

MicroRNAs: New players in endometriosis

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Abstract

Endometriosis is an estrogen-dependent inflammatory disorder that limits the quality of life of affected women. This pathology affects 10% of reproductive-age women, although the prevalence in those patients experiencing pain, infertility or both is as high as 35%-50%. Endometriosis is characterized by endometrial-like tissue outside the uterus, primarily on the pelvic peritoneum, ovaries and the pouch of Douglas. Despite extensive research endeavours, a unifying theory regarding the exact etiopathogenic mechanism of this high prevalent and incapacitating condition is still lacking, although it has been suggested that epigenetics could be involved. MicroRNAs (miRNAs), one of the epigenetic players, are small non-coding RNAs that can act as post-transcriptional regulators of gene expression, reducing the expression of their target mRNAs either inhibiting its translation or promoting its degradation. MiRNA expression profiles are specific of tissue and cell type. Abnormal miRNA expression has been described in different pathological conditions, such as a myriad of oncological, cardiovascular and inflammatory diseases and gynecological pathologies. In endometriosis, miRNA expression patterns of eutopic endometrium from patients and control women and from different endometriotic lesions have been described. These small non-coding molecules have become attractive candidates as novel biomarkers for an early non-invasive diagnosis of the disease, which could suppose a valuable benefit to the patients in terms of improvement of prognosis and reduction of the ratio of recurrence. In this systematic review we will focus on the role of miRNAs in the pathophysiology of endometriosis.

Key words: MicroRNAs; Endometriosis; Epigenetics;

Angiogenesis; Biomarkers

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Core tip: Endometriosis is an estrogen-dependent inflammatory disorder that limits the quality of life of affected women. Nowadays, a unifying theory regarding its exact etiopathogenic mechanism has not been achieved yet. Our objective is to review the current literature to better understand the role of microRNAs, one of the epigenetic players, in the pathophysiology of endometriosis and their potential as novel diagnostic biomarkers to guide therapeutic interventions in endometriosis.

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INTRODUCTION

Endometriosis is an estrogen-dependent inflammatory disorder that limits the quality of life of affected women^[1-3]. This pathology affects 10% of reproductive-age women, although the prevalence in those patients experiencing pain, infertility or both is as high as 35%-50%^[4]. The prevalence of this condition is estimated around 176 million worldwide, with an average diagnostic delay of 7 years^[5], being the mean age at diagnose 32.5-36.4 years, depending of the study population^[5,6].

Endometriosis is characterized by endometrial-like tissue outside the uterus, primarily on the pelvic peritoneum, ovaries and the pouch of Douglas. These extrauterine lesions are responsible for the main symptoms, pelvic pain and infertility^[1].

Despite extensive research endeavours, a unifying theory regarding the exact etiopathogenic mechanism of this high prevalent and incapacitating condition is still lacking. Several authors have reported a hormonal, immunity and a genetic base for this gynecological disorder. However, a growing body of evidence suggests that epigenetics could also be involved^[7], with an exponential increase of papers published on this issue in recent years.

Epigenetics refers to the study of mechanisms that control gene expression in a potentially heritable way without affecting DNA sequence. MicroRNAs (miRNAs), DNA methylation and modifications of the chromatin structure represent the different types of the known epigenetic modifications, exerting their regulatory effect additively^[8]. In this review we will focus on the role of miRNAs in the pathophysiology of endometriosis.

MiRNAs are small (19-22nt) non-coding RNAs that can act as post-transcriptional regulators of gene expression, reducing the expression of their target mRNAs either inhibiting its translation or promoting its degradation. MiRNAs usually regulate gene expression by binding to the 3' UTR (Untranslated Region) of their target mRNA. Importantly, several miRNAs can target a given mRNA and a single miRNA can target several mRNA, increasing the complexity of the regulatory mechanism mediated by these molecules^[9-13]. In malignancies, miRNAs can act as oncogenes or tumor suppressors, depending on their targets^[14-16]. It is important to highlight that the miRNA expression profiles are specific of tissue and cell type^[9]. To date, more than 1881 miRNA precursors, coding for more than 2500 mature miRNAs have been described in humans^[17].

