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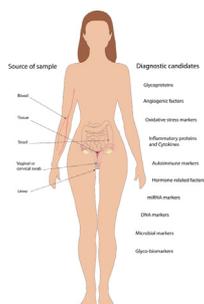
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Review

Novel diagnostic options for endometriosis – Based on the glycome and microbiome

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ABSTRACT

Background: Endometriosis is a chronic gynaecological disease whose aetiology is still unknown. Despite its prevalence among women of reproductive age, the pathology of the disease has not yet been elucidated and only symptomatic treatment is available. Endometriosis has high latency and diagnostic methods are both limited and invasive.

Aim of review: The aim of this review is to summarise minimally invasive or non-invasive diagnostic methods for endometriosis and their diagnostic efficiencies. Furthermore, we discuss the identification and diagnostic potential of novel disease biomarkers of microbial or glycan origin.

Key scientific concepts of review: Great efforts have been made to develop minimally invasive or non-invasive diagnostic methods in endometriosis. The problem with most potential biomarker candidates is that they have high accuracy only in cases of severe disease. Therefore, it is necessary to examine other potential biomarkers more closely. Associations between gastrointestinal and genital tract microbial health and endometriosis have been identified. For instance, irritable bowel syndrome is more common in women with endometriosis, and hormonal imbalance has a negative impact on the microbiome of both the genital tract and the gastrointestinal system. Further interrogation of these associations may have potential diagnostic significance and may identify novel therapeutic avenues. Glycomics may also be a potent source of biomarkers of endometriosis, with a number of glyco-biomarkers already approved by the FDA. Endometriosis-associated microbial and glycomic profiles may represent viable targets for development of innovative diagnostics in this debilitating disease.

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Introduction

Endometriosis (EMS) is a chronic gynaecological condition which affects at least 176 million women worldwide, around 6–10% of women of childbearing age. EMS is characterized by the growth of endometrial-like tissue outside the uterine cavity, and is associated with pelvic pain, dysmenorrhea and infertility. Despite its prevalence, however, the aetiology of the disease is still poorly understood [1]. In addition to fertility problems and reduced quality of life, this enigmatic disease also has serious economic consequences [2]. Direct healthcare costs for women with EMS are more than twice as high as women without the disease [3]. Furthermore, the EMS-related burden of illness cost has been valued as high as 54 million euros per year in European Union countries, representing a serious burden for society. This amount also includes additional costs beyond hospitalisation of the disease e.g. lost days at work, layoffs, having to change jobs, sick leave, time off for having surgery [4].

There are several hypotheses regarding the origin of the disease, which can be divided into the following four major groups: transport, coelomic metaplasia, embryonic cell rests, and immunological theories [5–7]. The theory of coelomic metaplasia can provide an explanation for the phenomenon of EMS described in men, while immunological theories give an answer to the persistence of the disease [8]. Based on our current knowledge, we assume that the development and persistence of the disease depend on several co-existing factors. Indeed it is likely that EMS is a condition of multifactorial aetiology; involving genetic predisposition, prenatal exposure to endocrine-disrupting chemicals, the microbiome, the immune system and sex hormones [9].

Diagnosis of EMS remains challenging, due in large part to the wide range spectrum of symptoms associated with the disease [10]. Definitive diagnosis is invasive, requiring laparoscopic surgery. Numerous attempts have been made to develop an effective and less invasive diagnostic method to date. The emerging discipline of glycomics holds promise as a new ‘omics’ approach to understanding complex diseases. As a result of the development of separation techniques, we are increasingly aware of the importance of glycosylation. Glycomics is a discipline for the study of carbohydrates and indirectly provides an opportunity to discover new glyco-biomarkers [11]. In this review, we present efforts to diagnose EMS and outline a microbiome and glycosylation profile-based approach as a potential new source of biomarkers.

Background information on endometriosis

Symptoms and presentation of disease

There are many symptoms of EMS, but the most common are severe dysmenorrhea, deep dyspareunia, ovulation pain, irregular uterine bleeding, infertility, chronic fatigue, pelvic tenderness and chronic pelvic pain; although none of these symptoms are specific for EMS. Endometriosis is strongly associated with infertility, with a 35–50% prevalence of EMS in women presenting with infertility [12]. However, evidence for the effect of EMS on likelihood of pregnancy has been conflicting [13,14]. Many women with EMS pursue pregnancy via Assisted Reproduction Technologies (ART) which is considered an effective treatment option for women with EMS [15]. However, even with the availability of ART, a negative impact can be seen on many parameters of in-vitro fertilization in women with EMS [16]. The likely mechanism for the impact of EMS on fertility, whether related to oocyte and subsequent embryo quality, or implantation, has also been debated. Some have found no difference in number or quality of embryos from patients with EMS compared to those without [17]. A reduced ongoing

pregnancy rate in those with EMS was noted, suggesting an altered endometrial receptivity. Others have noted a reduction in oocyte quality, fertilisation rates and embryo quality [18,19]. It is likely that EMS impairs fertility through multiple pathways [20].

Diagnosis

The diagnostic time for EMS is 4–11 years (average time ~ 7 years), in part due to the nonspecific disease symptoms and highly limited tools of diagnosis [21,22]. Diagnostic techniques such as two-, or three-dimensional ultrasound, magnetic resonance imaging and other imaging techniques may be effective to diagnose ovarian and deep infiltrating EMS [23,24]. These aforementioned imaging methods are only suitable for detecting severe and extended or clearly visible EMS, but histological confirmation is still required. The gold standard for confirmatory diagnosis of EMS is laparoscopic surgery with histologic examination after biopsy [25]. However, the surgical diagnosis has multiple drawbacks, such as risks inherent to the procedure and anaesthetic complications.

Pathogenesis and risk factors

Neither the exact pathophysiology of EMS nor the risk factors are fully elucidated, but a number of factors have been scientifically investigated [26,27]. The most widely accepted theory of the pathogenesis of EMS is that of retrograde menstruation, wherein endometrial tissue is expelled into the peritoneal cavity during menstruation [28]. Evidence for this theory is based on the increased incidence of EMS seen in women with outflow obstruction, such as cervical stenosis and uterine anomalies [29,30]. However, as 90% of women experience retrograde menstruation, it is likely that the eutopic endometrium itself in EMS is abnormal, predisposing to the formation of ectopic deposits [31]. A link between EMS and pelvic infection has been suggested. A retrospective study of data from over 14,000 individuals suggested that the risk of developing EMS was three times higher in those with pelvic inflammatory disease [32]. A growing number of studies suggest a link between EMS and other chronic and autoimmune diseases [33,34]. The incidence of EMS is over 2-fold higher among women whose mothers also suffered from the disease, however, genetic predisposition is not the only contributing factor for the development of EMS [35,36]. Additionally, lower birth weight, early age at menarche and shorter menstruation cycles (<26 days) have been associated with a higher risk of EMS [37–39]. Decreased pregnancy rates and nulliparity as a result of modern lifestyle contribute to an elevated incidence of EMS. However, pregnancy cannot be a strategy for managing symptoms and reducing the progression of the EMS, because there is a poor connection between the positive effects of pregnancy and EMS [40]. There is clear association between environmental toxins like polychlorinated biphenyl and dioxin [41,42] and other adulthood exposure (e.g. alcohol and caffeine intake) and higher risk of EMS [43–45]. There are many other indirect risk factors of EMS, like skin sensitivity, night shift work and certain dietary factors, but further investigations are needed to uncover clear associations [27,46].

