelet depletion therapy reduced depth of myometrial invasion, platelet aggregation and staining level of COX-2 and NF-κB p65. There were no adverse effects observed after ozagrel treatment. However, in the high-dose platelet depletion group, one mouse died and two appeared lethargic, while there were no apparent side effects in the low-dose depletion therapy group. Both treatment strategies resulted in significantly reduced platelet counts, depth of myometrial infiltration and staining level of COX-2 and NF-κB p65.

**Discussion**

**Main findings**

This study gives an overview of the angiogenesis-related markers in the endometrium of women with adenomyosis. Out of all outcomes of the included articles, MVD and VEGF were the most often studied parameters. The majority of articles that included these two markers reported an increase in levels in both eutopic and ectopic endometrium in adenomyosis patients compared with control patients. Increased VEGF leads to more angiogenesis, which in turn results in an increased MVD. The markers studied in the included 20 articles either directly or indirectly influence the expression of VEGF, MVD or capillary characteristics, suggesting their impact on angiogenesis in both ectopic and eutopic located endometrium (Table III and Fig. 2). This is in agreement with our hypothesis that adenomyosis patients have an increased MVD and display more angiogenic markers in the eutopic and ectopic endometrium than healthy individuals. Direct correlation with AUB or fertility outcomes has not been studied extensively, and agreement with our hypothesis that adenomyosis patients have an increased MVD and display more angiogenic markers between the proliferative or secretory phases of the menstrual cycle. Continuous regeneration and degeneration of the endometrium during the menstrual cycle under the influence of estradiol (E2) has been associated with VEGF expression in endometrial epithelial cells (Moller et al., 2001), causing a higher expression of VEGF in the secretory phase than in the early (Day 6–10) proliferative phase (Chen et al., 2010; Huang et al., 2014). This is surprising, since we expected an increased level of angiogenesis in the proliferative phase after the menses to rebuild the endometrial layer under the influence of increased E2 levels. The inconsistency with our findings may be explained by different expression of VEGF within the early, mid and late proliferative phases (Sugino et al., 2002), which were not taken into account in the included studies.

**Interpretation of the findings**

The potential role of angiogenesis in the pathophysiology of adenomyosis and its related symptoms is complex. It is a process that is suggested to be triggered by tissue injury and repair (TIAR) causing reactive EMT in response to hypoxic and hormonal stimuli (Leyendecker et al., 2009; Ibrahim et al., 2017). An interpretation of the findings of our review and their potential interaction is depicted in Fig. 2.

The underlying pathogenesis relies on the theory that endometrial cells invade the myometrium at sites in the junctional zone that are weakened, either from genetic predisposition or from uterine auto-traumatization and induced hypoxia (Benagiano et al., 2012). In this theory, hyper and non-synchronized peristalses of the uterus following physiological processes such as menstruation or sperm transport cause chronic damage to the junctional zone, where the endometrium lies adjacent to the myometrium (Leyendecker, 2015). When the endometrium invades these disruptions in the junctional zone, ectopic foci of endometrium establish and cause local inflammation and hypoxia. Exposure to ovarian estrogens, but also local estrogen production due to local estrogen sulfatase and aromatase activity in the endometric tissue, may play an additional role (Bulun, 2009; Yamamoto et al., 1993). These events are directly related to the increased angiogenesis in these tissues since they lead to the production of VEGF, thus causing angiogenesis (Goteri et al., 2009). Hypoxia is suggested to be a driving factor for physiological angiogenesis to induce endometrial repair during menstruation (Maybin et al., 2018). A vicious cycle is established when estrogen increases uterine peristalsis again and more auto-traumatization follows (Vannuccini et al., 2017).