MiRNAs were first described in 1993 by Lee *et al*^[10] in the worm *Caenorhabditis elegans*. Since then, studies about biogenesis, functions, roles and characterisation of the mechanism of action of miRNAs have grown considerably and nowadays they are considered as excellent biomarkers of some diseases such as coronary artery disease^[18-20], cancer^[21,22], and several gynecological pathologies, including endometriosis^[23,24].

PERITONEAL FACTORS AND ENDOMETRIOSIS

Endometriosis is a multifactorial disease in which endometrial and peritoneal factors such as those related to angiogenesis and proteolysis may be involved^[25-27]. Peritoneal fluid (PF) is a complex suspension containing large amount of macrophages as well as endometrial and red blood cells, small molecules diffused from plasma through the mesothelial wall and other components dependent on ovarian contribution and local secretion such as steroid hormones and growth factors, respectively^[28]. Because ectopic lesions located in the pelvic peritoneum are completely submerged in this fluid, their components have emerged as an important field of study^[28-31].

It is well documented that endometriosis is characterized by an important inflammatory process^[32-34] with and increased production of reactive oxygen species (ROS)^[35-37]. Berkes *et al*^[38] and Santulli *et al*^[39] have identified significantly increased levels of protein oxidative stress markers in the PF from women with deep infiltrating endometriosis when compared with endometriosis-free controls. On the other hand, NETosis describes the mechanisms by which activated neutrophils expel their entire chromatin, serving as catch and kill scaffold against microorganisms, a structure designated as neutrophil extracellular traps (NETs). Furthermore, it is known that ROS are the major activator of NETosis. The involvement of NETosis in endometriosis was studied Berkes *et al*^[38], who observed the presence of NET formation in virtually half of the patients with endometriosis, primarily in the

stage I and II group and rarely in controls, suggesting that NETosis is implicated in the initiation of the disease.

The contribution of immune system disorders to endometriosis has been proposed by several authors^[2,40-42]. In this context, macrophage migration inhibitory factor (MIF) is arousing growing interest. MIF is a major pro-inflammatory factor found elevated in PF from women with endometriosis. Apart from its effect on activating and inhibiting macrophage mobility, it is also considered a critical upstream activator of innate immunity. MIF may be required for ectopic endometrial tissue growth and progression of endometriosis lesions *in vivo*^[43]. Interestingly, miR-451 has been postulated to target MIF^[44]. By using a murine model, Nothnick and coworkers^[45] concluded that disruption of miR-451 expression in endometrial tissue impairs the ability of this tissue to establish ectopically. These authors also found elevated expression levels of miR-451 and diminished of MIF in ectopic endometriotic lesions (mainly peritoneal lesions) when compared with matched eutopic tissue. In addition, *in vitro* luciferase assays corroborated MIF as a target of miR-451 and forced expression of miR-451 reduced MIF and cell survival. Consequently, the aforementioned authors hypothesized that miR-451 over-expresses in ectopic lesions in an attempt to curtail endometriotic lesion/cell survival^[46].

MIRNAS IN ENDOMETRIOSIS

Abnormal miRNA expression has been described in different gynecological pathologies, including malignancies^[47-49], benign conditions as leiomyoma^[50], adenomyosis^[51], and endometriosis^[11,52-54]. Among gynecological tumors, ovarian cancer represents the second most prevalent and the most lethal malignancy in developed countries^[55,56], what could be explained by the difficulty of its diagnosis at early stages and the lack of effective treatments^[49]. Recently reviewed by Davidson *et al*^[57], miRNAs could be an invaluable tool to overcome the above mentioned limitations, regarding their potential role in diagnosis and progression of ovarian carcinoma as well as prediction of response to chemotherapy. For instance, miRNAs of the miR-200 family, the miR-199/14 cluster and the let-7 paralogs have emerged as potential therapeutic targets in ovarian cancer^[49]. In addition, Lee *et al*^[58] found that higher expression of miR-181d, miR-30c, miR-30d, and miR-30e-3p was associated with significantly better disease-free or overall survival in this condition. These both miR-30 and miR-200 families have also been associated with endometrial cancer, the most frequent gynecological malignancy^[56,59]. In a recent work, Kong *et al*^[59] reported miR-30c to be a tumor suppressor *via* the miR-30c-MTA-1 signaling pathway, with a decreased expression of this miRNA in tumor cells.