Biomarkers

Given the severe and debilitating outcomes of EMS, there is a high demand for a less invasive diagnostic biomarker. First, we need to understand what a biomarker is and its significance. A biomarker is defined as a specific attribute that is measured as an indicator of normal biological processes, pathogenic processes, or response to exposure or intervention, including therapeutic interventions [47]. A diagnostic biomarker is a characteristic that can

be used to detect or confirm disease or condition, or to identify individuals with a subtype of the disease. All available evidence must be gathered from a biomarker candidate and the potential benefits and risks of use must be presented objectively [48]. Clinical and analytical validation are distinct processes; however, these two parts of the validation process are interrelated (Fig. 1). A reliable measurement method must be developed and analytically validated before determining a cut-off value. These circumspect measures improve the biomarker candidate's clinical validation success in the clinical trial phase [48]. To choose biomarker candidates, it is very important to select an adequate control group. Most candidates perform well as a potential marker when compared to a healthy control group. Endometriosis is a disease with a very heterogeneous appearance and as such, it can be misdiagnosed as a number of other inflammatory gynaecological or urological conditions. Therefore, it is important to select a heterogeneous control group that meets the criteria of the highly specific diagnosis of EMS [48]. Cochrane studies have concluded that there are currently no non-invasive biomarker candidates that can replace invasive laparoscopic surgery in clinical practice [49]. In light of this landscape, we will herein briefly summarize the scientific information to date and outline how microbial and glycosylation patterns may hold promise as novel biomarkers of EMS.

Putative non-invasive candidate biomarkers

In recent years, many studies have targeted the pathomechanism of EMS adopting mostly molecular biological studies of endometrial lesions and healthy endometrial tissues. Another major area of research is the search for potential new biomarkers and several blood-derived candidates have been tested (Fig. 2). Below we present promising minimally or non-invasive biomarker candidates and evaluate their suitability as a potential replacement for laparoscopy diagnosis.

Glycoproteins

Cancer antigen (CA)-125 is a membrane glycoprotein, member of the mucin family and a component of the epithelium of the

female reproductive tract, which inhibits the adhesion of infectious agents to epithelial cells [50]. The FDA approved the measurement of CA-125, in combination with other factors, to estimate the risk of epithelial ovarian cancer and monitor response of the treatment [51,52]. CA-125 had been identified as a very promising candidate in EMS [52] (Table 1), displaying menstrual levels almost 200% higher in women with EMS compared to healthy controls [53]. However, further clinical studies revealed that it is alone a poor diagnostic marker of EMS [54]. While CA-125 is effective for indicating inflammatory, benign, and malignant transformations of the genital tract, its ubiquitous presence renders it poorly specific as a marker for EMS. The efficacy of CA-125, CA 19–9, CA 72–4 and human epididymis protein 4 have been tested individually or in combination, but neither method has proven sensitive enough to be a viable biomarker in early EMS [55–57]. CA-125 has also been tested in combination with other potential candidates to promote higher accuracy, but these could be expensive or difficult to apply in clinical practice [58] (Table 1). The endometrial glands produce Glycodelin-A during the secretory phase, which has an immunosuppressive function, is involved in the regulation of angiogenesis and apoptosis and may play an important role in the fetomaternal protective mechanism [59,60]. Glycodelin-A levels have been demonstrated to increase in the bloodstream of patients with EMS. It is an unsuitable biomarker candidate alone [61,62]; but in combination with other factors may form an effective biomarker panel [63,64]. While CA-125 and Glycodelin-A glycoproteins are promising biomarkers in severe EMS versus healthy controls, none are sufficiently sensitive enough to replace the gold standard of diagnostic surgery.

Angiogenic factors

Since angiogenesis has an essential role in the progression of ectopic lesions during EMS, vascular endothelial growth factor (VEGF) is the most studied pro-angiogenic factor in EMS [65]. It is widely accepted that VEGF is a major stimulus of angiogenesis and permeability in this disease [66] (Table 1). VEGF A, VEGF 121 and VEGF 189 factors were significantly overexpressed during menstruation in EMS patients compared to the control group, which may help to explain the pathomechanism of the disease and provide potential biomarkers, but further investigations are required [67]. Pigment epithelium-derived factor (PEDF) is a glycoprotein that is potentially involved in a variety of biological processes, possessing potent antiangiogenic, neuroprotective, anti-inflammatory and immunosuppressive properties. Previous studies found that the level of PEDF is decreased in peripheral blood of EMS patients [68] but further studies are needed to determine whether PEDF may be a suitable biomarker candidate. Research findings on angiogenic factors are encouraging, but more data are needed to estimate their specificity and sensitivity efficacy in EMS. In addition, studies should be performed in patients with gynaecological tumour disease, as these angiogenic factors are also of great significance in tumour development and a sensitive biomarker candidate has to distinguish between EMS from other gynaecological tumour.

Oxidative stress markers

Oxidative stress markers were found to be altered significantly in EMS patients and monitoring them was considered a promising avenue to identify new biomarkers. Blood levels of superoxide dismutase and glutathione peroxidase were measured with commercially available assay kits, but their combined sensitivity was 78%, which is not accurate enough for clinical use [69]. The marker soluble tumour necrosis factor-alpha receptor (sTNFR-I) was shown to detect early stage EMS with 75% specificity, which is notable as

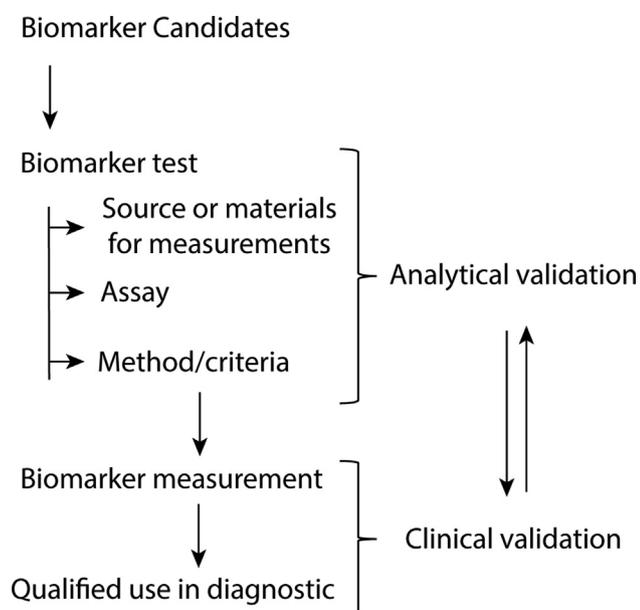


Fig. 1. Biomarker selection and validation approach. Clinical and analytical validation are distinct processes; however, these two parts of the validation process are connected.

