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Review Article

Novel methods in diagnosis of endometriosis in future

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ABSTRACT

Endometriosis causes severe pain and infertility affecting quality of life. According to ASRM it is a chronic inflammatory disease that requires life-long management plan and surgery has to be restricted only once in the life time of the patient. Earlier, the diagnosis of endometriosis was confirmed by surgical method and histo-pathological examination. There is often a diagnostic delay up to 7 years or even beyond, which will affect the patients getting earlier treatment. Recently, lot of non-invasive techniques for diagnosis of endometriosis have come into vogue so that, treatment can be planned without surgical diagnosis. Apart from imaging through USG, CT or MRI, earlier lesions can be picked up by biomarkers like IL-6, IL-8, CA 125, HE4, neutrophil-lymphocytic ratio, Hs-CRP, Tumour necrosis alpha and mi RNA, neural elements in endometrium, glyco-proteins like CA-125, CA-19.9, CA-15.3, CA-73, AFP and CEA. Urocortin, activin and follistatin are growth factors and VEGF, TNF-alpha, NK cells, i-SCAM-I, MCP-1 are immunologic markers for diagnosis of endometriosis. Circulating endometrial cells are also present in the peripheral blood of patients. miRNA in endometriosis is found to be a potential biomarker in the recent years and also associated with altered vaginal microbiome. There has been up-regulation and down-regulation of certain miRNAs in endometriosis patients. In patients with symptoms of endometriosis, miRNA study in peripheral blood can be used as a biomarker for confirmation of diagnosis. There is a strong association between mitochondrial DNA variation and endometriosis which is found to be rational biomarkers.

Keywords: Non-invasive diagnosis, Imaging USG, MRI, Glycoproteins, Mitochondrial DNA, miRNA

INTRODUCTION

Endometriosis is defined as the presence of endometrial glands and stroma implanted other than the endometrial cavity. Implants grow and invade the tissue in their vicinity, causing inflammatory reactions according to the statement given by Davila et al. Endometriosis should be viewed as a chronic inflammatory disease which is estrogen dependent and progesterone resistant that requires a life-long management plan with the goal of maximizing the use of medical treatment and avoiding repeated surgical procedures according to American Society for Reproductive Medicine (ASRM). Treatment goals are to alleviate pain, promote fertility, prevent recurrence and improve the quality of life. As per recent estimates of about 190 million women suffer from Endometriosis globally (WHO) of these, 50 million are in India. Younger the age of occurrence of symptoms, the severity of the lesion is very high 25-45% of women with infertility, 75% of women with chronic pelvic pain and 40-

70% of women with severe dysmenorrhea suffer from endometriosis.[1] Endometriosis commonly affects the genital tract, though extra genital sites are also the victims of endometriosis. It affects the peritoneum, ovaries, POD, utero-sacral ligament, bladder, ureters, bowel, appendix and vagina. It is occasionally seen over the lungs, umbilicus, brain, nose etc., only spleen is supposed to be protected from endometriosis as it has very high reticuloendothelial system which will wash away the deposits. The average delay in diagnosis varies according to the country and it may range between 5 years to more than 12 years.[2] Likewise, patients would have visited 8-9 consultants before the diagnosis is made. Natural course of the disease varies, 30% regresses, 40% progresses and 30% remain static. Various phenotypes of endometriosis are superficial peritoneal, ovarian, deep infiltrative and adenomyosis. Diagnosis of endometriosis is based on history, clinical examination imaging by USG, CT and MRI, biomarkers and laparoscopy (Figure 1 and 2).[2] Histopathology is not necessary to diagnose endometriosis. Treatment can be started without histopathology based on clinical and imaging diagnosis. Laparoscopy is the best modality for diagnosis and treatment of endometriosis. Once clinical diagnosis is made, trial of COCs/GnRH analogues/Dienogest can be used.

For a definitive diagnosis of endometriosis, visual inspection of the pelvis at laparoscopy is the gold standard investigation, unless the disease is visible in the vagina or elsewhere (Figure 1 and 2).