Regarding endometriosis, miRNA expression patterns of eutopic endometrium from control women and pati-

ents^[53,60] and ectopic lesions from patients have also been described^[11,53,61]. Although endometriosis is a benign condition, it shares common mechanisms with tumors (e.g., tissue invasion, inflammation, reduced apoptosis and aberrant angiogenesis)^[55]. In this context, the relationship between endometriosis and ovarian cancer, specially endometrioid and clear cell ovarian carcinoma, has been long reviewed^[62-66], but recent literature on this issue points that existing data is not enough to establish a doubtless causality^[55].

Among the pioneering studies addressing the miRNA expression patterns in endometrial and endometriotic tissues was the work published by Burney *et al*^[67]. Four endometrial samples from women with endometriosis and three from endometriosis-free women in the early secretory phase of the menstrual cycle were assessed for miRNA expression by means of microarray analysis. After real time quantitative polymerase chain reaction (qRT-PCR) validation, the authors reported a downregulation of four miRNAs (miR-34c-5p, miR-34b*, miR-9 and miR-9*) belonging to two miRNA families (miR-34 and miR-9, respectively) in the eutopic endometrium of women with endometriosis compared to endometrium from control women. Notably, members of the miR-34 family mediate the p53-dependent suppression of proliferation^[68].

Furthermore, Laudanski *et al*^[60] conducted a study enrolling 25 endometriosis-free women and 21 patients with ovarian endometriosis in the proliferative phase in which the expression of 667 human miRNAs was examined. Validation of array results led to the corroboration that miR-483-5p, a regulator of IGF2, and miR-629-3p, involved in inflammation, were down-regulated in the eutopic endometrium of patients in comparison to controls. The authors pointed to the idea that dysregulation of these genes could contribute to the overgrowth of endometrial tissue outside the uterus.

Human endometrium is a unique tissue that undergoes complex molecular, cellular, and functional changes on a cyclic basis under ovarian hormone regulation^[69,70]. These changes are essential for uterine receptivity and can be grouped in three distinct phases: Proliferative, secretory and menstrual^[71]. Thus, some authors hypothesized that miRNA expression could vary across the menstrual cycle^[11,72]. For instance, Kuokkanen *et al*^[72] showed that miRNA expression profiles of human endometrial epithelium were under hormonal regulation and, therefore, varied across the physiological phases of the menstrual cycle. Particularly, miRNAs targeting several cell cycle regulators were over-expressed in the midsecretory phase. Conversely, others have identified no effect on menstrual cycle phase on endometrial miRNA expression^[11,53]. These discrepancies could be explained by the cell-type specificity in the response to sex steroid hormones of the human endometrium^[72] and the different type of cellular populations studied in each study.

Filigheddu *et al*^[61] described a set of miRNAs dif-

ferentially expressed in ovarian endometriomas in comparison to paired eutopic endometrium. By means of microarray technology, 84 significant differently expressed miRNAs were identified. In addition, the use of bioinformatic tools allowed researchers to identify the predicted targets of these dysregulated miRNAs, as well as the molecular networks and the biological function they affected. Interestingly, one of the most significantly up-regulated miRNAs was found to be miR-202-3p. In a recent report^[53], our research group corroborated these results, with a 200-fold over-expression of miR-202-3p in ovarian endometriomas in comparison to paired eutopic endometrium. With regards to miR-202-3p, it has been reported^[73] that this miRNA targets the glioma-associated oncogene homolog 1 (GLI1) transcription factor, a strong positive activator of downstream target genes involved in proliferation, migration, invasion and angiogenesis, such as BCL-2, CD24, metalloproteinase-2 (MMP-2) and MMP-9^[74]. GLI1 also regulates the transcription of vascular endothelial growth factor A (VEGF-A), which has been postulated as the main regulator of angiogenesis^[74-77]. Thus, the over-expression of BCL-2 in the eutopic endometrium of patients with endometriosis^[4,67] could be a consequence of the GLI1 regulation by miR-202-3p.

Using a Next Generation Sequencing approach, Hawkins *et al.*^[78] found 10 miRNAs up-regulated (miR-100, -193a-3p, -193a-5p, -202, -29c, -485-3p, -509-3-5p, -574-3p, -708) and 12 miRNAs down-regulated (miR-10a, -34c-5p, -141, -200b, -200c, -200a, -203, -375, -429, -449b, -504, -873) in ovarian endometriomas in comparison to control endometrium, suggesting that miRNAs could also play a significant role in these ovarian lesions. Interestingly, one of the most dysregulated miRNAs in ovarian endometrioma was miR-29c, in agreement with our own data^[53].