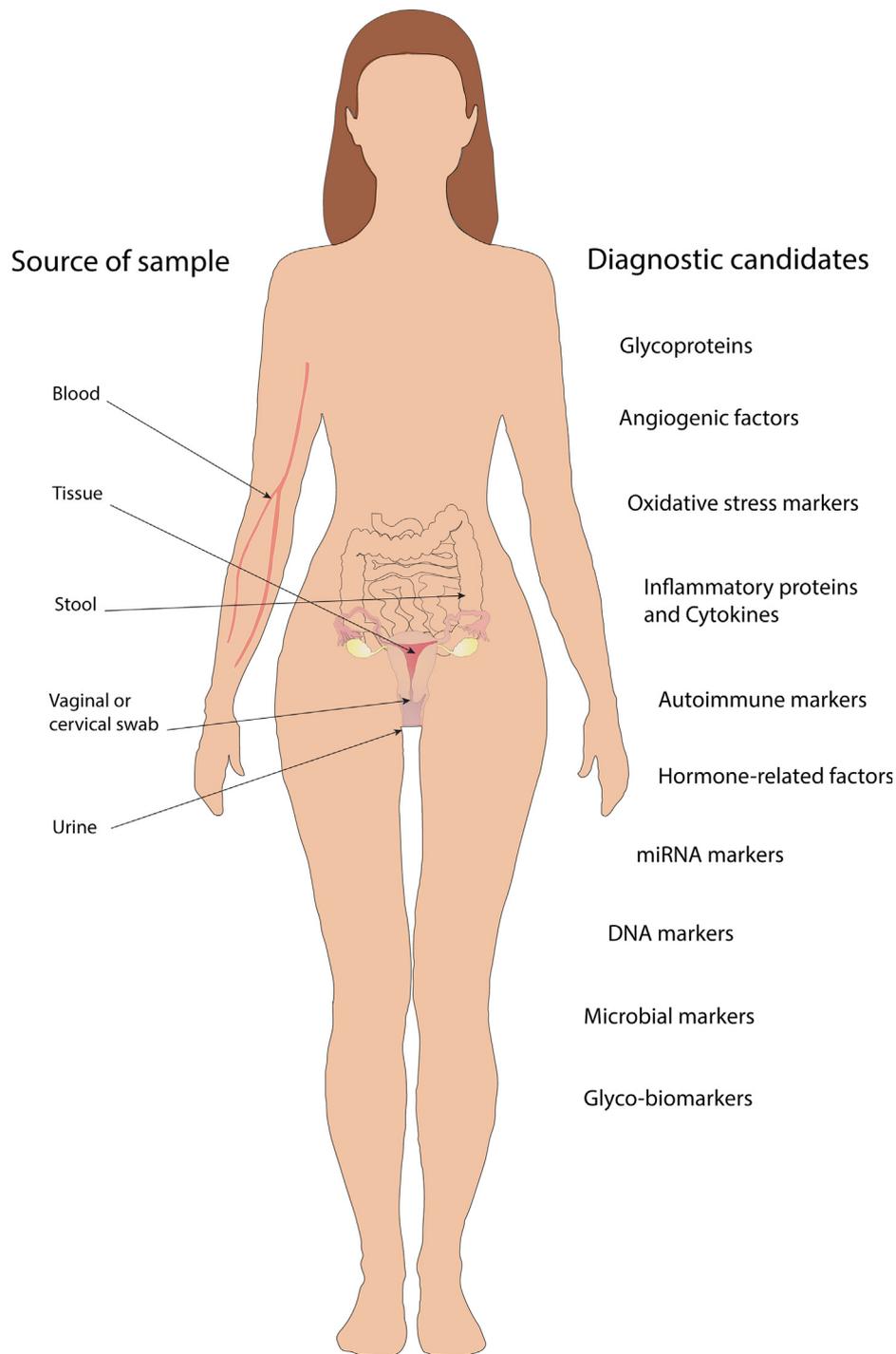


Fig. 2. Summary of sample sources and potential biomarker candidates for endometriosis diagnosis that could replace highly accurate but invasive laparoscopic surgery. The presented promising minimally or non-invasive biomarker candidates' suitability as a potential replacement for laparoscopy diagnosis are questionable. A large pool of biomarker sources (blood, tissue, urine, stool, vaginal or cervical swabs) and a glycomic or microbiology approach open up further perspectives for identifying new candidates.

most candidates are only able to indicate an advanced stage of EMS [70] (Table 1). Research findings on these stress markers are encouraging, but larger clinical studies are necessary to estimate their actual efficacy as potential diagnostic markers of EMS.

Inflammatory proteins and cytokines

Immunomodulatory factors, such as Galectin-1, Galectin-3 and Galectin-9 have been investigated, but most of them require more

data to assess usability and some have already proven to be inadequate biomarker candidates for EMS [71]. Galectins bind β -galactoside sugars and are involved in the regulation of apoptosis and they may play an indirect role in the abnormal survival of endometrial cells outside the uterus. The results of a Galectin-9 pilot study were very promising because of its high area under the curve (AUC) value, although this has yet to be repeated with a larger study cohort [72] (Table 1). Changes in several other immunomodulatory molecules have been reported in women with

Table 1
Reported sensitivity, specificity and other features of promising biomarker candidates in endometriosis.

Analyte	Source of the analyte	Limitation	Sensitivity	Specificity	Cut-off value	Reference
CA-125	serum	for diagnosis of moderate or severe EMS	52%	93%	≥ 30 units/mL	Hirsch et al. 2016
CA-125 + 50 IU/mL	peripheral blood	unknown	92.2%	81.6%	CCR1/HPRT + MCP-1 1.16 pg/mL 140 pg/mL	Agic et al. 2008
VEGF	serum	unknown	74%	80%	>3.88 pg/mL	Kressin et al. 2001
sTNFR-I	serum	unknown	60.7%	75%	351.22 pg/ml	Othman et al. 2016
Galectin-9	serum	only compared to healthy, normal pelvic control	94%	93.75%	132 pg/mL	Brubel et al. 2017
Urocortin	plasma	specific diagnosis only in ovarian EMS	76%	88%	46 pg/mL	Pergialiotis et al. 2019
let-7b + let-7c + let-7d + let-7e	serum	hormonal phase affects the result	83.3%	100.0%	0.823 pg/mL	Cho et al. 2015
miR-122	serum	unknown	95.6%	91.4%	3.24 pg/mL	Maged et al. 2018
ccf nDNA and ccf mtDNA	peripheral blood	unknown	70%	87%	416 ccf nDNA genome equivalent/ml	Zachariah et al. 2009

