Vasculogenesis: a new piece of the endometriosis puzzle

M.W. Laschke*, C. Giebels, and M.D. Menger

Institute for Clinical & Experimental Surgery, University of Saarland, D-66421 Homburg/Saar, Germany

*Corresponding address. Tel: +49-6841-162-6554; Fax: +49-6841-162-6553; E-mail: matthias.laschke@uks.eu

Submitted on March 8, 2011; resubmitted on April 6, 2011; accepted on April 18, 2011

Table of Contents

- Introduction
- Methods
- Mechanisms of blood vessel formation
- Vasculogenesis in the endometrium and endometriotic lesions
- Mechanisms of EPC mobilization
- Mechanisms of EPC recruitment
- Future perspectives
- Conclusions

Background: Endometriosis is a complex disease with a multifactorial pathogenesis, which is crucially dependent on the development of new blood vessels. Based on the current literature, the present review highlights the fact that the neovascularization of endometriotic lesions is not only driven by angiogenesis, but also involves de novo formation of microvessels from circulating endothelial progenitor cells (EPCs). This process, termed post-natal vasculogenesis, is a characteristic of various pathogenic conditions, such as tumour growth and atherosclerosis, and typically comprises the activation, mobilization and recruitment of bone marrow-derived EPCs to the sites of tissue hypoxia.

Methods: Literature searches were performed in PubMed, MEDLINE and ISI Web of Knowledge for publications focusing on vasculogenesis in the endometrium and endometriotic lesions.

Results: Recent studies indicate that up to 37% of the microvascular endothelium of ectopic endometrial tissue originates from EPCs, partly controlled by the stromal-cell-derived factor-1/chemokine receptor type 4 axis. Accordingly, blockade of EPC recruitment effectively inhibits the formation of microvascular networks in developing endometriotic lesions, indicating that vasculogenesis represents an integral part of the pathogenesis of endometriosis.

Conclusions: The involvement of vasculogenesis in endometriosis may offer the exciting opportunity for the future establishment of novel diagnostic and therapeutic strategies for this frequent gynaecological disease.

Key words: endometriosis / vasculogenesis / angiogenesis / endothelial progenitor cells / stem cells

Introduction

Endometriosis is a frequent gynaecological disease, which is defined as the presence of endometriotic lesions outside the uterine cavity, consisting of proliferating endometrial glands and stromal tissue (Galle, 1989). According to the classical implantation theory of Sampson (1927), these lesions originate from endometrial fragments, which are retrogradely shed through the Fallopian tubes into the peritoneal cavity during menstruation. However, retrograde menstruation is a physiological event, which can be found in up to 90% of women (Halme et al., 1984). Therefore, there must be other mechanisms, which are involved in the initiation of the disease. They may include
a defective immune surveillance in the peritoneum (Vinatier et al., 1996; Christodoulakos et al., 2007), a disturbed balance between reactive oxygen species and antioxidants in the peritoneal fluid (Jackson et al., 2005; Augoulea et al., 2009), or the spread of endometrial epithelial progenitor cells and mesenchymal stem cells (Sasson and Taylor, 2008; Gargett and Masuda, 2010; Masuda et al., 2010).

Although the mechanisms contributing to the establishment of endometriotic lesions are still a matter of debate, there is no doubt that long-term survival and proliferation of these lesions are crucially dependent on the formation of new blood vessels, which guarantee oxygen and essential nutrient supply (Groothuis et al., 2005; Laschke and Menger, 2007; May and Becker, 2008; Taylor et al., 2009). In fact, endometriosis lesions are typically characterized by a dense vascularization (Nisolle et al., 1993; McLaren, 2000; Giudice, 2010). So far, it has been assumed that this vascularization exclusively occurs by the ingrowth of new blood vessels from the surrounding host tissue via the process of angiogenesis. Accordingly, many research groups have focused on angiogenesis in endometriosis, which offers the opportunity to develop anti-angiogenic treatment strategies (Ferrero et al., 2006; Laschke et al., 2007, 2008; Van Langendonckt et al., 2008; Xu et al., 2009; Katayama et al., 2010; Zhang et al., 2011).