Different miRNA profiles have been described in peritoneal lesions compared to paired eutopic endometrial tissues^[11]. Through miRNA microarray analyses and *in silico* studies, the authors identified 22 differently expressed miRNAs that putatively regulated the expression of 673 differently expressed mRNA targets. Of them, 14 were up-regulated in peritoneal lesions (miR-1, -29c, -99a, -99b, -100, -125b, -125a, -126, -143, -145, -150, -194, -223, -365) and 8 were down-regulated (miR-20a, -34c, -142-3p, -141, -196b, -200a, -200b, -424) compared to paired eutopic endometrial tissue. Interestingly, the mRNAs targets of these miRNAs had been previously related to endometriosis-associated molecular pathways, including cell death, cell proliferation and angiogenesis^[11].

More recently, Saare *et al.*^[79] identified five over-expressed miRNAs (miR-34c, -449a, -200a, -200b, -141) in peritoneal endometriotic lesions in comparison to eutopic endometria using a high-throughput miRNA sequencing approach. This set of miRNAs allowed the discrimination of peritoneal lesions from the healthy surrounding tissue. Finally, they concluded providing

a note for caution when evaluating peritoneal lesions, due that analyses carried out in biopsies also containing healthy surrounding tissues could mask aberrant miRNA expression intrinsic of peritoneal endometriotic tissues.

Although efforts have been made in the identification of the role of miRNAs in the pathogenesis of endometriosis, we are aware that future research will provide new regulatory functions for known miRNAs and that new identified miRNAs will expand our knowledge of this condition. Hence, several authors are focusing on the discovery of new miRNAs associated with human female reproductive tract disorders. For instance, Creighton *et al.*^[80] performed a next generation sequencing of over 100 tissues or cell lines derived from human female reproductive organs in both healthy and pathological states. As a result, 7 confirmed and 51 highly confident predicted novel miRNAs were identified.

Even though the involvement of miRNAs in the pathophysiology of endometriosis requires further investigation, nowadays these small non-coding molecules are considered as putative biomarkers for an early non-invasive diagnosis of the disease, which could suppose a valuable benefit to the patients in terms of improvement of prognosis and reduction of the ratio of recurrence, as recently demonstrated in other miRNA regulated diseases^[81-83].

ANGIOGENESIS-RELATED MIRNAS IN ENDOMETRIOSIS

The involvement of angiogenesis in the physiopathology of endometrisis has been long discussed, as the endometrial tissue migrated to the peritoneum requires a blood supply in order to survive, proliferate, invade the extracellular matrix and establish the endometriotic lesion^[84]. VEGF represents one of the most potent angiogenic factors. Several studies have reported an increase in VEGF-A levels in endometriosis and it has been suggested that VEGF-A plays an important role in the progression of the disease^[84,85]. Regarding angiogenesis inhibitors, alterations of thrombospondin-1 (TSP-1) expression has been reported to be involved in endometriosis, in which vascularisation is mandatory for the survival of migrated tissues^[85].

In previous publications, our research group has found and up-regulation of the expression of angiogenic and proteolytic factors in endometrial tissue from patients with endometriosis^[85,86] and we have suggested that this increase might contribute to the invasive potential of endometrial cells.

The miRNA regulation of angiogenesis has been long reported in several pathologies, including endometriosis^[12,53,54]. The miR-17-92 cluster, also known as oncomir-1, encodes six mature miRNAs (miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a)^[87] and has been reported to play an important role in the tumor neovascularisation^[27]. Two miRNAs encoded in this cluster, miR-17-5p and miR-20a, have been found

to be down-regulated in ovarian endometriotic cysts in comparison to eutopic endometrium^[11,61]. As miR-17-5p targets TSP-1, a decrease of the miR-17-5p levels in ovarian cysts could repress the down-regulation of TSP-1 expression and provide an explanation for the clinically observed low invasion grade of these endometriotic lesions to the surrounding ovarian tissue.