EMS; β 1-integrin and other cell adhesion molecules [73], intercellular adhesion molecule-1 (ICAM-1) [74], E-cadherin [75] and matrix metalloproteinases (MMP) 2 and 9 [76]. C-reactive protein (CRP) is a general marker for inflammatory processes and correlates well with the presence of EMS, but its specificity is not high enough to be a potent biomarker for EMS. A more accurate method is to measure high-sensitivity C-reactive protein (hsCRP); however, hsCRP of plasma is not effective enough to make a definitive diagnosis of EMS [77,78]. Due to the heterogeneous nature of this illness, none of the candidates or combinations mentioned herein is specific enough to make a definitive diagnosis. Inflammatory factors, as a panel of tumor necrosis factor- α , interleukin-1 β , interleukin 6, interferon- γ and soluble ICAM-1, are not specific enough to diagnose early phase EMS, although there is ample evidence that they are involved in the pathomechanism of EMS [64,79]. Many conditions, including EMS, can cause an increase in the level of inflammatory factors. Indeed, a number of these conditions can also co-present with EMS. Therefore, further studies are needed to prove the practical usage of these factors.

Autoimmune markers

Several autoimmune markers have been tested as potential biomarker candidates, but none have worked as expected except for a panel of anti-tropomodulin (TMOD)3b, anti-TMOD3c, anti-TMOD3d, anti-tropomyosin (TPM)3a, anti-TPM3c and anti-TPM3d. This panel showed an AUC of 0.869 with quite high specificity (80%), but further validation is required with a larger study group [80]. The contribution of dysregulated inflammatory processes to the pathomechanism of EMS is widely accepted, but none of the factors involved have proven thus far to be viable biomarkers.

Hormone-related factors to properly estimate efficacy

It is also well established that EMS is an estrogen-dependent condition, and the attempt to identify a hormone-based biomarker was, therefore, a logical approach. Urocortin (UCN) is a member of the corticotrophin-releasing hormone family and is expressed by eutopic and ectopic human endometrial tissue; its level can be measured in both tissue and blood. Serum UCN level in EMS patients suggested that it could be a novel biomarker of a subtype (endometrioma) of EMS, with sensitivity and specificity of 80%.

Although UCN seems a promising candidate for endometrioma identification and follow-up, more studies are required to determine its efficacy [81] (Table 1). Activin A is a growth factor expressed by endometriotic tissue that participates in the regulation of the menstrual cycle and whose actions are controlled by the binding protein follistatin. Both proteins are traceable in serum and their concentrations increase in women with EMS. The AUC of activin A was 0.700, while AUC of follistatin was 0.620 (95% confidence interval: 0.510–0.730) for the diagnosis of ovarian endometrioma. However, the combination of activin A and follistatin did not improve their diagnostic accuracy, and provided no diagnostic value for peritoneal or deep infiltrating EMS [82]. In summary, hormone-related factors, such as UCN, have proven to be highly sensitive diagnostic candidates for ovarian endometrioma, however, they are less able to identify other manifestations of EMS.

Epigenetics: miRNA markers

One of the most promising areas of EMS diagnosis is genomics and epigenomics. In one study, women with EMS had significantly downregulated microRNA levels of miR-17-5p, miR-20a and miR-22 compared to the control group, although combined AUC value of these miRNAs was 0.74 which does not meet the biomarker accuracy criterion [83]. The miRNA let-7 is reportedly involved in abnormal endometrial growth and EMS [84]. Cho *et al.* quantified miRNA let-7a-f and miR-135a,b from 24 patient and 24 control blood samples. The combined AUC of miRNA let-7b, let-7c, let-7d and let-7e during the proliferative phase was 0.929. These results suggest let-7d may be a reliable candidate with high accuracy; nevertheless, the results should be treated with caution due to the small number of study participants [85] (Table 1). Other microRNAs are also dysregulated in EMS patients, including miR-122 and miR-199. Eighty women were enrolled in an Egyptian study that examined microRNAs extracted from blood samples. The results showed a positive correlation between miR-122 and interleukin 6 (IL-6) levels and accuracy of miR-122 was 93.75% (sensitivity 95.6% and a specificity of 91.4%). However, more validation studies are required with an extended case number [86]. It is clear that the miR-200-family has a different expression pattern in endometrial lesions compared to eutopic tissues, nevertheless only one study examined miR200-family member - miR-141* in EMS patients. MiR-200a, miR-200b and miR-141* were isolated from peripheral

blood and the results indicated that the combined AUC value of the above-mentioned microRNAs was 0.76. Notably, however, this value was dependent on sampling time. All three miRNAs had lower levels in the blood samples collected in the morning which associated with the circadian clock. Other studies also concluded that sampling time is an important aspect of miR-200 levels. This important caveat should be taken into account in future studies and should be explored as a potential cause of fluctuation (e.g. circadian rhythm) [87]. The collection and processing of miRNA samples require expertise, and the conditions of a collection can affect the result, making practical application difficult.

Genetics: DNA markers

Screening of EMS-associated mitochondrial DNA (mtDNA) deletions may be an effective way to identify novel non-invasive markers. Mutation frequency in mtDNA is high and repair capability is limited, making mtDNAs an excellent source of biomarker candidates. Creed *et al.* selected the following genomic regions for PCR analysis: CO2 to ATP6 (1.0 kb deletion); ATP6 to ND3 (1.2 kb deletion); ATP8 to ND4 (2.4 kb deletion); ATP6 to ND5 (3.7 kb deletion); ATP8 to ND5 (5.0 kb deletion); CO1 to ND5 (6.5 kb deletion); and CO2 to CytB (7.7 kb deletion), two of these deletions showed moderate accuracy. The sensitivity of the 1.2 kb deletion assay was 81.8% and specificity was 72.2%, while the diagnostic value of the 3.7 kb deletion assay was less accurate with sensitivity and specificity of 85.1% and 57.9%. Interestingly, there is a minimal correlation between menstrual stage and mtDNA deletion, suggesting that menstrual phase should be taken into account in future expanded mtDNA genomic analyses [88]. Circulating cell-free DNA has offered novel possibilities for non-invasive diagnosis and monitoring of many diseases, including EMS. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyses a key step in glycolysis and has recently been implicated in several non-metabolic processes, including transcription activation and initiation of apoptosis. A study reported that concentrations of circulating cell-free GAPDH gene sequence were significantly increased in plasma from EMS patient's sample [89] (Table 1).

Familial clustering of EMS suggests the importance of hereditary factors, but evidence indicates that genetic predisposition is multifactorial and not linked to a single mutation [90]. Mutations in the genes for DNA mismatch repair proteins, cell adhesion proteins and tumour suppressors have been identified in patients with EMS, but none of these mutations are specified as appropriate biomarkers in EMS [91]. Despite numerous studies conducted, none have yet identified clinically applicable non-invasive genetic or epigenetic biomarker candidates for EMS.