However, besides angiogenesis, there also exists the possibility that new blood vessels develop from circulating endothelial progenitor cells (EPCs), which are recruited and incorporated into the sites of neovascularization (Asahara et al., 1997). Based on the current literature, the present review highlights that this process, termed post-natal vasculogenesis, is an integral part of the pathogenesis of endometriosis, which may have important implications for the establishment of novel diagnostic and therapeutic strategies for this frequent gynaecological disease.

Methods

Literature searches were performed in PubMed, MEDLINE and ISI Web of Knowledge for publications written in the English language focusing on vasculogenesis in the endometrium and endometriotic lesions. The searches included the key words ‘endometrium’, ‘endometriosis’ and ‘endometriotic lesions’, which were paired with the key words ‘endothelial progenitor cells’, ‘stem cells’ and ‘vasculogenesis’. The searches included both animal and human studies. No restriction was set for the publication date.

Mechanisms of blood vessel formation

The development of new blood vessels occurs via two principal processes, i.e. angiogenesis and vasculogenesis. Angiogenesis is defined as the formation of new microvessels from pre-existing ones and occurs via sprouting angiogenesis and intussusception. Sprouting angiogenesis is a tightly regulated multi-step process, which comprises the release of pro-angiogenic growth factors, matrix degradation by proteases, endothelial cell migration and proliferation, sprout and network formation as well as vessel maturation (Risau, 1997; Carmeliet, 2000) (Fig. 1A). In contrast, intussusception represents the internal splitting of a vessel in two by transluminal invagination and pillar formation (Burri et al., 2004; Demir et al., 2010). This permits the rapid expansion of the endothelial surface area for metabolic exchange and contributes to the optimization of the local vascular branching geometry. Thus, intussusception is of particular importance in vascular remodelling and creation of a local organ-specific angioarchitecture (Makanya et al., 2009).

Vasculogenesis was originally defined as de novo blood vessel formation by migration and differentiation of angioblastic progenitor cells during embryonic and fetal development (Kässmeyer et al., 2009). However, based on the pioneering work of the groups around Shi et al. (1994) and Asahara et al. (1997), it is now well accepted that vasculogenesis also contributes to the formation of new blood vessels in the adult. This so-called post-natal vasculogenesis is characterized by the mobilization of bone marrow-derived or tissue-resident EPCs into the bloodstream in response to certain cytokines or tissue ischaemia. These circulating EPCs are then recruited into sites of neovascularization, where they are incorporated into the vascular endothelial lining and differentiate in situ into endothelial cells (Murasawa and Asahara, 2005) (Fig. 1B).

During the last few years, circulating EPCs have been shown to be involved in vascularization during wound healing (Asahara et al., 1999; Liu et al., 2010), limb ischaemia (Hur et al., 2007), remodelling after acute myocardial infarction (Kumar and Caplice, 2010), atherosclerosis (Sata et al., 2002; Xu, 2006), fracture healing (Lee et al., 2008; Matsumoto et al., 2008) and tumour growth (Peters et al., 2005; Ding et al., 2008). Due to their vessel-forming capacity, they are a promising cell source for the establishment of therapeutic approaches in the field of vascular medicine and tissue engineering (Asahara and Kawamoto, 2004; Kusuma and Gerecht, 2010).

EPCs are often characterized by the combined expression of different surface markers, including CD34, CD133, stem cell antigen-1 and vascular endothelial growth factor receptor (VEGFR)-2, as well as their ability to take up Dil-labelled acetylated low-density lipoprotein (Ac-LDL) and to bind Ulex europaeus or Bandeiraea simplicifolia-1 under culture conditions (Rafii and Lyden, 2004). Therefore, late EPCs are thought to have different roles in neovasculogenesis and vascular repair. Early EPCs mainly secrete angiogenic growth factors, which support the recruitment of resident mature endothelial cells and induce their proliferation and survival, whereas late EPCs exhibit a high proliferation activity and are directly incorporated into the endothelium of newly forming vessels (Hur et al., 2004). Therefore, late EPCs have to be considered as endothelial progenitors in the classical sense, whereas early EPCs are rather a pro-angiogenic cell population, which indirectly support the development of new blood vessels by local stimulation of tissue-resident endothelial cells.
Vasculogenesis in the endometrium and endometriotic lesions