Hereby, this TIAR theory provides insight into the role of the immune system and inflammation in adenomyosis. The reciprocal relationship between angiogenesis and the immune system has long been recognized (Griffioen and Molema, 2000). On the one hand, it is known that angiogenesis can be regulated by immune cells (Dirkx et al., 2006; Sica et al., 2008; Dings et al., 2011) and cytokines produced by immune cells (Castermans et al., 2008; Yao et al., 1999). On the other hand, immunity is heavily regulated by angiogenesis (Griffioen et al., 1996; Griffioen et al., 1996; Dirkx et al., 2003; Dirkx et al., 2006). This makes the separation between angiogenesis and immunity difficult, also reflected by the cytokines in Table III, and the correlation with increased macrophage number in adenomyotic tissue (Nie and Liu, 2016). Most leukocytes produce a series of angiogenic factors, including VEGF, TGF-β and ILs (Carmeliet, 2003;
Angiogenesis and abnormal uterine bleeding (AUB) are closely associated with adenomyosis. Various studies have demonstrated that estrogen and progesterone are potent stimulators of COX-2, which in turn leads to increased levels of prostaglandins (Maia et al., 2005). The presence of EMT was evident by the increased expression of TGF-beta and vimentin in the epithelial cells of adenomyotic endometrium but negative in the control endometrium (Liu et al., 2016; Cai et al., 2018). TGF-beta derived from platelets is known to activate transcription markers involved in EMT (Zhang et al., 2016), and its presence was previously reported in adenomyotic mice (Ribatti, 2017; Shen et al., 2016). eIF3e, a eukaryotic initiation factor that seems to have an anti-angiogenic effect [silencing of eIF3e has previously been associated with increased angiogenesis through HIF-1α activation (Chen et al. 2010)] was studied for its supposed role in EMT in adenomyosis. Indeed, decreased expression of eIF3e was related to increased TGF-beta and vimentin levels in the ectopic endometrium (Cai et al. 2018). Staining of vimentin appeared in the cytoplasm of stromal cells in control and adenomyosis groups. It was stained positive in the cytoplasm of epithelial cells in the ectopic adenomyotic endometrium but negative in the control endometrium (Liu et al., 2016; Cai et al., 2018). Thus, vimentin may serve as a marker for adenomyotic tissue. α-SMA is a molecular marker for pericytes (used to determine the maturity of vessels) and predominates within vascular smooth muscle cells (Morikawa et al., 2002). Decreased levels of α-SMA expression were reported in women with menorrhagia compared with controls (Abberton et al., 1999), suggesting an increase in immature leaky vessels. Our expectation was contradicted in this review since increased α-SMA expression was reported in adenomyosis patients. However, the increase in TGF-β1 and α-SMA was only reported in the endometrial epithelium and stromal cells (Liu et al., 2016) and only provides evidence of fibrogenesis, which might have evolved secondary to angiogenesis in the myometrium (Liu et al., 2016).

Estrogen is also a potent stimulus for COX-2, which in turn leads to increased levels of prostaglandins in the uterus (Bulun et al., 2000; Chen et al., 2010). Symptoms of AUB and dysmenorrhea are associated with increased prostaglandin levels (Maia et al., 2005), while COX-2 was associated with angiogenic markers in vitro (Leung et al., 2003). However, this could not be confirmed in vivo, since COX-2 expression was not different between the ectopic and control endometrium (Tokyol et al., 2009).
The other angiogenic markers identified in this review, such as IL-6, IL-22, LPA (-1,-6), SLIT/ROBO, activin A, follistatin, myostatin and pSTAT3, stimulate endometrial stromal cells to produce VEGF (Carrarelli et al., 2015; Rocha et al., 2012; Wang et al., 2014; Wang et al., 2016) and induce autocrine activity, attraction, migration and proliferation of vascular endothelial cells (Wu et al., 2005; Kozian et al., 1997; Dickinson and Duncan, 2010; Wang et al., 2003; Wang et al., 2008; Yang et al., 2017). However, previous studies reported no positive correlation of SLIT/ROBO with MVD (Wang et al., 2003), and when LPA was added to a culture of endometrial stromal cells, there were no differences in the release of VEGF between cases and controls (Yang et al., 2017). The presence of activin A was previously linked to the development of AUB and dysmenorrhea since it increased VEGF in endometriosis patients (Rocha et al., 2012). Activin A has been linked to physiological processes, such as embryo implantation, and pathologies including endometrial carcinoma, where it acts by activating MMP-2 and -9 (Jones et al., 2006). MMPs are known for their great impact on tissue degradation during menstruation (Salamonsen and Woolley, 1996), and a link with increased angiogenesis in tumors has been reported (Bergers et al., 2000; Fang et al., 2000; Tang et al., 2005). MMP-2 and -9 were increased in adenomyosis patients, and the positive correlation with VEGF indicates increased angiogenesis (Li et al., 2006). STAT3 is reported to upregulate VEGF expression and stimulate growth of tumors through angiogenesis (Niu et al., 2002). By inhibiting STAT3, GRIM-19 can downregulate the VEGF expression. IL-10 is a known anti-angiogenic and anti-inflammatory factor that is produced by uterine natural killer cells and macrophages. IL-10 inhibits angiogenesis through downregulation of COX-2 and MMP-2 and -9 (Moore et al., 2001; Zhihong et al., 2016). A significantly lower expression of IL-10 reported in the adenomyosis endometrium compared with the control endometrium indicates an upregulation of angiogenesis.