Overall, there are many different approaches for developing an effective and sensitive non-invasive diagnostic method. Although we sought to present the most promising candidates, it should be taken into account when evaluating the results that in the presented studies, samples from patients with severe EMS were compared with a healthy control group. Further sophisticated studies have indicated that these biomarker candidates may only be sensitive enough to identify severe EMS. Given the complex aetiology of EMS, it seems more likely that a panel of clinical markers will be necessary.

Microbiome in endometriosis: Diagnostic potential

In the study of the interaction between EMS and microbiome, there are two distinct but interacting microbial ecosystems: one in the gut and one in the female genital tract.

The presence of metabolically inactive, naturally cell-wall-deficient (CWD) or L-form bacteria, those which adopt a CWD state

under stress [92–94], may play a significant role in the development of chronic diseases such as EMS [95]. These CWD and L-forms of pathogens (such as *Chlamydia*, *Mycoplasma*, *Streptococcus*, *Enterococcus*, and *E. coli*) are metabolically inactive, non-culturable forms of intracellular bacteria to allow them to survive non-conductive conditions for growth, characterized by slow growth and absence of cell wall [96]. They are able to evade the immune response and antibiotics, survive and persist long term in the host and then reactivate under more favourable conditions [95]. They integrate into host cells using the host pathways and their peptides then display structural similarity with self-peptides which can activate autoreactive T-cells and cause autoimmunity [95]. These pathogens are able to alter host energy metabolism and glucose balance, dysregulate autophagy, induce epigenetic modifications, and make changes in blood clotting and impaired wound healing to promote their spread [95]. In the developed infection the T-cells get exhausted and appropriate T-cell response is lost [95]. Immune cells could also be regulated by dopamine levels, which these bacteria use [95]. Some pathogens produce saccharide structures on their surface that mimic human carbohydrates which may help the bacteria to avoid recognition by the human immune system [95]. These factors all contribute to long term pro-inflammatory, autoimmune status in the host, which enables these pathogens to survive, and also alter the environment around them for their benefit.

In patients with EMS, several microbial abnormalities have been identified, such as higher amounts of *Gardnerella*, *Streptococcus*, *Enterococci*, and *E. coli* than in healthy women. This abnormality was associated with increases in vaginal pH and hormonal status [97,98] (Table 2). These pathogens could be also attracted by the altered pH, which was originally generated by intracellular CWDs [95]. Hormones, especially estrogen, can regulate a number of anti-microbial peptides [95]. Bacteria can spread through endometrial influx in retrograde menstruation [95], when hormone levels are low. LPS/endotoxin from these bacteria was found to promote growth of EMS through TLR4 [99].

Therefore, EMS could be initiated and regulated by these pathogenic microbes.

The identification and treatment of microbial infections could be an effective treatment strategy for the symptoms of EMS. In this section, we present the available evidence for both gut and genital tract microbiome with regards to EMS.

Microbial composition of the genital tract

The relevance of the vaginal microbiota to health and homeostasis of the female genital tract is well known and its alteration has been observed in many gynaecological diseases including EMS [100,101] (Table 2). The conventional theory of sterility of the uterus and upper reproductive tract, as well as the abnormal presence of the microbiome that can be detected there, are debatable in the light of new evidence and improved detection techniques [102–104]. Although there is a need to standardize and harmonize sample collection methods, it is clear that the microbiome is related to the functioning of the female genital tract [105]. There are several differences in the composition of the genital tract and gastrointestinal system microbiome between EMS and healthy female groups. These alterations and their potential effects are not well-characterized (Fig. 2), but they may help to further interrogate the underlying pathomechanism of this condition and to develop more efficient treatment guidelines. Dysbiosis of the upper genital tract microbiome has been described under inflammatory conditions such as EMS or adenomyosis [101] (Table 2) compared to healthy controls. The members of the control group are volunteers who meet pre-established criteria (e.g., members of a healthy population that do not have the particular disease to be analysed

Table 2
Microbiome changes in human female patients and animal models of endometriosis.

Genital tract microbiom				Gut microbiome			
Sample source/type	Detection technique	Results	Reference	Sample source/ type	Detection technique	Results	Reference
sample of lower and upper genital tract/ human	16S rRNA amplicon sequencing	in relation to hysteromyoma <i>Lactobacillus</i> sp. were found to be more abundant in the samples of control group, while <i>L. iners</i> was more abundant in the patient group	Chen et al. 2017	fresh stool samples/human	16S rRNA gene amplification	<i>Shigella</i> and <i>Escherichia</i> dominant stool microbiome in severe EMS	Ata et al. 2019
endometrial and cystic fluid samples/ human	16S rDNA and sequence analysis	<i>Enterobacteriaceae</i> and <i>Streptococcus</i> spp. were significantly overrepresented in EMS samples compared to the healthy controls	Khan et al. 2016	faeces and peritoneal macrophage collection/mice	16S V4 gene region amplification; hematoxylin-eosin and immunofluorescent staining	<i>Firmicutes</i> and <i>Bacteroidetes</i> ratio was elevated in EMS mice	Yuan et al. 2018
vaginal fluid, eutopic endometrium and endometriotic lesion tissue samples/human	16S rRNA amplicon sequencing	the microbial diversity of the endometriotic lesion had a higher diversity which had shifted towards <i>Alishewanella</i> , <i>Enterococcus</i> , <i>Ureaplasma</i> and <i>Pseudomonas</i>	Hernandes et al. 2020				
rectal and vaginal samples/human	16S rRNA amplicon sequencing	vaginal microbiome (e.g. <i>Firmicutes</i> / <i>Bacteroidetes</i> , <i>Anaerococcus</i> , <i>Lactobacillus</i> alterations) was predictive of endometriosis rASRM stages	Perrotta et al. 2020	faeces sample/ mice; eutopic endometrium and endometriotic lesions	16S rRNA gene amplification; enzyme-linked immunosorbent assay	broad-spectrum antibiotic that reduced the size of endometriotic lesions; gut microbiome may promote inflammation in EMS	Chadchan et al. 2019
vaginal and endometrial smear samples/human	bacterial vaginosis scores in Gram-stained vaginal samples, immunohistological test, measure of intra-vaginal pH	<i>Lactobacillaceae</i> , <i>Streptococcaceae</i> , <i>Staphylococaceae</i> and <i>Enterobacteriaceae</i> were significantly increased in EMS samples compared to the control group of samples	Khan et al. 2014				
vaginal and endocervical swab samples/human	16S rRNA gene amplification	absence of <i>Atopobium</i> genus in the vaginal and cervical microbiota in severe EMS	Ata et al. 2019	fresh faecal sample/Macaca mulatta	microflora cultivation on different types of agar	lowered <i>Lactobacillus</i> and higher <i>Gram-negative</i> bacteria ration in EMS samples	Bailey and Coe 2002