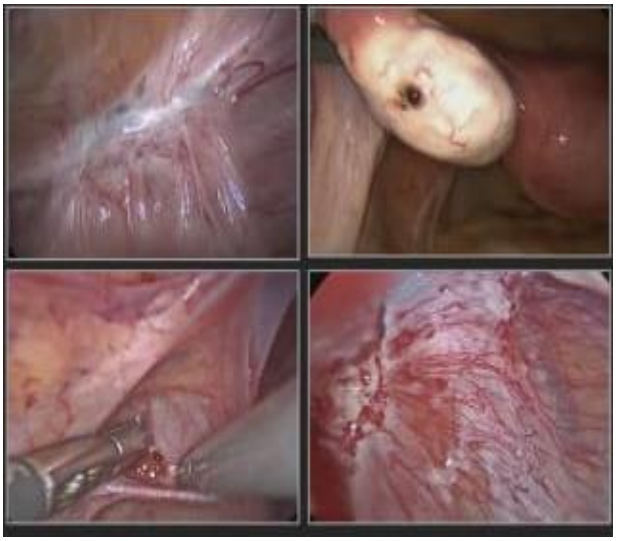

Figure 1: Laparoscopy view of endometriosis.

The symptoms of endometriosis are dysmenorrhea, dyspareunia, dyschezia, AUB, dysuria, diffuse abdominal pain, infertility which ultimately leads to depression and fatigue. Thorough clinical examination should be done including speculum, per vaginal examination preferably during menstruation. According to Professor Charles Chapron et al in 2019 there is no need for laparoscopy for diagnosis of endometriosis and medical management should be the first line of treatment based on the symptoms. Similarly, treatment for endometriosis can be started without histopathological confirmation according to ESHRE, ASRM and SOGC guidelines.[3-7]

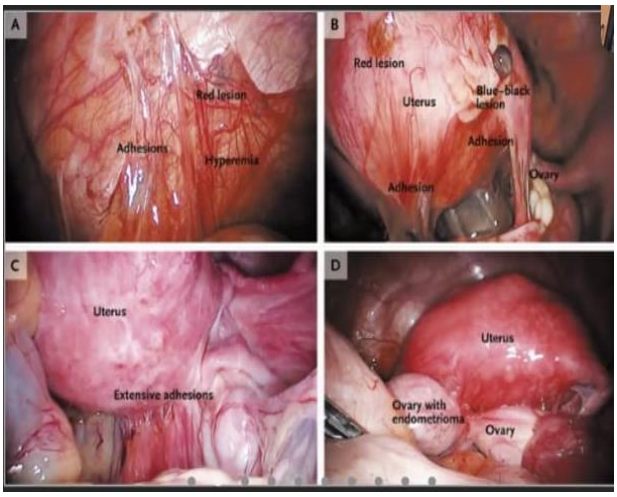

Figure 2: Laparoscopy view of endometriosis.

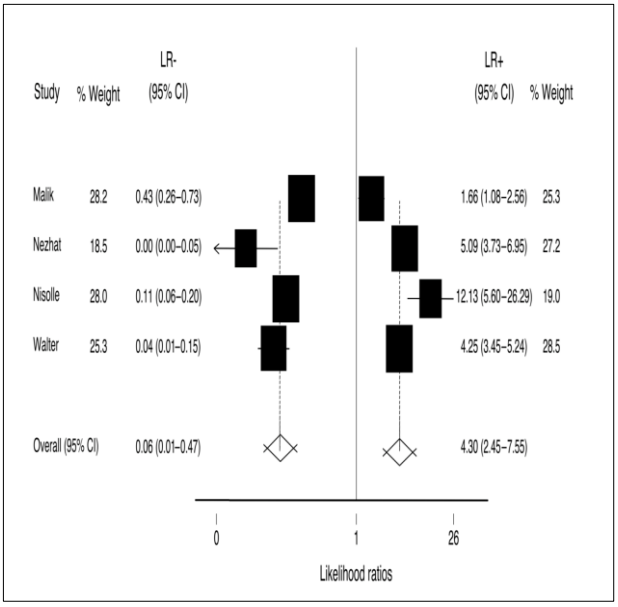

Figure 3: Accuracy of laparoscopy.