The endometrium is a highly dynamic tissue, which undergoes proliferation, differentiation and tissue breakdown in each menstrual cycle throughout a woman’s reproductive life. Accordingly, the endometrium exhibits an exceptional regenerative ability, which has long been attributed to the existence of endometrial stem cells, although no proof had been provided. This changed in 2004, when the Gargett lab demonstrated for the first time that human endometrium contains rare populations of clonogenic epithelial and stromal stem cells with high proliferative potential (Chan et al., 2004; Gargett, 2007). In the same year, Taylor (2004) analysed the endometrium from HLA-mismatched bone marrow transplant recipients and detected donor-derived endometrial epithelial and stromal cells, indicating that circulating bone marrow-derived stem cells can contribute to endometrial regeneration. Later on, these findings were confirmed experimentally in different murine models (Bratincsák et al., 2007; Du and Taylor, 2007; Zhou et al., 2011) and also clinically in female patients, who received bone marrow transplantation from male donors (Ikoma et al., 2009) (Table I). However, it remained unknown, whether bone marrow-derived EPCs are involved in endometrial neovascularization.

To clarify this issue, Masuda et al. (2007) analysed the uteri of ovariectomized and lethally irradiated nude severely compromised immunodeficient mice, which were reconstituted with bone marrow from transgenic mice overexpressing β-galactosidase (lacZ) or enhanced green fluorescent protein (eGFP) under the transcriptional control of the Tie2 promoter. By immunohistochemistry, they could demonstrate that bone marrow-derived EPCs are incorporated into the microvessels of the endometrium after 17β-estradiol (E2) administration. Interestingly, they further found that this is mainly the case for EPCs expressing estrogen receptor (ER)-α, but not ER-β. Additional EPC culture assays revealed an E2-dependent mobilization of EPCs from the bone marrow into the peripheral circulation. Taken together, these findings provided the first evidence for physiological post-natal vasculogenesis in the uterus, which seems to be regulated by systemic E2 levels (Table I).

Mints et al. (2008) took advantage of the extremely rare occasion of a pregnancy and subsequent normal menstruations in a 30-year-old female patient, who had undergone non-myeloablative allogeneic bone marrow transplantation, with cells from a brother. They obtained endometrial biopsies during the Caesarean section and on a follow-up 1 year later. They performed immunohistochemical analyses for the detection of CD34/VEGFR-2-positive endothelial cells and FISH probes for the identification of donor cells. Of interest,
they found that up to 14% of the patient’s endometrial endothelial cells are donor-derived, demonstrating that incorporation of bone marrow-derived EPCs also plays a substantial role in the vascularization of human endometrium (Mints et al., 2008) (Table I).

Previous studies indicate that eutopic endometrium and endometriotic lesions are different tissues with respect to gene expression, the type and degree of differentiation, and hormone dependency (Schweppe, 1989; Hudelist et al., 2005; Meola et al., 2010). Moreover, the eutopic endometrium is part of a tightly regulated physiological organ system, whereas endometriotic lesions can only survive if they adapt to their surrounding environment. Nonetheless, both tissue types are crucially dependent on the formation of new blood vessels, suggesting that post-natal vasculogenesis may also play a crucial role in the pathogenesis of endometriosis. To test this hypothesis, we recently induced intraperitoneal endometriotic lesions by suturing uterine tissue samples to the abdominal wall of irradiated FVB/N mice, which were reconstituted with bone marrow, from FVB/N-TgN (Tie2/GFP) 287 Sato mice (Laschke et al., 2011) (Fig. 2). Because the development of spontaneous endometriosis is dependent on menstruation and thus restricted to humans and subhuman primates, surgical induction of endometriotic lesions is a common approach in murine endometriosis studies (Becker et al., 2006, 2008; Körbel et al., 2010; Laschke et al., 2010). Nonetheless, while interpreting the results of these studies, it should be kept in mind that surgically induced endometriotic lesions do not reflect authentically the situation of human endometriosis. In our study, we analysed the vasculization and recruitment of GFP-positive bone marrow-derived EPCs in the lesions by means of intravital fluorescence microscopy and immunohistochemistry. Interestingly, we found that in line with the data of Mints et al. (2008), ~18% of all endothelial cells were found to be GFP-positive at Day 7 after the induction of endometriotic lesions (Table I). Throughout the observation period of 28 days, this cell fraction slightly decreased to 13%, indicating that the incorporation of EPCs into the microvasculature may be an important mechanism in particular during the engraftment of endometriotic lesions. Using the FVB/N-TgN (Tie2/GFP) 287 Sato mouse, it has to be considered that the Tie2-GFP-positive cell population of this strain is clearly heterogeneous. In fact, studies identified a subset of Tie2-GFP-positive cells in the bone marrow, which co-express the haematopoietic marker CD45 as well as the endothelial markers CD34, VEGFR2 and CD31 (Shaw et al., 2004). This shows that there may be a common haemangioblastic precursor cell, which gives rise to both EPCs and haematopoietic stem cells that subsequently differentiate into lymphoid and myeloid cells. Accordingly, we observed in our study that not all circulating GFP-positive cells were incorporated as EPCs into the microvascular endothelium of endometriotic lesions, but a small fraction was also located within the endometrial stroma surrounding the microvessels. Although not proved, these cells may have represented macrophages rather than EPCs. Finally, we could demonstrate that inhibition of EPC recruitment markedly reduced the vascularization of endometriotic lesions. This indicates that vasculogenesis is essential for vascularization in endometriosis. Most probably, vasculogenesis and sprouting angiogenesis occur in parallel in endometriotic lesions (Fig. 1B). These conclusions are strongly supported by a recent study of Becker et al. (2011), reporting in a comparable murine model of surgically induced endometriosis that even up to 37% of all endothelial cells in endometriotic lesions were bone marrow-derived 1 week after endometrium transplantation into the peritoneal cavity (Table I).