Extracellular matrix remodelling is a crucial process in the regulation of angiogenesis^[88] and plays a critically important role in the establishment of the endometriotic lesion^[89]. mRNA levels of key components of the metallo-proteinase systems have been reportedly observed to be deregulated in eutopic and ectopic endometria of patients with endometriosis^[85,86,89,90]. In a recently published paper^[53], we observed that miR-29c-3p was up-regulated in several endometriosis tissues (ovarian endometrioma, peritoneal lesions and rectovaginal nodule). Provided that miR-29c-3p regulates different genes of the extracellular matrix, our results are in agreement with previously published studies^[11,78] accounting for the coordinated role of several miRNAs in the remodelling process necessary for the implantation of migrated endometria in ectopic locations and the establishment of endometriotic lesions.

Taking into consideration the importance of angiogenesis in the pathophysiology of endometriosis, several therapies targeting VEGF as blockers or inhibitors have been proposed aiming to decrease the number of lesions, inhibit growth and reduce vascular density. In this context, soluble truncated VEGF receptors (Flt-1), antibodies to human VEGF and bevacizumab^[91], among others, have been tested in murine models of endometriosis. Although results from these studies are promising, it should be taken into account that the use of an animal model that neither menstruate nor develop spontaneous endometriosis is a major limitation. Furthermore, the use of molecules that might block the expression or mimic functions of angiogenesis-related miRNAs could represent new therapeutic approaches in the treatment of endometriosis as recently demonstrated in other miRNA-regulated diseases^[92].

CLINICAL UTILITY OF MIRNAS AS BIOMARKERS OF ENDOMETRIOSIS

Despite the fact that endometriosis is one of the most common benign gynecological diseases, there is a lack of non-invasive or semi-invasive diagnostic test that overcomes the need for the current surgical diagnosis^[53,67]. Laparoscopy with histological confirmation, the gold standard for diagnosis, is a minimally invasive procedure. However, patients usually undergo general anesthesia and a certain degree of expertise from clinicians is necessary and it is a costly procedure. Additional concerns are related to the delay in the diagnosis of the disease, which has been estimated of around 7 years^[32,93]. This may be due to multiple reasons including non-specific symptoms of

the disease (pelvic pain and infertility), which leads to multiple tests for differential diagnosis^[45]. As a consequence, patients are diagnosed at advanced stages of the disease, which impairs the prognosis and increases the risk of recurrence. For all these reasons, there is a great interest among researchers to find a non-invasive or semi-invasive test for the diagnosis of endometriosis that would ideally diagnose patients in initial steps of the disease and overcome the need for an invasive procedure.

Since they were first described to be present in blood^[82], circulating miRNAs have become interesting biomarkers in different conditions^[16,23,24,94,95]. The presence of miRNAs in different biofluids, including blood^[96], could be explained by different mechanisms: (1) passive release of miRNAs from broken cells and tissues following tissue injury, chronic inflammation, cell apoptosis or necrosis, or from cells with a short half-life, such as platelets; (2) active secretion *via* cell-derived microvesicles (including exosomes and shedding vesicles); and (3) active secretion by cells as RNA-binding-protein conjugated complexes. Mechanisms (2) and (3) also offer an explanation for their highly elevated stability in plasma, despite the presence of elevated amounts of RNases^[97]. Although so far the biological functions of circulating miRNAs remain to be completely defined, some authors have proposed a role into cell-to-cell communication for these short nucleic acids^[97-100]. In any case, it is clear that their presence in plasma/serum and the distinct advantages that they offer over other biomarkers (for instance and unlike mRNAs, miRNAs show high stability in blood, can be both amplified and detected with high sensitivity and specificity^[101] and are highly resistant to storage handling^[97,101]) offers an opportunity to use them as biomarkers.

