Noninvasive diagnosis of endometriosis: Review of current peripheral blood and endometrial biomarkers

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A B S T R A C T

A noninvasive biomarker-based test could help shorten the diagnostic delay for endometriosis. The most investigated biomarker sources are peripheral blood and endometrium. Discovery of endometriosis biomarkers is often hypothesis-driven, i.e. when one or a few biomarkers are investigated based on their role in the disease pathogenesis. Alternatively, a hypothesis-generating approach has been followed using the “omics” technologies. A variety of biomarkers for endometriosis have been investigated, but no biomarker has been validated for clinical use. Many challenges lie ahead in the endometriosis biomarker field. In the future, harmonized collection and reporting methods should allow large-scale international collaboration for highly powered studies.

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Introduction

Endometriosis is a common gynaecological disorder, affecting about 10% of women of reproductive age and up to 50% of women with pelvic pain and/or infertility [1]. The gold standard for the diagnosis of endometriosis is laparoscopic visualization of the lesions with histological confirmation [2]. The time between onset of symptoms and diagnosis can add up to 8–10 years [3]. This diagnostic delay can be attributed to the need for laparoscopy for diagnosis, the variability of disease symptoms and the overlap in symptoms with other diseases [1,3]. Transvaginal ultrasound can identify deep nodules and ovarian endometriotic cysts [4], but is operator-dependent and shows limited accuracy for the detection of superficial peritoneal lesions [5]. Therefore, biomarker research has been defined as a research priority by the World Endometriosis Society (WES) and World Endometriosis Research Foundation (WERF) [6]. A panel of women with endometriosis, healthcare practitioners and researchers in the United Kingdom and Ireland have ranked finding a noninvasive diagnosis number four in the top ten of research priorities for endometriosis [7]. Despite decades of research, no biomarker has been validated for the diagnosis of endometriosis [8,9].

Peripheral blood is a useful source of biomarkers, as it is easily obtained and minimally invasive [10]. Much research has been done into blood-based biomarkers for endometriosis [11]. In a recent Cochrane review, meta-analyses were performed on the published data sets of anti-endometrial antibodies, interleukin-6 (IL-6), cancer antigen-19.9 (CA-19.9) and CA-125, but none of the investigated peripheral blood biomarkers showed sufficient accuracy for a replacement test (sensitivity ≥ 94%, specificity ≥ 79%) nor a triage test to rule endometriosis out (sensitivity ≥ 95%, specificity ≥ 50%) in case of a negative test result or rule it in (sensitivity ≥ 50%, specificity ≥ 95%) for a positive result [8].

According to the retrograde menstruation theory, endometriotic lesions originate from endometrial fragments that are sloughed through the fallopian tubes into the peritoneal cavity during menses [12]. Endometrium, as the tissue where the disease originates, has therefore been investigated as a source of biomarkers [13]. However, despite it being a relatively simple procedure, endometrial sampling is more invasive than taking a blood sample and has therefore been described as semi-invasive rather than noninvasive [4]. The procedure can be uncomfortable and sometimes even painful for the patient. Gupta et al. reviewed the literature on endometrial biomarkers and found that most studies were of poor methodological quality [9]. Only Protein Gene Product 9.5 (PGP9.5) and CYP19 had sufficient data for meta-analysis [9]. While PGP9.5 showed promising results, inter-study variability is too high to consider it a valid biomarker.

Endometriosis biomarker studies are often based on an underlying assumption regarding the pathogenesis and pathophysiology of the disease, a strategy which can be termed as “hypothesis-driven”. In contrast, most studies employing “omics” technologies aim to find general differences between patients, without restricting the search to a specific set of markers, a so-called “hypothesis-generating” approach [14] (Fig. 1). The main goal of this review is to provide an overview of recent biomarker studies in peripheral blood and endometrium that have been published since the Cochrane

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**Biomarker discovery approaches**

<table>
<thead>
<tr>
<th>Hypothesis-driven</th>
<th>Hypothesis-generating</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Definition</strong></td>
<td>A hypothesis about the pathogenesis of endometriosis leads to the investigation of a pre-specified amount of markers</td>
</tr>
<tr>
<td><strong>Analytical methods</strong></td>
<td>Single (or small-scale multiplex) ELISA, qPCR, immunohistochemistry</td>
</tr>
<tr>
<td><strong>Statistics</strong></td>
<td>Mostly univariate (ROC curve analysis), sometimes multivariable</td>
</tr>
</tbody>
</table>

Fig. 1. Biomarker discovery approaches.
reviews by Nisenblat et al. and Gupta et al. [8,9]. We also reflect on the challenges faced by the endometriosis biomarker research community.