Pathology of angiogenesis in adenomyosis in relation to AUB
Increased angiogenesis resulting in an increased amount of leaky fragile vessels may be the cause of AUB in women with adenomyosis (Leyendecker et al., 2009) (Fig. 3). The correlation between AUB and the MVD through staining of vWF, and increased levels of TF and NF-κB was the only evidence found to directly support this theory. TF is a glycoprotein in the cell membrane that is involved in the clotting cascade and platelet activation (Liu et al. 2011; Krikun et al., 2008). Increased levels of TF with the co-occurrence of enlarged and widened blood vessels in bleeding sites of the endometrium were linked to angiogenesis in the endometrium (Krikun et al., 2009; Krikun et al., 2008; Liu et al., 2011). The expression of TF was positively correlated with AUB and dysmenorrhea (Liu et al., 2011). The NF-κB heterodimer belongs to a family of nuclear transcription markers and is known to be involved in the degradation of basement membrane and the remodeling of the extracellular matrix, which is one of the earliest processes in angiogenesis. Also, it has been suggested to play a pivotal role in endometriosis (Guo 2007). However, the exact role of NF-κB in angiogenesis seems to differ among cell types and is inconclusive (Tabruyn and Griffioen 2008; Tabruyn et al. 2009). Thus, suggesting that NF-κB’s role in angiogenesis explains its association with dysmenorrhea and the severity of AUB in adenomyosis patients is disputable (Nie et al., 2010; Li et al., 2013). TNF-α, a known proangiogenic factor, can activate NF-κB, which translocates and binds to the nucleus of its target genes (Gilmore, 2006) and leads to higher protein levels of COX-2, VEGF and TF. This binding activity was associated with the severity of dysmenorrhea in adenomyosis patients (Li et al., 2013).

Pathology of angiogenesis in adenomyosis in relation to subfertility
VEGF and angiogenesis are crucial for embryonic implantation, decidual vascularization and the early stages of development (Burton et al., 2009). Any disruption in this process might lead to fertility problems. Unfortunately, a direct relationship between angiogenic markers and subfertility has not been studied in adenomyosis patients and could therefore not be established in this review. According to our hypothesis, there is indirect evidence to support the relationship between the increase in angiogenic markers in the endometrium of adenomyosis patients summarized in this review and the observed lower probability of clinical pregnancy and a 2-fold risk of miscarriage in this population undergoing IVF (Campo et al., 2012; Vercellini et al., 2014). One study reported increased levels of the angiogenic factor IL-6 and decreased IL-10 during the window of implantation in adenomyosis patients versus control patients after ovarian stimulation with GnRHa, indicating that increased angiogenesis might be involved in the observed compromised endometrium receptivity (Zhihong et al., 2016). No pregnancy outcomes were available to support this hypothesis.