and meet the selection criteria) and serve as a reference point for evaluation. Microbial composition of endometrial swab samples was sequenced using next-generation sequencing techniques and significant alterations were identified between control and EMS groups. In this study, the proportion of *Enterobacteriaceae* and *Streptococcus* spp. were significantly overrepresented in EMS women compared to the healthy controls [106] (Table 2). 16 s rDNA sequencing of vaginal fluid, eutopic endometrium and EMS lesion samples were recently analysed, microbiome alpha and beta diversity did not change significantly with *Lactobacillus*, *Gardnerella*, *Streptococcus* and *Prevotella* dominating the vagina fluid. However, the microbiome of the endometriotic lesions had a higher diversity which had shifted towards *Alishevanelle*, *Enterococcus*, *Ureaplasma* and *Pseudomonas* [107] (Table 2). An observational pilot study discovered that changes in the composition of the vaginal microbiome can predict the revised American Society for Reproductive Medicine (rASRM) stage of EMS patients [104]. This study was performed with a small patient group and the results are very promising, but further investigations are needed to confirm the previous findings [108] (Table 2). Endometriosis is an estrogen-dependent disease and a common choice of treatment is the use of Gonadotropin-Releasing Hormone agonist (GnRHa), which inhibits estrogen production thereby relieving symptoms. It should be noted that GnRHa treatment influences the microbial composition of the upper genital tract and ovaries, demonstrating the potential role for hormones in influencing the microbiome [106,109]. The proportion of *Lactobacillaceae*, *Streptococcaceae*, *Staphylococaceae* and *Enterobacteriaceae* were significantly increased in endometrial swabs and endometrioma/non-endometrioma cystic fluid EMS samples, compared to a control group of samples from women without EMS. This study further revealed an increase in endometrial colonization concurrent with endometritis in EMS. An intra-vaginal pH ≥ 4.5 was associated with endometritis in both the control and EMS groups, while treatment with GnRHa significantly increased prevalence of acute endometritis in both groups [98] (Table 2). The microbiota of stool samples, vaginal and endocervical swabs from women with severe EMS (stage 3 and 4) compared to healthy controls were analysed during an observational study. There was a not significant difference in microbiome diversity between control and patient's samples but the proportion of some bacteria shifted in patient samples. The reduction of *Atopobium* spp. increased the proportion of *Gardnerella* in vaginal and cervical swab samples. Furthermore, the presence of *Sneathia*, *Gardnerella*, *Streptococcus*, *Escherichia/Shigella* and proportion of *Ureaplasma* were higher, while *Alloprevotella* was decreased in cervix microbiome. Fecal *Escherichia* and *Shigella* was also found to be higher in severe EMS patients compared to healthy controls [100,110] (Table 2).

The scientific results available so far suggest that the composition of the genital microbiome changes during EMS. Perrotta *et al.* found that microbial alteration is associated with the severity of EMS (based on rASRM classification). Based on these findings, we believe that the further study of the genital microbiome is of great importance in future EMS treatment.

Microbiome of the gastrointestinal tract

There is substantial evidence pointing to the role of the gut microbiome in modulating extra intestinal host health [111]. Indeed, changes in circadian rhythms and immune function have been linked to intestinal microbial processing and the resulting metabolites like short-chain fatty acids [112,113]. Patients with EMS had reduced microbiome diversity and an increased proportion of potentially pathogenic microbes in both genital and gastrointestinal samples, compared to healthy women without EMS [100] (Table 2). Composition of the gut microbiome was moni-

tored in a mouse model of EMS and it was found that, 42 days after EMS induction, the *Firmicutes* and *Bacteroidetes* ratio in the stool with increased *Ruminococcaceae*, *Bifidobacterium* and *Parasutterella* genera suggesting that EMS could induce dysbiosis [114] (Table 2). Targeting of gut microbiota in EMS mice using a broad-spectrum antibiotic reduced the size of endometriotic lesions and inflammatory processes highlighting the microbial role in this disease. However, more in-depth studies are needed to understand how the gut microbiome contributes to pelvic inflammatory processes and EMS progression [115] (Table 2). Insight from *in vivo* mouse studies using transplantation of uterine tissue fragments does not suggest a significant impact on gut microbial composition, at least in the acute phase of EMS lesion formation [116]. Furthermore, lower *Lactobacillus* and higher *Gram-negative* microbial loads were identified in the intestinal microbiome of *Rhesus macaque* with EMS and subjects with EMS had an increased prevalence of gastrointestinal inflammation [117] (Table 2). This finding is consistent with clinical observations related to EMS; women with EMS are more likely to be diagnosed with irritable bowel syndrome, even after reaching a definitive diagnosis of EMS [118]. Estrobolome may play a role in EMS through β -glucuronidase secreted by gut microbiome. β -glucuronidase releases conjugated estrogen, thereby increasing the level of free estrogen, which may have a direct effect on EMS [109]. While further studies are required to fully delineate the relationship between the gut microbiome, the reproductive tract microbiome and EMS, this rapidly evolving field presents novel opportunities not only for diagnostic development, but also for therapeutic intervention using pro- or prebiotics [97,119]. A double-blind, placebo-controlled clinical study found that *Lactobacillus gasseri* OLL2809 reduced menstrual pain and dysmenorrhea without side-effect in EMS patients [120]. A pilot placebo-controlled randomized clinical trial showed some positive influence of lactobacillus administration on endometriosis-related pain ease [121].

There is also evidence for changes in the intestinal microbiome in EMS and estrobolome may have a significant impact on EMS. In case of EMS affecting the intestinal tract, there may be a more direct interaction between the microbial composition of the intestinal and genital tract and EMS. Further investigations are needed to identify the importance of the microbiome in the pathomechanism and possible diagnostic or treatment usage of EMS. Nevertheless, it is likely that examination of the microbial composition of the intestinal and genital tracts as a complex system may yield the most effective results in the future.

Glycosylation and gynaecological diseases

Carbohydrates, also known as 'glycans', play a significant role in signal transduction and metabolic processes and are important in determining the properties of proteins and in immunological responses [11]. Glycosylation is one of the most common post-translational modifications of eukaryotic cells, which is important for normal biological functions. Only carbohydrates that have been studied in gynaecological research are discussed below.