**Hypothesis-driven approach for biomarker discovery and validation**

The majority of biomarker research has been hypothesis-driven, meaning that changes in marker expression are anticipated either as a cause or a consequence of the disease. Biomarkers that capture pathophysiological events could be present in the eutopic endometrium or secreted into the blood stream (overview in Fig. 2).

Sampson’s theory of retrograde menstruation is the most widely accepted theory for the pathogenesis of endometriosis [12], but retrograde menstruation is a common phenomenon and endometrial cells can be found in the peritoneal cavity of women with and without endometriosis [15]. Therefore, other pathways must play a role in the disease development. Endometriosis is regarded as a chronic inflammatory disorder, with a crucial immune component [16]. Immune cells are recruited to the peritoneal cavity to clean up menstrual debris, but sometimes endometrial fragments can survive, evade the immune system, attach to the peritoneum, invade it and eventually establish endometriotic lesions with their own blood supply and neural ingrowth [1,17].

**Peripheral blood**

*Inflammation and oxidative stress*

Endometriotic lesions go through monthly cycles of bleeding, much like the eutopic endometrium. This bleeding elicits an inflammatory response, which in turn propagates lesion growth [16,18]. Ruptured erythrocytes cause an accumulation of iron which evokes oxidative stress [19]. Disruption of the balance between reactive oxygen species and antioxidant defence has been proposed as a mechanism supporting endometriotic lesion development and growth [20].

**CA-125**

The most commonly described endometriosis biomarker is CA-125, a marker of peritoneal inflammation. CA-125 has been evaluated at different cut-offs, each generating a separate set of sensitivities and specificities for the diagnosis of endometriosis. In the meta-analysis by Nisenblat et al., none of the cut-off thresholds met their criteria for a replacement or triage test [8]. The cut-off

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**Fig. 2.** Overview of promising molecules in peripheral blood discussed in this review found by hypothesis-driven approach.
interval of >16.0–17.6 U/ml approached the criteria of a rule-in test the most, with a mean sensitivity of 56% (95% confidence interval (CI) 24–88%) and mean specificity of 91% (95% CI 75–100%) [8]. In contrast, a meta-analysis by Hirsch et al. concluded that measuring CA-125 at a cut-off of >30 U/ml could act as a rule-in test, showing a sensitivity of 52.4% (95% CI 37.9–66.4%) and specificity of 92.7% (95% CI 89.4–95.1%) [21]. The different conclusions from both meta-analyses can be attributed to different definitions of a rule-in test, the studied cut-off intervals and different criteria for study inclusion in the meta-analysis. Overall, CA-125 seems to be hampered in its sensitivity because it is mostly elevated in the advanced endometriosis stages as opposed to all stages, while its specificity can be poor because of its rise in other gynaecological diseases [21,22]. Despite these drawbacks, CA-125 is often included as a benchmark molecule in studies investigating other biomarkers [23–26].

**Cytokines and inflammatory proteins**

A number of cytokines have been investigated as endometriosis biomarkers, but the results have been conflicting [11]. IL-6 has been the most widely studied and was available for meta-analysis by Nisenblat et al. [8]. When considering 3 studies, an average sensitivity of 63% (95% CI 52–75%) and specificity of 69% (95% CI 57–82%) was found for a cut-off value of >1.90–2.00 pg/ml. For other cut-off values not enough data were available for meta-analysis [8]. A recent study utilized an ELISA kit for IL-6 with a detection limit of 3.91 pg/ml [27]. The majority of serum samples had IL-6 values below this detection limit [27], highlighting the importance of choosing an adequate ELISA kit.

Cytokine measurements are complex due to their short half-life, circadian pattern and variables concerning sample collection [28]. IL-35, an inhibitory cytokine produced by regulatory T-cells and possibly involved in the mediation of immune tolerance after the initial inflammatory reaction, was found to be upregulated in patients with endometrioma versus controls with infertility or benign ovarian tumours, showing an area under the ROC curve (AUC) of 0.694 (95% CI: 0.551–0.837) [29]. In contrast, three independent studies have shown the limited use of cytokines and chemokines for the diagnosis of endometriosis [30–32].