Other studies have explored the relation between VEGF and abortions or miscarriages. Compared with control patients, there was an increased VEGF-A expression in women with spontaneous miscarriages. In women with recurrent miscarriages, decreased VEGF-A and VEGF-C with increased levels of VEGF-R1 and -R2, TGF-β1 and α-SMA staining and reduced levels of IL-10 were reported (Lash et al., 2012; Plevyak et al., 2002). Another study reported a decreased GRIM-19, resulting in an increased STAT3 expression in villous samples of patients with miscarriages compared with controls (Chen et al., 2015). They reported a lower VEGF expression in women with miscarriages in the same study (Chen et al., 2015). Suppression of angiogenesis by long-term downregulation with GnRHa, which might indirectly reduce VEGF expression, resulted in improvement in IVF outcomes with no difference in clinical pregnancy rates and early pregnancy loss in comparison to controls (Mijatovic et al., 2010; Costello et al., 2011). These results suggest that abnormal levels of VEGF (subtypes) may lead to a disbalance in the angiogenesis process that may play a role in a higher miscarriage rate. According to Lash et al. (2012), women with recurrent miscarriage have more matured vessels in the endometrium compared with controls, which contradicted our hypothesis of less matured vessels being the cause of infertility. This contradiction might be explained by the following: for an ongoing pregnancy, a certain balance of vessel maturity should be achieved. Possibly both overly matured vessels and less matured vessels can lead to miscarriages. In case of more matured vessels, less production of blood vessels or less adaptation of blood flow to the implanted fetus might be the cause of a higher risk for miscarriage. However, to the best of our knowledge there is no literature to further explore this theory.

Strengths and limitations
This is the first review of available literature describing possible angiogenic-related markers in the adenomyotic endometrium (both...
ectopic and eutopic) and relating the pathophysiology of angiogenesis to clinical symptoms of adenomyosis, such as AUB and fertility problems. Strengths of this review include the systematic approach in accordance with the Prisma guidelines using two databases (PubMed and Embase), its registration in the PROSPERO International prospective register of systematic reviews and the use of quality checklists to assess the included articles for risk of bias. Another strength of the review is the stratified presentation of the results concerning the outcomes of vascular characteristics (MVD), (anti-)angiogenic markers, type of cells and menstrual phase. However, not all articles reported the menstrual phase, age or parity of the participants or adjusted the results accordingly. Neither were other potential confounders, such as medication use or comorbidity, most importantly endometriosis, taken into account in most articles. The lack of information on these factors seriously limits the interpretability of the observed differences in the angiogenic profile of the endometrium and prevents further insight into the possible correlation of these individual factors with angiogenesis in the endometrium. Despite the limitation of the unknown effect of concomitant endometriosis, the presence of angiogenesis in the eutopic endometrium in adenomyosis seems most relevant for the association with AUB or subfertility (Vercellini et al., 2014).

Other limitations of the articles include the relatively small sample size and the heterogeneity of the study population and of the angiogenic markers. Most of the studied markers were only studied and reported in a few articles, hampering the ability to draw solid conclusions on all individual factors in relation to angiogenesis in adenomyosis. Some possible relevant angiogenic markers, such as IFN-alpha and TNF-alpha, were not studied in the endometrium of adenomyosis patients. Additionally, some articles included other endometrial abnormalities in their control group, and these abnormalities may share pathological pathways with adenomyosis (Tokyol et al., 2009). Also, many articles lacked a pre-reported definition of adenomyosis or a description of the location of the biopsies taken. Differences in depth of the endometrial invasion may lead to different angiogenic results. The additional search on potential anti-angiogenic therapy for adenomyosis further strengthens this review and stresses the clinical relevance of the topic. However, the heterogeneity of the anti-angiogenic agents and (animal) models used severely limits the interpretability of these results. Moreover, the animal studies lacked a complete description of the applied methods and the results. For example, incomplete reporting of physiological parameters of the animals limits the understanding of the side-effect profile of the applied therapy.