### Table I Overview of studies focusing on the contribution of bone marrow-derived stem cells or progenitor cells to the regeneration or vascularisation of different types of endometrial tissue.

<table>
<thead>
<tr>
<th>Study</th>
<th>Tissue type</th>
<th>Cell type</th>
<th>Cell characterization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Becker et al. (2011)</td>
<td>Murine endometrium, murine endometriotic lesions</td>
<td>Bone marrow-derived EPCs</td>
<td>CD31+/VEGFR+/CD45−/GFP</td>
</tr>
<tr>
<td>Bratincsá et al. (2007)</td>
<td>Murine endometrium</td>
<td>Bone marrow-derived haematopoietic stem cells</td>
<td>CD45+, GFP</td>
</tr>
<tr>
<td>Du and Taylor (2007)</td>
<td>Murine endometrium, murine endometriotic lesions</td>
<td>Bone marrow-derived stem cells</td>
<td>Y chromosome in females, lacZ</td>
</tr>
<tr>
<td>Ikoma et al. (2009)</td>
<td>Human endometrium</td>
<td>Bone marrow-derived stem cells</td>
<td>Y chromosome in females</td>
</tr>
<tr>
<td>Laschke et al. (2011)</td>
<td>Murine endometriotic lesions</td>
<td>Bone marrow-derived EPCs</td>
<td>Tie2/GFP</td>
</tr>
<tr>
<td>Masuda et al. (2007)</td>
<td>Murine endometrium</td>
<td>Bone marrow-derived EPCs</td>
<td>Tie2/lacZ, Tie2/eGFP</td>
</tr>
<tr>
<td>Mints et al. (2008)</td>
<td>Human endometrium, murine endometrium</td>
<td>Bone marrow-derived EPCs</td>
<td>Y chromosome in females, CD34+/VEGFR2+</td>
</tr>
<tr>
<td>Taylor (2004)</td>
<td>Human endometrium</td>
<td>Bone marrow-derived stem cells</td>
<td>HLA type</td>
</tr>
<tr>
<td>Zhou et al. (2011)</td>
<td>Murine endometrium</td>
<td>Bone marrow-derived stem cells</td>
<td>Y chromosome in females</td>
</tr>
</tbody>
</table>