Glycosaminoglycans (GAGs) are linear and heterogeneous sulphated glycans. Although GAGs are structurally complex, the backbones of these polysaccharides are simply made up of repeating disaccharide building blocks composed of alternating uronic acid and hexosamine units. Proteoglycans are made of GAGs covalently attached to the core proteins. They are found in all connective tissues, extracellular matrix and on the surfaces of many cell types [11,122]. In mammals, there are two main types of glycosylation by which carbohydrates can attach to a protein. O-linked glycans are attached to proteins via the hydroxyl group of serine or threonine through post-translational modifications. N-glycans are

attached to proteins via the asparagine residues due to co- and post-translational modifications [11]. Both *N*- and *O*-glycosylation have the same biological relevance in humans, but the study of *O*-glycosylation is hampered by a lack of specific enzymes. In the case of *N*-glycans, the availability of endo- and exoglycosidases has facilitated investigation [123,124]. Many diseases are associated with impaired or deficient glycosylation such as galactosemia, congenital muscular dystrophies and rheumatoid arthritis or ovarian cancer [125]. Given that ovarian cancer has the highest mortality rate among gynaecological diseases and has limited early diagnostic tools, the glycomics approach is of great importance. Serum glycoproteins of sLe^x level were increased in both breast and ovarian cancer patients, with an increased proportion of fucosylated structures in the ovarian cancer group [126]. The degrees of galactosylation, sialylation, and the ratio of bisecting structures decrease in patients' serum IgG, which directly affects inflammatory and antibody-dependent cellular cytotoxicity processes [127–129]. Alterations in glycosylation patterns and their potential importance as biomarkers in gynaecological diseases are supported by several studies. Serum mannose levels have been studied in women with polycystic ovary syndrome (PCOS) and it has been demonstrated that elevated mannose levels may be a good diagnostic marker. When mannose levels were combined with total testosterone levels, the accuracy increased to 83.3% compared to the original 72.5% [130]. The subtype of clear cell carcinoma of epithelial ovarian cancer is difficult to detect and is associated with poor prognosis. Studies aimed at enhancing the efficacy of the current diagnostic test have been performed, and have shown that the combination of *Wisteria floribunda* lectin analysis with the CA-125 marker may be an effective early biomarker of the disease [131]. Serum IgG glycosylation pattern is an effective marker in the early detection of breast cancer. Two glycan structures (*m/z* 1591 = FA2 (core fucosylated biantennary glycan) and 1794 = FA2B (core fucosylated biantennary bisected glycan)) are significantly altered in early phase breast cancer patients compared to healthy controls and their AUC values are 0.944 and 0.921, indicative of high accuracy [132].

In summary, glycans play an essential role in the functioning and regulation of living organisms. Under pathological conditions, in addition to changes at the proteomic, metabolic, and genetic levels, glycan structures are also modified. This finding has been validated in the case of gynaecological diseases by many studies, providing an opportunity to develop new less-invasive diagnostic methods based on the measurement of glycosylation changes.

Glycosylation and endometriosis

There is little published research on EMS and glycosylation, and most studies are conducted with a tissue or peritoneal liquid samples, collected by invasive means. As outlined above, Galectin-1 is an endogenous lectin expressed in human stromal and endothelial cells of EMS lesions and contributes to vascularization and growth of EMS lesions independently of other vascularization factors. Using a mouse EMS model, inhibition of Galectin-1 by monoclonal antibody decreased the size and vascularization area of EMS lesions and identified this lectin as a potential target of therapy [133] (Table 3). A study found that peritoneal dendritic cells of EMS tissue expressed significantly higher levels of mannose receptors than the healthy control tissue, which contribute to phagocytosis of dead EMS cell and participate in EMS lesion formation [134] (Table 3). The elevated mannosylation level of the patients' sample also could be a possible target of a diagnostic test. Human endometriotic cyst stromal cells and normal endometrial stromal cells were analysed by *Wisteria floribunda* (WFA) agglutinin binding lectin microarray. The WFA-binding *N*-glycan level was decreased in human endometriotic cyst stromal cells and *N*-

acetyl-galactose-aminy transferase expression was repressed in these cells [135] (Table 3). Transforming growth factor- β 1 cytokine increased the adhesion of endometrial cell to mesothelium due to the increased α 2-6 sialylation; however, the inhibition of β -galactoside α 2-6 sialyltransferase 1 and 2 decreased the establishment of EMS lesions [136] (Table 3). Another study analysed α 2-6-sialyltransferase expression in eutopic and ectopic endometria of 102 women with EMS and 72 healthy women. Expression of α 2-6-sialyltransferase was repressed in EMS lesions compared to the healthy control samples and the hyposialylated endometriotic cells might contribute to the formation of early EMS lesions [137] (Table 3). An accurate measurement of sialic acid level would be a possible diagnostic tool in the future. Experiments conducted with *Dolichos biflorus* agglutinin showed that EMS tissue glycosylation is different in an EMS patient group compared to controls and that was confirmed by gene expression study. Endometrial tissue shows delayed and abnormal differentiation mainly in Stage IV EMS patients, which may be directly associated with glycosylation and gene expression alterations. Deficiency of endometrial epithelial differentiation and glycosylation may also be related to EMS infertility and inappropriate endometrial receptivity [138–140] (Table 3). Glycodelin-A is expressed by endometrial tissue with different glycoforms and immune localization in women with or without EMS. Glycodelin-A expression significantly increased in luteal phase of EMS tissue and displayed a different *N*-glycan profile. These alterations may contribute to infertility in women with EMS, as blastocyst implantation is associated with the adequate epithelial glycocalyx of endometrial cells [141] (Table 3). Autoantibodies react with Thomsen-Friedenreich antigen-bearing proteins and behave as autoantigens in EMS, highlighting the association between glycosylation and EMS [142] (Table 3). Peritoneal haptoglobin, which is secreted by endometriotic tissues, is an analogue of hepatic haptoglobin and its glycosylation is different between women with or without EMS. The level of sialylation is decreased in peritoneal haptoglobin and this phenomenon can be explained by carbohydrate-deficient glycoprotein syndrome type I. Therefore, this glycoprotein may be a potential target for immunotherapy or a specific biomarker of EMS, as it is a tissue-specific factor and probably has an immune-modulating function similar to that of hepatic haptoglobin [143] (Table 3). There are very few articles regarding blood or urine glycosylation in EMS, although they would be ideal sources for diagnostic markers. Blood collection is minimally invasive and urine collection is a non-invasive and painless procedure. An Iraqi study draws attention to the importance of serum sialylation. Serum sialylation is dramatically changed in EMS patients after zoladex therapy, indicating that changes in serum sialylation may be a new biomarker of EMS [144] (Table 3). A study of plasma *N*-glycan profile revealed a significant decrease of monosialylated and an increase of tri- and tetra-antennary glycans in patients with mild to severe EMS, while a biantennary glycan structure significantly decreased only in deep infiltrating EMS compared to healthy controls. The authors were unable to reveal which changes in serum proteins caused the changes identified at the serum level, but suggested that glycosylation changes related to IgG and transferrin proteins contributed to the global change [145] (Table 3). While glycosylation of urine in EMS has not been studied so far, published work on urinary glycosylation in other gynaecological conditions can serve as a reference point for EMS studies. In a study of endometrial cancer, the urinary level of zinc α -2 glycoprotein and α 1-acid glycoprotein were significantly increased, while the fragment of nebulin and the CD59 levels were much lower in the cancer group compared to the control group. Western blot of *O*-glycan-binding champedac assay was used to investigate the *O*-glycan content of urinary proteins. Urine *O*-glycosylation was significantly different in endometrial cancer patients, compared with healthy controls. Six clusters of *O*-glycoproteins were

Table 3
Summary of *N*- and *O*-glycosylation changes associated with endometriosis.