**Conclusion**

The increased expression of angiogenic markers and decrease in anti-angiogenic markers, as well as an increase in MVD and capillary characteristics in the ectopic and eutopic endometrium in comparison with the endometrium in control patients without adenomyosis, support our hypothesis that increased angiogenesis plays a role in the pathogenesis of adenomyosis.

**Future perspectives**

The implicated role of these markers in AUB and subfertility outcomes, such as lowered pregnancy rates and increased miscarriage rates during IVF, is only based on indirect evidence. Direct evidence requires future studies focusing on the relation between angiogenesis and these clinical outcomes in adenomyosis patients. Confirmation of this hypothesized relation might give future tools for the development of targeted selective therapies. When a positive correlation between ongoing angiogenesis and the occurrence of adenomyosis can be confirmed, it is tempting to speculate on the use of angiostatic drugs for treatment of adenomyosis. Similar to endometriosis, where the benefit of angiostatic therapy has been validated (Laschke and Menger, 2012; Zheng et al., 2018), it is suggested that adenomyosis can be efficiently treated with inhibitors of angiogenesis as well. To date, studies of anti-angiogenic treatment regimens for adenomyosis are still lacking. Available literature on the treatment of adenomyosis report on hormonal therapies, some of which (in)directly influence angiogenesis, on preclinical studies or on the effects of herbal compounds which appear to inhibit vascular growth factors (Table II) (Streuli et al., 2014; Benagiano et al., 2009). Since endometriosis has been recognized as an angiogenic-dependent disease for a long time, anti-angiogenic compounds have been studied in endometriosis to a greater extent and a number of in vitro and in vivo studies show promising results (Nap et al., 2005; Nap et al., 2004; Korbel et al., 2018; Hu et al., 2017). However, there are no clinical studies on the effectiveness of such therapies for adenomyosis-related AUB or subfertility. Treatment that is targeted at attenuating angiogenesis has been studied most extensively in the field of cancer research (Wentink et al., 2015; Hurwitz et al., 2004; Dings et al., 2006; Wu et al., 2019). Angiostatic approaches in the field of cancer, however, have only limited effects on patient survival. This is due to the fact that cancer cells have the capacity to become resistant to angiogenesis inhibitors, e.g. through drifting towards dependency to alternative growth factors (van Beijnum et al., 2015; Bani et al., 2017; Huijbers et al., 2016). Inhibition of angiogenesis may be extremely attractive for non-malignant diseases in which cells are not genetically unstable, as was observed in ophthalmology applications. Treatment of age-related macula degeneration, for example, appears to have resulted in a renaissance of therapeutic intervention (Rosenfeld et al., 2006). Therefore, such treatment also seems attractive for reversing or inhibiting angiogenesis in adenomyosis. Nonetheless, the clinical applicability of these compounds in the population of adenomyosis patients is much more restricted than in the patients with cancer, whose compounds were originally developed for: in the latter population, a life-threatening disease needs to be treated, and for that reason, the safety requirements differ completely from the requirements for a drug that treats a benign disease in a population that wants to preserve fertility and accepts fewer side effects (Laschke and Menger, 2012). Since angiogenesis in the reproductive tract is one of the only physiologically occurring angiogenesis mechanisms in the human body, this function would need to be preserved. Local application of a therapeutic agent would ideally reduce angiogenesis and adenomyosis-related AUB and fertility difficulties without disrupting physiological angiogenesis in the reproductive organs.

**Supplementary data**

Supplementary data are available at Human Reproduction Update online.

**Authors’ roles**

M.H.: conceptualization, data analysis and writing. C.W.: conceptualization and data analysis. F.G.: supervision, review and editing. V.M.:...
review and editing. W.H.: review and editing. J.H. and A.W.G.: supervision, review and editing. All authors also contributed to the critical revision of the intellectual content and approved the final version of the paper.

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**Conflict of interest**

The authors have no conflicts of interest to declare.

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