### Mechanisms of EPC mobilization

Under steady-state conditions, only low numbers of EPCs continuously recirculate in the bloodstream. Most progenitor cells are localized in the bone marrow, where they are retained in a quiescent state by high SDF-1 expression induced by the physiological hypoxic microenvironment (Ceradini and Gurtner, 2005). However, in course of tissue damage and/or tissue hypoxia, the quantity of circulating EPCs may increase rapidly. This EPC mobilization can be initiated by the up-regulation of endogenous factors in the blood, which leads to the activation of haematopoietic stem cells. They
E2 induces proliferation, migration and mobilization of EPCs (Matalliotakis et al., 2010). May also been shown to be up-regulated in the course of endometriosis (Bolego et al., 2006). Vascular endothelial growth factor-2 (Fontaine et al., 2003) and fibroblast growth factor-2 (Fontaine et al., 2006) (Fig. 3). Importantly, these factors have also been shown to be up-regulated in the course of endometriosis (Matalliotakis et al., 2003; Bourlev et al., 2006; Xavier et al., 2006; May et al., 2010). Moreover, several studies have demonstrated that E2 induces proliferation, migration and mobilization of EPCs (Iwakura et al., 2003; Strethrow et al., 2003; Masuda et al., 2007; Bolego et al., 2010). Considering the fact that endometriosis is an E2-dependent disease (Rizner, 2009), these findings imply that hormonally regulated EPC mobilization may also play a crucial role in endometriosis (Fig. 3). On the other hand, we recently reported that surgically induced endometriosis in mice is not associated with increased EPC levels in the blood, bone marrow and spleen, indicating that the physiological pool of circulating EPCs may already be sufficient to contribute to the vascularization of endometriotic lesions (Laschke et al., 2011). However, for our experiments, we used C57BL/6 mice. This strain also exhibited only slightly increased blood levels of EPCs after induction of endometriosis in a study of Becker et al. (2011). In contrast, 129/Sv mice with endometriotic lesions, which are known to be one of the most angiogenic strains (Rohan et al., 2000), presented with massively increased EPC levels when compared with sham-operated and healthy control animals (Becker et al., 2011). Of further interest, this increased mobilization of EPCs could be effectively suppressed by treatment with the anti-angiogenic compound Lodamin, the non-toxic form of the fumagillin derivate TNP-470, while inhibiting the growth of endometriotic lesions (Becker et al., 2011). Thus, it may be speculated that numbers of circulating EPCs are elevated in patients with highly active endometriotic lesions or with severe stages of endometriosis. If so, EPCs would represent an attractive novel biomarker for the diagnosis or staging of the disease.

**Mechanisms of EPC recruitment**

The central characteristic in environments where post-natal vasculogenesis occurs is hypoxia. During recent years, sophisticated studies have demonstrated that hypoxia regulates the recruitment of EPCs to ischaemic sites via the stromal-cell-derived factor (SDF)-1/chemokine receptor type (CXCR)4 axis (Ceradini and Gurtner, 2005). SDF-1 (CXCL-12) is a small molecular weight chemokine, which binds exclusively to CXCR4. Under hypoxic conditions, SDF-1 expression is up-regulated by the transcription factor hypoxia-inducible factor (HIF)-1α (Ceradini et al., 2004), resulting in an increased homing of CXCR4-positive EPCs. Accordingly, experimental studies have shown that blockade of either CXCR4 on infused progenitor cells or SDF-1 in the host circulation is associated with a massive reduction in the number of EPCs that are recruited to ischaemic tissues (Ceradini and Gurtner, 2005).

Considering the pathogenic mechanisms, which have been proposed to be involved in the development of endometriosis, it is apparent that SDF-1/CXCR4-mediated EPC homing may be important for vasculogenesis in endometriotic lesion formation. In fact, ectopic endometrial tissue, which is retrogradely shed through the Fallopian tubes into the peritoneal cavity, initially lacks its own blood supply and, thus, suffers from hypoxia (Fig. 3). Accordingly, early endometriotic lesions are characterized by an increased expression of HIF-1α (Becker et al., 2008) and SDF-1 (Laschke et al., 2011). Moreover, treatment with the SDF-1/CXCR4 axis antagonist AMD3100 significantly decreases the recruitment of circulating EPCs in endometriotic lesions (Laschke et al., 2011).