Inflammatory proteins that have been investigated in other diseases have been examined in endometriosis. Serum YKL-40 was significantly higher in women with endometriosis than in fertile controls [26]. Total circulating cell-derived microparticles (cMPs) and circulating tissue factor-containing microparticles (cMP-TF) have been investigated as markers of inflammation and coagulation [33]. Only cMPs were significantly elevated in stage III–IV endometriosis, especially in the subgroup of cases presenting with deeply infiltrative endometriosis. Soluble tumour necrosis factor receptor I (sTNFR-I) and sTNFR-II can antagonize the effects of TNF-alpha by sequestering the active ligand [34]. sTNFR-I serum levels were significantly higher in women with all-stage and stage I–II endometriosis than in controls without endometriosis [34]. The AUC of sTNFR-I was 0.67 (95% CI 0.53–0.80) for stage I–II endometriosis and 0.62 for all endometriosis. At a cut-off of 351.22 pg/ml, sTNFR-I had a sensitivity of 60.7% and a specificity of 75% to detect stage I–II endometriosis [34]. sTNFR-II levels were only significantly elevated when comparing stage I–II endometriosis cases with controls, which resulted in a borderline significant (p = 0.05) AUC of 0.63 (95% CI 0.53–0.80) [34].

**Oxidative stress markers**

As a marker of oxidative stress and inflammation, total and active levels of myeloperoxidase (MPO) have been investigated [35]. Specific MPO activity (active MPO divided by total MPO) only showed a difference between women with endometriosis versus controls with benign gynaecological disorders, but not versus controls with a normal pelvis [35]. This result reflects the influence of the inflammatory component in control women with other disorders and highlights the importance of the choice of control group [35]. Two other oxidative stress markers that have been investigated in the context of endometriosis are superoxide dismutase (SOD) and glutathione peroxidase (GPx) [19]. Activity of SOD (an anti-oxidant) was significantly altered in endometriosis, while GPx (a peroxide-scavenging enzyme) showed no difference. The AUC for SOD and GPx combined was 0.78, with 68.75% sensitivity a 80.77% specificity for the discrimination of endometriosis patients from women with no
complaints from an outpatient setting [19]. It remains uncertain whether the diagnostic value of SOD and GPx would hold up in a clinical flow with symptomatic controls.

**Immune markers**

Galectin-9 is an immunomodulatory protein that might be a marker for endometrial receptivity and has therefore been examined as endometriosis biomarker [36]. In a study comparing mostly stage III–IV endometriosis cases with healthy controls, Galectin-9 showed an AUC of 0.973 (95% CI: 0.900–0.977). At a cut-off of 132 pg/ml this resulted in a sensitivity of 94% and a specificity of 93.75% [36]. Despite this promising high accuracy, there are a number of caveats concerning this study, such as the low number of stage I–II endometriosis cases included and the calculation of diagnostic accuracy based on the comparison with healthy control women. In fact, women with other benign gynecological disorders (i.e., leiomyoma, ovarian cysts, unexplained infertility, etc) had serum Galectin-9 values that were significantly higher than the healthy control group and that were not significantly different from the endometriosis cohort [36], which suggests a much lower diagnostic accuracy in the clinical reality.

Glycodelin is a protein with immunosuppressive properties through inactivation of T cells and natural killer (NK) cells that has been investigated in endometriosis either alone or in combination with other biomarkers [8,37,38]. In a study by Mosbah et al., glycodelin was upregulated in endometriosis cases versus controls that underwent tubal ligation [38]. The AUC was 0.96 and at a cut-off of 108.5 ng/ml, this resulted in a sensitivity of 91.7% and a specificity of 75% [38]. This promising result may have been a consequence of patient selection (inclusion of cases with mostly advanced endometriosis versus a presumably healthy control group), because past studies have suggested only little clinical value in using glycodelin as a biomarker on its own [8].