In the field of gynecological pathologies, several authors have explored this possibility^[94,102,103]. In ovarian cancer, miRNA expression profiles have been analyzed in whole blood and sera from patients, either as free-circulating miRNAs or encapsulated in exosomes. An example of the last is the study conducted by Taylor *et al*^[95] in serum exosomes from patients with serous papillary adenocarcinoma of the ovary. Eight miRNAs (miR-21, -141, -200a, -200b, -200c, -203, -205, -214) were found to be up-regulated in tumor-derived exosomes compared with serum from benign ovarian disease patients. Interestingly, these 8 miRNAs showed a high correlation between their cellular and exosomal levels. In another study, published by Resnick and coworkers^[104], 8 miRNAs were found to be deregulated (miR-21, miR-29a, miR-92, miR-93 and miR-126 up-regulated and miR-99b, miR-127 and miR-155 down-regulated) in serum obtained from 19 patients with epithelial ovarian carcinoma (serous, clear cell, endometrioid and mucinous) in comparison to miRNAs analysed in sera from 11 controls. Interestingly, three out of the five up-regulated miRNAs (miR-21, miR-92

Table 1 Current studies assessing the clinical utility of circulating miRNAs as biomarkers of endometriosis

Sample	Anticoagulant	Main results	Participants (patients/controls)	Ref.
Serum	-	↓ let-7b and miR-135 ^a let-7d and let-7f showed a tendency towards down-regulation ^a	<i>n</i> = 24/ <i>n</i> = 24 ^b	[102]
Plasma	EDTA	↓ miR-17-5p, miR-20a and miR-22	<i>n</i> = 23/ <i>n</i> = 23 ^c	[103]
Serum	-	↑ miR-122 and miR-199a ↓ miR-9*, miR-141*, miR-145* and miR-542-3p ^a	<i>n</i> = 60/ <i>n</i> = 25 ^d	[106]
Plasma	EDTA	↓ miR-200a-3p, miR-200b-3p and miR-141-3p ^a	<i>n</i> = 61/ <i>n</i> = 65 ^e	[110]

Down-regulated (↓) and (↑) up-regulated miRNAs in samples from patients in comparison to control women. ^aCombination of miRNAs in bold yielded the best diagnostic value; ^bControl group presented dermoid cysts (*n* = 10), serous cystadenoma (*n* = 5), mucinous cystadenoma (*n* = 3), simple ovarian cysts (*n* = 3) and paratubal cysts (*n* = 1); ^cControl group presented uterine leiomyoma (*n* = 14), mature teratoma (*n* = 4), simple cysts (*n* = 3) and unexplained infertility (*n* = 2); ^dMain diagnosis: Infertility due to tubal factors; ^eThirty-five endometriosis-free women with primary (*n* = 10) or secondary (*n* = 15) infertility, suspicion of endometriosis (*n* = 5), polycystic ovaries (*n* = 3) and pelvic pain (*n* = 2) and 30 self-reported healthy women. EDTA: Ethylenediaminetetraacetic acid.

and miR-93) were overexpressed in 3 patients with normal CA-125 levels. This finding could be explained by the high sensitivity and accuracy of the RT-PCR quantification, suggesting that miRNAs could provide an advantage as biomarkers in terms of sensitivity in comparison to those in current clinical use.

Häusler *et al.*^[105] analysed the miRNA expression profile in whole blood from 24 patients with epithelial carcinoma (mainly serous histotype) and from 15 healthy donors. As a result, the expression of miR-30c1* was found to be up-regulated and the expression of miR-181a*, miR-342-3p and miR-450b-5p down-regulated in patients in comparison to controls, enabling a discrimination between populations.

Regarding endometriosis, an interesting recent review from Fassbender *et al.*^[23] pointed to the possibility of developing a semi-invasive test for endometriosis from PF obtained *via* transvaginal ultrasound-guided aspiration. Although this is an interesting approach, current research is mainly focused on developing serum/plasma biomarkers as a noninvasive diagnostic tool. Jia *et al.*^[103] explored this possibility, conduct a study that enrolled 23 women with histologically proven endometriosis and 23 endometriosis-free controls. RNA from plasma was extracted to perform a miRNA microarray profiling. Three out of the six miRNAs selected for qRT-PCR (miR-17-5p, miR-20a and miR-22) were proven to be significantly down-regulated in patients and useful to discriminate women with endometriosis from patients. Wang *et al.*^[106] performed a circulating miRNA profiling with a different approach. For miRNA profiling, 2 pools of sera from 10 endometriosis patients and 10 control women, respectively, were prepared. Results from array were validated by qRT-PCR in sera from 60 patients and 25 control women, finding that miR-199a and miR-122 levels were up-regulated and miR-145*, miR-141*, miR-542-3p and miR-9* down-regulated in samples from patients in comparison to control women and could therefore serve as biomarkers of the disease. In a very recent study, Cho *et al.*^[102] quantified the levels of miR-135a,b and let-7a-f in sera of 24 endometriosis patients and 24 disease-free women. The selection of these miRNAs was based on their previous association with endometriosis^[107,108]. Employing a logistic regression