Sample source/type	Detection technique	Results	Reference
endometriotic lesions and eutopic endometrium/human endometriotic lesions/mice	immunofluorescence and immunohistochemistry of tissue samples	Galectin-1 inhibition with monoclonal antibody therapy decreased the size and vascularization area of EMS lesions in mice	Baston et al. 2014
endometrial stromal cells, monocyte-derived dendritic cells from peritoneal fluid/human	isolation, culture, staining, and cell preparation of the cells; flow cytometry; RT-PCR	rate of mannose receptor-positive myeloid dendritic cells was higher in EMS samples than in the control group; inhibition of mannose receptors reduced phagocytosis of dead endometrial stromal cells	Izumi et al. 2017
endometriotic cyst stromal cells and normal endometrial stromal cells from endometrial tissue/human	protein extraction for lectin microarray; lectin histochemistry; western blot analysis; RT-PCR	<i>Wisteria floribunda</i> agglutinin binding glycans decreased in the stromal components of the ovarian endometriotic cysts; <i>N</i> -acetyl-galactose-aminyl transferases were downregulated	Hirakawa et al. 2014
immortalized endometriotic epithelial cells, endometrial cells from adenocarcinoma, endometrial stromal cells, peritoneal mesothelial cells/human; <i>in vivo</i> endometriosis model/mice	cell adhesion assay; lectin blot analysis; lectin fluorescence-activated cell sorting analysis; western blot analysis; RT-PCR; gene knockdown with siRNA	transforming growth factor- β 1 increased adhesion of endometrial cells to the mesothelium through induction of α 2-6 sialylation	Choi et al. 2018
endometrial and ectopic endometriotic cells from tissue and peritoneal fluid/human	enzyme-linked immunosorbent assay; RT-PCR; lectin immunoblot; transwell migration assay	ST6GALNAC1 expression decreased and ST6GALNAC5 expression increased in EMS; reduced α -2,6 sialylation in the peritoneal fluid and endometriotic cells; higher migration capacities of desialylated eutopic endometrial stromal cells of EMS group	Maignien et al. 2019
endometrial tissue/human	electron microscopy; endometrial morphometry; lectin histochemistry;	lack of <i>Dolichos biflorus</i> agglutinin-binding glycans in severe EMS tissue; unusual ultrastructure of proliferative phase phenotype and delayed maturation	Jones et al. 2009,
endometrial tissue/human	lectin histochemistry; image analysis	<i>Dolichos biflorus</i> agglutinin-binding glycans significantly decreased in severe EMS tissue and moderated diminish of <i>Vicia villosa</i> agglutinin-binding glycans	Miller et al. 2010
endometrial tissue/human	two-dimensional electrophoresis; western blot; immunofluorescence;	both epithelial and stromal cells produce Glycodelin-A with several glycoforms; abnormal expression of Glycodelin-A in EMS patients during the cycle	Focarelli et al. 2018
serum, ectopic and eutopic endometrium/human	hematoxylin/eosin-staining; immunoblot; SDS-PAGE; modification of carbohydrate epitopes on glycoproteins	autoantibody responses in EMS tissue; lack of glycan moiety of antigens result in loss of antibody binding; autoantibodies react with other Thomsen-Friedenreich antigen-bearing proteins like IgA, haemopexin; autoimmune response may play a direct role in EMS	Lang et al. 2001
serum, endometriotic lesions, non-affected serosal peritoneal tissue/human	recombinant gene over-expression; lectin binding assays;	peritoneal and hepatocellular haptoglobin deviate in <i>N</i> -glycan moiety and mainly in sialylation; increased interaction of peritoneal haptoglobin with <i>Maackia amurensis</i> and <i>Lotus tetragonolobus</i> lectin	Piva et al. 2002
serum/human	quantitative sandwich enzyme immunoassay	systemic level of inflammation with different sialylation status in EMS patients	Rasha Z. Jasim et al. 2014
serum/human	HPLC <i>N</i> -glycan characterisation; collection of fasting glucose and epidemiological data	decrease of GP2 (A2B, A1G1, FA2) peak and increase of GP14 (A2BG1, A2G1, M4A1G1, FA2G1, FA2BG1, A1G1S1, FA2G1, M6D1), GP17 (A4F1G4S4, A4G4LacS4) and GP18 (A4F2G4S4) peaks in EMS patients; decrease of GP1 (A2) peak in deep infiltrating EMS samples	Berkes et al. 2013
urine samples/human	two-dimensional electrophoresis with silver staining; on-membrane digestion; MALDI-ToF MS; western blot; CGB lectin affinity separation and LC-MS/MS	level of zinc alpha-2 glycoprotein and alpha 1-acid glycoprotein were significantly increased and the fragment of nebulin and the CD59 levels were decreased in the endometrial cancer group; <i>O</i> -glycosylation of nebulin was barely visible in patients	Mu et al. 2012

identified in the urinary samples of both patient and control groups, but the *O*-glycosylated nebulin spot was well visualized only in control samples while it was barely visible in cancer patient samples [146] (Table 3). In addition, studies have confirmed that GAGs, proteoglycans and free oligosaccharides of urine can serve as potential biomarkers in many diseases [147,148].

As is apparent from the above literature review, the number of studies that examine glycosylation changes during EMS is negligible. Most studies have examined tissue or peritoneal fluid glycosylation (in many cases in an animal model) to better understand the pathomechanism of the disease. Although we see examples of

human serum and urine glycosylation assays, they were conducted with a low number of participants. The results are exciting and promising, but further research is needed on the subject. We have not found an example of an approach to glycans as a possible diagnostic tool; however, glycomics may be a promising source for potential biomarker candidates.

Discussion and future perspective

Efforts to discover possible EMS biomarkers have drawn from the diverse fields of genomics, transcriptomics, proteomics, and

metabolomics. So far, none of the biomarker candidates have proven sensitive enough to compete with laparoscopic diagnosis. The importance of commensal microbes for human health and homeostasis has come to the fore in recent years. The microbiome of patients with EMS is different compared to healthy women, therefore changes in the genital and gut microbial profile may help to further illuminate the pathomechanism of the disease and provide potential biomarker candidates. The high sensitivity of analytical chemistry platforms supports the significant potential of glycomic research. The few glycosylation studies of EMS published to date have primarily used tissue-based approaches. Blood and urine tests for EMS biomarkers have extraordinary clinical diagnostic potential. Most importantly, these samples can be collected minimally or non-invasively and present abundant opportunities for identification of novel glycosylation patterns and biomarkers.

Compliance with ethics requirements

This article does not contain any studies with human or animal subjects.

CRediT authorship contribution statement

Zsuzsanna Kovács: Investigation, Writing - original draft, Visualization, Funding acquisition. **Louise Glover:** Writing - review & editing. **Fiona Reidy:** Writing - review & editing. **John MacSharry:** Writing - review & editing. **Radka Saldova:** Conceptualization, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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