**Autoimmune markers**

Among the immune/inflammatory mechanisms at play in endometriosis are abnormalities in immune cell numbers and activation/function (e.g., increase in numbers of activated macrophages, abnormal NK cell function, polyclonal activation of B cells), and in cytokine levels [39]. Among these are cytokines (IL-27, IL-6 and TGF-β) and T cell subsets (e.g., IL-10 + Th17 cells) that regulate autoimmune mechanisms [40]. Because autoantibodies (AAbs) are circulating, relatively stable molecules they have a great diagnostic potential. Notably, levels of these AAbs were among the few markers amenable for a meta-analysis in the review by Nisenblat et al. [8]. However, AAbs did not meet the criteria for either a replacement nor triage test [8]. A recent study by Gajbhiye et al. tested the sensitivity and diagnostic accuracy of 11 autoimmune markers using ELISA [41]. They also compared the results with that of previously reported endometriosis markers such as CA-125, CA-19.9, and AAbs to syntaxin 5, PDIK1L, and alpha-enolase. The sensitivity of 6 AAbs was higher than the other markers for diagnosis of stage I-II endometriosis, and logistic regression analysis showed that as a panel they could be useful for the noninvasive diagnosis of endometriosis [41]. This panel consisted of anti-tropomodulin (TMOD)3b, anti-TMOD3c, anti-TMOD3d, anti-tropomyosin (TPM)3a, anti-TPM3c and anti-TPM3d, and showed an AUC of 0.869 with a sensitivity of 79% and a specificity of 80% [41]. These results are promising, but external independent validation is warranted.

**Cell survival, adhesion and migration**

The survival of endometrial cells in the pelvic cavity depends on their ability to evade apoptosis and, after attachment to the peritoneum, on the formation of new blood vessels. CD95/FAS is a receptor of Fas Ligand (FasL). The soluble form of this receptor can prevent FasL from binding their receptor on cells, thereby preventing apoptosis. In a study comprising serum of 30 endometriosis patients and 30 healthy controls, CD95/FAS was elevated in endometriosis in a stage-dependent way [42]. Of the two investigated angiogenesis markers, HIF-1α but not Tie-2 was increased in endometriosis [42]. mRNA of survivin, an apoptosis inhibitor, has been detected in serum of 20/30 endometriosis patients versus 2/10 controls, resulting in a sensitivity of 66.7% and specificity of 80% [25]. VEGF mRNA was upregulated and a cut-off of dCt <9.5 (with GAPDH as housekeeping gene) resulted in a sensitivity of 80% and a specificity of 70% [25].
Endometriosis has been described as a cancer-like process, involving cell migration and invasion. In a study by Kuesel et al., sVCAM-1 was significantly upregulated in endometriosis, while sICAM-1 was downregulated [43]. The ratio of sVCAM-1/sICAM-1 showed an AUC of 0.929 (CI: 0.864–0.994). At a ratio cut-off of 1.55, this resulted in a sensitivity of 90.3% and a specificity of 86.7% [43]. However, another study showed a lack of diagnostic power for sICAM in endometriosis [38]. Systemic MMP-9 concentrations were higher in women with endometriosis than in controls, and this was correlated with disease severity [44].

Since endometrial cells can migrate to ectopic locations, it has been proposed that they can be found in the circulation. Cells staining for vimentin (stromal cells), pan-cytokeratin (CK, epithelial cells), and estrogen or progesterone receptor (ER, PR) and negative for CD45 (leukocyte marker) have been identified as circulating endometrial cells (CECs) [23]. CECs were found at a higher frequency in peripheral blood of women with endometriosis (17/19; 89.5%) than in controls (6/40; 15.0%) [23]. However, no quantification was possible and the investigated markers are not specific for endometrial cells, e.g., vascular endothelial cells also express CK and ER/PR [23].

Urocortin-1 can influence endometrial adhesion and angiogenesis [45] and also promotes endometrial differentiation and decidualization [46]. It could discriminate between endometriosis patients and women with no other lesions, but not women with other benign gynaecological disorders [46], thereby limiting its biomarker potential in a clinical setting.

The apoptosis marker Annexin V, angiogenic marker VEGF, and cell adhesion molecule ICAM-1 have all been included in a biomarker panel for endometriosis along with CA-125 and glycodelin, while investigated cytokines were not included in the panel [37]. These results indicated that apoptosis and angiogenesis markers may show better prospects for the diagnosis of endometriosis, than cytokines and inflammatory factors [37].