approach, researchers found that a combination of let-7b, let-7d and let-7f during the proliferative phase yielded the highest area under the curve value in discriminating patients with endometriosis from control women. Of note, several miRNAs were found to be differently expressed depending on the phase of the menstrual cycle in patients but not in controls, in agreement with previous reports^[109]. Finally, Rekker *et al.*^[110] performed the last published study regarding circulating miRNAs as biomarkers of endometriosis. Based on previous literature, authors selected 3 miRNAs from the miR-200 family (miR-200a-3p, miR-200b-3p and miR-141-3p) whose expression was assessed in plasma samples from 61 patients and 65 control women. The expression of all 3 miRNAs was down-regulated in patients and miR-200a-3p and miR-141-3p showed the highest potential as noninvasive biomarkers for this benign condition. Remarkably, authors also analyzed variations of the levels of the three miRNAs of interest with time of sampling (morning/evening) finding lower levels in evening samples, perhaps due to circadian fluctuations in their expression. This is an interesting approach and points to the time of sampling as an important factor to be taken into account when performing circulating miRNAs studies. All these studies on the role of circulating miRNAs as biomarkers of endometriosis are summarized in Table 1.

Importantly, it should be noted that the circulating miRNA pool is not a mirror of tissue miRNAs content^[83,111] and that changes in tissue miRNA will not be reflected in the same extent in the circulating miRNA profile^[101]. Therefore, the aforementioned differences in endometrial miRNA expression profiles found in endometriosis should be considered in the context of a semi-invasive diagnosis of endometriosis by means of endometrial biopsy, because of the low probability of finding such differences in serum or plasma from the same patients.

CONCLUSION

MiRNAs, one of the epigenetic players, are small non-coding RNAs that can act as post-transcriptional regulators of gene expression reducing the expression of their target mRNA. The involvement of miRNAs in

different pathological conditions has been well established and miRNA expression profiles have been performed in biopsies from different conditions, including gynaecological pathologies as endometriosis. Despite being a benign gynaecological pathology, endometriosis deeply impairs the quality of life of affected women in terms of pain and infertility. The prevalence of endometriosis in reproductive-age women is estimated around 1 out of 10 and raises to 5 out of 10 in patients experiencing both pain and infertility. Research endeavours are being conducted in order to find a non-invasive or semi-invasive biomarker of the disease that ideally diagnosis the disease at initial stages and overcomes the need for the current laparoscopy gold standard diagnosis. In this area, circulating miRNAs have emerged as attractive molecules to be considered as biomarkers. Up to date, only few studies have been performed in order to obtain a circulating miRNA-based diagnostic tool. However, differences in experimental design among them make it difficult to compare results. From our point of view, there is a need for standardization of clinical data annotation, sample collection and handling among research projects that takes into account several aspects: (1) surgical and non-surgical data; (2) type of sample (serum/plasma) and processing protocols. In the case of plasma, the choice of anticoagulant is not a minor feature in experimental design and must be carefully addressed; (3) time of sampling is also an important factor and a decision has to be made between morning fasting samples or evening samples, as demonstrated by Rekker *et al.*^[110]; and (4) number of participants in circulating miRNAs as biomarkers of endometriosis studies is scarce and usually control population is heterogeneous, including self-reported endometriosis-free women, patients with different benign gynaecological conditions and infertile women due to tubal factors. For all these reasons, we encourage researchers in the field to follow recommendations from the World Endometriosis Research Foundation^[112-115] in order to solve the observed heterogeneity in experimental designs and improve reproducibility between studies. In addition, validation of experimental algorithms in different cohorts is needed so as to improve quality of research and reach the ultimate goal, benefit patients with an earlier diagnose of endometriosis and avoiding unnecessary assisted reproductive techniques in those women whose fertility is not affected by the disease. To achieve this ambitious objective, we do encourage researchers to collaborate and synergistically add efforts to be able to recruit larger cohorts of patients and endometriosis-free women for circulating miRNAs studies, adopt standardized protocols and improve research outcomes.

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