However, there may be additional processes involved in EPC homing during endometriosis (Fig. 3). The establishment of endometriotic lesions is typically associated with drastic microhaemodynamic changes within their developing microvasculature, including decreasing diameters and increasing blood flow velocities of individual microvessels (Laschke et al., 2005, 2006a, b). Thus, shear-stress-induced
up-regulation of adhesion molecules of the selectin and integrin family may promote EPC recruitment to the endothelium, as already described in the models of vascular injury (Hristov et al., 2007; Zampetaki et al., 2008). These adhesion molecules may include P- and E-selectin, which have been shown to interact with P-selectin glycoprotein ligand-1 expressed on the surface of EPCs (Vajkoczy et al., 2003; Foubert et al., 2007), as well as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule-1 binding to lymphocyte function-associated antigen-1 and very late antigen-4 on EPCs (Duan et al., 2006; Silverman et al., 2007). On the other hand, endothelial adhesion molecules may be up-regulated in consequence of the inflammatory activity of endometriotic lesions. For instance, González-Ramos et al. (2007) could demonstrate that red lesions, which are highly vascularised, exhibit a significantly increased nuclear factor-κB (NF-κB) activation and ICAM-1 expression when compared with less-active black lesions. Moreover, circulating EPCs may be captured from the bloodstream by activated platelets, which adhere to the endothelium of ischaemically injured microvessels (Lev et al., 2006). It is further possible that EPCs may home to endometriotic lesions via the expression of specific hormone receptors, such as ER-α (Masuda et al., 2007).

Finally, certain environmental factors may determine the extent of EPC recruitment into endometriotic lesions. Zhou et al. (2011) recently demonstrated that cigarette smoking can adversely affect bone marrow-derived stem cell recruitment to and differentiation in endometrial tissue. In line with these findings, it has been reported that smoking is associated with a decreased incidence of endometriosis (Cramer et al., 1986).

**Future perspectives**

Research on vasculogenesis in endometriosis is still in its infancy. Nonetheless, recent progress in the field of cardiovascular and oncology research indicates that circulating EPCs are attractive candidates for the establishment of future diagnostic and therapeutic approaches.

EPCs may be used as novel biomarkers for the prediction of disease risk and the determination of disease progress. For instance, Werner et al. (2005) reported that the number of circulating CD34/VEGFR-2-positive EPCs can be used to predict cardiovascular outcome in patients suffering from coronary artery disease. In cancer patients, elevated levels of EPCs positively correlate with disease activity (Zhang et al., 2005). In addition, the analysis of EPC levels during different chemotherapy and anti-angiogenic regimens has shown an association between EPC levels and the efficacy of the therapy (Georgiou et al., 2008). Accordingly, EPC levels may be used not only to detect an angiogenic disease, such as cancer or endometriosis, but also to identify the best anti-angiogenic treatment strategy and to determine the optimum biological dose of the used drugs. Finally, EPCs have been suggested as a possible vehicle for systemic delivery of cancer gene therapy (Dudek, 2010). For this purpose, isolated EPCs are expanded in vitro and transduced with a gene encoding for a therapeutic protein. Subsequently, these modified EPCs are injected back into the patient, where they are specifically recruited into the newly developing tumour microvasculature via the process of vasculogenesis. In contrast to local, intratumoral delivery of gene therapy, this cell-based approach bears the major advantage that it is capable of targeting tumours and metastases, which cannot be easily detected and directly accessed.

Taken together, these examples demonstrate that the steadily increasing knowledge of the mechanisms of post-natal vasculogenesis contributes to the development of promising diagnostic and therapeutic strategies. Future studies have to clarify now, whether these strategies can also be transferred to the diagnosis and treatment of endometriosis.

**Conclusions**

During the last decade, post-natal vasculogenesis has been shown to be a characteristic of various pathogenic conditions, such as tumour growth and atherosclerosis. Recent studies now provide the first evidence that post-natal vasculogenesis is also involved in the complex, multifactorial pathogenesis of endometriosis. In fact, bone marrow-derived EPCs could not only be detected in developing endometriotic lesions, but have been shown to be essential for the establishment of a dense lesion microvasculature. These findings offer the exciting opportunity to develop novel EPC-based diagnostic and therapeutic approaches.
approaches for endometriosis. For this purpose, it will at first be necessary to carefully analyse the mechanisms, which regulate the mobilization and recruitment of EPCs into endometriotic lesions, and to assess the correlation between vasculogenesis and the activity and progression of the disease. Scientists dealing with this challenging task may significantly benefit from the impressive advances, which could be achieved in the field of stem cell research during the last decade.

Authors’ roles

M.W.L. and C.G. were involved in manuscript drafting, image acquisition and critical discussion; M.D.M. was involved in the manuscript drafting and critical discussion.

Funding

No funding was sought or obtained for this study.

References