**Pain**

As pain is a common symptom of endometriosis, pain pathways have been examined for the diagnosis of endometriosis. Plasma brain-derived neurotrophic factor (BDNF) was shown to be increased in women with endometriosis compared with controls [24]. BDNF was also upregulated in stage I–II endometriosis versus the control group for which it showed an AUC of 0.75 and, at a cut-off of 1000 pg/ml, a sensitivity of 91.7% and a specificity of 69.4%. This study also investigated NGF, NT4/5, CA-125, and CRP, all of which did not show significant differences between the endometriosis and control group [24]. Plasma BDNF levels have also been correlated with pain and were useful to discriminate between women with endometrioma and women with other benign gynaecological disorders, showing an AUC of 0.72 [47]. However, BDNF was not useful to detect peritoneal endometriosis or deeply infiltrative endometriosis [47]. More research is necessary to decide whether BDNF is associated with one particular endometriosis phenotype and if the association is endometriosis-related rather than pain-related.

**Endometrium**

A systematic review by Gupta et al. summarised the investigated endometrial biomarkers for endometriosis [9]. Since most proteins were examined in individual studies, meta-analysis was only possible for CYP19 and PGP9.5. CYP19 is a cytochrome P450 isoform and a major component of aromatase, the enzyme responsible for a key step in the estrogen biosynthesis [1]. CYP19 showed an average sensitivity of 77% (95% CI 70–85%) and specificity of 74% (95% CI 65–84%) [9], which did not meet their criteria of a replacement nor a triage test.

Meta-analysis of nerve fiber density using PGP9.5 as a pan-neural marker, met the criteria of a replacement test with a mean sensitivity of 96% (95% CI 91–100%) and specificity of 86% (95% CI 70–100%) [9]. Great inter-study variability existed and one outlier study [48] was excluded from the analysis. An additional three other studies were not included in the meta-analysis. One study had controls that were deemed to be outside the scope of the analysis (either adenomyosis or leiomyoma). This study related the presence of nerve fibers to the occurrence of pain rather than the presence of endometriosis [49]. Furthermore, two studies that showed no nerve fibers in endometrium were also excluded from meta-analysis [50,51]. One had a poorly defined patient population and archived
samples [51], while the other was excluded based on the suspicion that their PGP9.5 assay might not have been sensitive enough to detect nerve fibers in the endometrium [50]. These outcomes underline the variability that exists in the nerve fiber literature for endometriosis. Methodological issues concerning endometrial biopsy sampling and the PGP9.5 immunohistochemistry (IHC) assay are important issues to take into consideration when assessing these varying results. Al Jefout et al. introduced the Endosampler technique as a measure to provide a full-thickness, narrow and deep high-quality strip of tissue and suggested that it is a useful tool to allow detection of endometrial nerve fibers [52]. However, the same endometrial biopsy technique was employed by Ellett et al. who found an overall low prevalence of nerve fibers in both cases and controls [53]. The rigorous sample collection method combined with the difficulty in optimizing the PGP9.5 assay renders this test currently unsuitable for diagnostic purposes [54].

EWI-2, the immunoglobulin superfamily member 8 (IGSF8), has been investigated in endometriosis because of its role in fertilization, cell proliferation and cell migration [55]. EWI-2 was reduced in endometrium of women with endometrioma on mRNA and protein level in both the proliferative and secretory phase of the cycle [55].

Reduced levels of glycodelin and its glycoforms were found in eutopic endometrium of women with endometriosis versus tubal sterilization controls during the window of implantation and the proliferative phase, while an 10-fold increase was observed in the late-secretory phase of the menstrual cycle [56].

**Non hypothesis-driven (“hypothesis generating”) approach: systems biology approach for biomarker discovery (“omics”)**

“Omics”-based research allows scientists to generate new hypotheses and are therefore useful for a systems biology approach. Especially for a complex disorder such as endometriosis this approach can be interesting to discover potential biomarkers, while simultaneously learning about the disease pathophysiology. The two most commonly used techniques for proteomics in endometriosis have been Surface-enhanced laser desorption/ionization (SELDI) and Two-Dimensional Difference Gel Electrophoresis (2D-DIGE) [57]. Studies regarding protein or peptide fingerprints in peripheral blood and endometrium have shown promising results [8–10], but due to differences in analytical methods, overlap in results has been limited. Currently, the SELDI-TOF method is no longer available, due to a variety of limitations [57]. Other mass spectrometry (MS)-based methods, such as the Orbitrap analyser, could replace SELDI as a platform for biomarker discovery and validation.

mRNA microarray data have suggested that the variation in gene expression is larger between different phases of the menstrual cycle than between endometriosis cases and controls [58]. However, differences in endometrial gene expression between women with and without endometriosis have been described in other studies [59,60]. Using microarray and a large-scale qPCR chip, classifiers could be developed separating endometriosis patients from the controls [59]. mRNA sequencing of secretory phase endometrial samples revealed 72 dysregulated genes in endometriosis [60]. Four upregulated proteins (matrix metallopeptidase 11, dual specificity phosphatase 1, Fos proto-oncogene and serpin family E member 1) and one downregulated protein (adenosine deaminase 2) were validated using qPCR [60]. However, sample size was small and the sample selection was restricted to women with stage III–IV endometriosis and control women coming into the clinic for hydrotubation.

Sequencing and microarray technology have allowed to investigate systemic levels of miRNA [61] and long non-coding RNA [62]. Mass and 1H nuclear magnetic resonance (NMR) spectrometry methods enable scientists to unravel the peripheral blood metabolome [63,64]. Using mass spectrometry methods, several acylcarnitines were shown to be increased while trimethylamine-N-oxide was decreased in endometriosis [63]. A panel of acylcarnitines had a specificity of 88.9% and a sensitivity of 81.5% for the detection of endometriosis [63]. The metabolic profiles of endometriosis patients reflected an imbalance in energy production and oxidative state of cells [63]. NMR spectrometry methods revealed that valine, fucose, choline-containing metabolites, lysine/arginine and lipoproteins were upregulated in endometriosis (mostly advanced stages), while creatinine was downregulated.
However, multivariate analysis did not allow the construction of a panel of metabolites that could cause a clear separation between endometriosis cases and controls [64].

More studies should be undertaken using the “omics” technologies. After initial discovery, follow-up in an independent validation study is crucial.

Challenges in biomarker validation

Despite numerous attempts at biomarker discovery, validation has been sparse. Due to its complexity, endometriosis biomarker research is often hampered by subpar quality studies. One way of assessing the quality of biomarker studies is following the Quality Assessment of Diagnostic Accuracy Studies (QUADAS) criteria [65,66]. These were adapted specifically for endometriosis studies [8,11]. It is a set of rules, specifying criteria for patient selection, methodology and analysis.

Patient selection

It is recommended to consider the endometriosis ASRM stage (I to IV) or phenotype (superficial, deep or endometrioma) when analysing biomarker results [8,11]. Each subcategory may present with a unique molecular signature and therefore their own biomarkers. The most investigated phenotype has been endometrioma, e.g. for the study of IL-35 [29], pro-inflammatory cytokines [32], SOD and GPx activity [19] in peripheral blood and EWI-2 in endometrium [55]. While a blood test for every endometriosis phenotype would be useful, endometrioma and deep nodules can usually be detected on ultrasound. Therefore, focus should be on discovery of biomarkers for peritoneal or ultrasound-negative endometriosis [67]. However, to date studies on peritoneal endometriosis specifically are lacking.

Another important factor in endometriosis research is the choice of an adequate control group. Controls should have laparoscopically confirmed absence of endometriosis [11] and clear mentioning of concomitant disorders [8]. Furthermore, controls should be symptomatic (pelvic pain and/or infertility) and should comprise women that would be tested in the clinical reality. On the other hand, the other gynaecological disorders that these women might have, potentially come with their own set of biomarkers [35]. Therefore, it would be useful to always include symptomatic women with a laparoscopically proven pristine pelvis.

Blood or tissue collection

Blood can be processed as whole blood, plasma or serum. Plasma is obtained after collection of blood with an anticoagulant with subsequent centrifugation to remove cells [68]. Commonly used anticoagulants are ethylenediamine tetraacetic acid (EDTA), which chelates Ca\(^{2+}\) ions, and heparin which activates antithrombin [68]. Heparin can interfere with downstream analyses such as qRT-PCR because of enzyme inhibition, but it is the anticoagulant of choice for metabolomics studies [63,64,69] and MMP measurements [44]. In the absence of an anticoagulant, serum can be obtained from whole blood after clot formation and centrifugation [70]. Serum and plasma are fundamentally different because during clotting fibrinogen and interacting proteins are removed from the sample, while other factors are released from the platelets into the serum [68]. VEGF is one such protein and therefore has higher values in serum than in plasma [71].

Endometrium is a complex tissue, because of its cyclicity and because it contains a variety of cell types, including endometrial stromal cells, epithelial cells, perivascular cells, endothelial cells, and their progenitors. Endometrium may be collected in fixative, snap-frozen, placed in an RNA-stabilizing solution, or used fresh, depending on the downstream use [72]. Studies on endometrial nerve fibers in particular have emphasized the importance of the collection method [73].

At least some of the controversial biomarker results in endometriosis research can be attributed to low sample sizes and to the lack of standardization in sample handling and storage [4]. To stimulate international collaboration, the World Endometriosis Research Foundation (WERF) has distributed standard operation procedures (SOPs) for collection of blood [70] and tissue [72].
Experimental variables and analysis methods

The variability between ELISA kits should be taken into consideration when comparing ELISA results from different manufacturers and even different batches from the same manufacturer [74]. Data analysis for biomarker research should not be limited to finding a significant difference, but ideally includes ROC curve analysis with the choice of cut-off giving a certain sensitivity and specificity. For more complex large-scale studies, multivariate statistical analysis is indispensable. One pitfall of multivariate statistics is the risk to overfit the model to the data, which happens when the sample size is not high enough for the amount of tested markers [75]. As a rule of thumb, every experimental group should contain at least 10 subjects per variable [76]. Ideally, an independent validation cohort is included in the same experiment to test model performance.

Future perspectives

For a complex disease such as endometriosis, a biomarker panel will most likely be more accurate than any single biomarker. Biomarker panels with good sensitivity and specificity have been reported for peptide peaks [58], proteins [37], metabolites [63], but validation has been lacking. New “omics” technologies and multiplex immunoassays could help discover more biomarker panels [28]. Furthermore, it can be interesting to combine noninvasive methods [77]. Promising panels should be analysed in a large sample set, ideally using standard procedures to allow other laboratories to replicate the data.

Summary

Many biomarkers for endometriosis have been investigated using either a hypothesis-driven or a hypothesis-generating (no prior hypothesis) approach, but no biomarker has been validated in peripheral blood nor endometrium. A panel of biomarkers will most likely be necessary to diagnose a complex disease such as endometriosis. Patient selection, sample collection and analytical variability should be taken into consideration when initiating a biomarker study or assessing other studies. Biomarker validation in an independent cohort is a crucial step in the development of a noninvasive test.

Conflict of interest

Author T.D. reports only conflicts of interest outside the scope of the paper. He has served as advisor for Bayer Pharma, Proteomika, Pharmaplex, Astellas, Roche Diagnostics, Actavis, has received grants from Ferring, Merck Serono, MSD, Besins, Pharmaplex, and has received travel support from Ferring, Merck Serono and MSD. From October 1st 2015, Thomas D’Hooghe is Vice-President and Head of Global Medical Affairs Infertility for the Multinational Pharmaceutical company Merck Serono (Darmstadt, Germany) and continues on a part time basis his academic appointment as Professor of Reproductive Medicine at the University of Leuven (KU Leuven) in Belgium.

Practice points

- No biomarkers currently exist for endometriosis. In peripheral blood, CA-125 is the most commonly investigated biomarker, but no consensus on the cut-off generating the best specificity and sensitivity has been reached. In endometrium, the study of nerve fiber growth in the endometrium has shown promising results, but the results have been variable.
- Biomarker discovery can be hypothesis-driven or hypothesis-generating, with the latter often employing “omics” technologies.
- Good endometriosis research should minimize bias introduced by patient selection, sample collection and analytic methods.
Research agenda

- Focus on development of a noninvasive test for endometriosis in symptomatic women with pelvic pain and/or infertility, without ultrasound evidence of endometriosis (i.e., women without ovarian endometrioma or deep endometriotic nodules that can be observed by vaginal ultrasound) [67].
- Adapting SOPs and forming international collaborations to reach highly powered studies
- Need for well-designed studies further investigating promising markers such as CA-125 and biomarker panels or finding new biomarkers using the “omics” technologies
- Using multivariate analysis to find biomarker panels and combine molecular markers with ultrasound and other clinical factors
- Investigating different patient populations, such as women using hormonal medication

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