IMAGING TECHNIQUES

Professor Iva Brosens, suggested imaging modalities like USG, colour Doppler, CT and MRI can be used for diagnosis and follow up of endometriosis. Associate Professor Luk Rombauts, fertility specialist and research director at Monash IVF says "*The key paradigm shift in the management of women with endometriosis is that we now have specialists who can diagnose endometriosis on ultrasound"*. He says, USG is almost like a pre-operative map of chocolate cyst or endometrioma but also deep endometriosis can be picked up by training. International deep endometriosis analysis (IDEA) group has developed

systematic sonographic approach for evaluation of suspected endometriosis (Table 1). Dynamic ultrasonography is when the operator performing the ultrasound examination assesses both the pelvic organs and their mobility in real-time.[8]

Table 1: USG diagnosis of DIE.

USG diagnosis	
Routine evaluation of uterus and adnexa (+sonographic signs of adenomyosis/ presence or absence of endometrioma)	First step
Evaluation of transvaginal, sonographic 'soft markers (i.e.; site-specific tenderness and ovarian mobility)	Second step
Assessment of status of POD using real-time ultrasound-based 'sliding sign'	Third step
Assessment of DIE nodules in anterior and posterior compartments	Fourth step

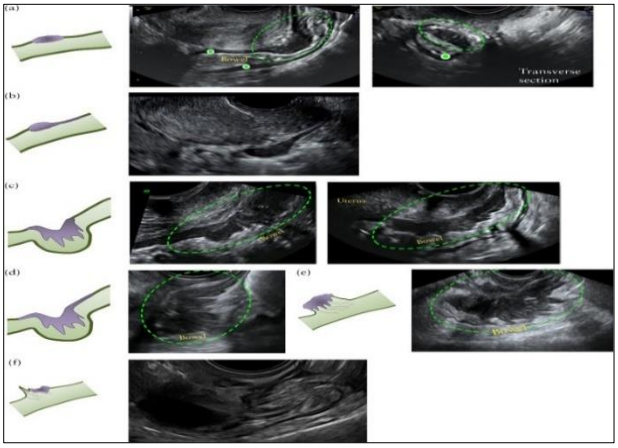

Figure 4: USG showing multiple signs which are indirect signs of DIE

There are 4 steps in diagnosis based on sliding sign and soft markers. Comet' sign, Moose antler's sign, Indian headdress sign and pulling sleeve' sign are various signs to assess deep endometriosis (Figure 5).[8] Sono-POD-Graphy is a newer ultrasound technique were in saline is instilled into the uterine cavity via intra uterine balloon catheter, so that through the trans-tubal spill saline will enter into the POD.[9] Fluid in the POD will create an acoustic window between the ultrasound probe and surrounding structures. The presence of superficial endometriosis can be diagnosed through this acoustic window. Superficial endometriosis was identified by sonography in 24/42 (57.1%) and in 37/42 (88.1%) participants by direct visualization at laparoscopy. The overall diagnostic performance of Sono-POD-Graphy has an accuracy of 69.1%, sensitivity of 64.9%, specificity of 100.0%, and positive predictive value of 100.0% and negative predictive value of 27.8%. Amongst those without deep endometriosis, endometriomas and pouch of Douglas obliteration, the diagnostic performance had an accuracy of 80.0%, sensitivity of 77.7%, and specificity of 100.0% positive predictive value of 100.0% and negative predictive value of 33.3% .10 MRI plays a major role in diagnosis of endometriosis especially whenever there is a doubt in diagnosis. MRI helps in characterization of lesion and can differentiate from dermoid and malignancy. Superficial endometriosis may not be picked up by MRI but indirect evidence can be identified.

Endometriomas, deep infiltrative endometriosis, extra genital endometriosis can be diagnosed by MRI. Recent study by Crestani et al, 2021 has described anogenital distance (AGD) from posterior fourchette to anterior anal verge is found to have a cut off of value of 20 mm. It is shortened in endometriosis patients irrespective of the stage of endometriosis. Since, MRI has sensitivity of only 42% in diagnosing stage I endometriosis- anogenital distance (AGD) measurement will be helpful for these patients.[11]

BIOMARKERS

Biomarkers have a clear impact in reducing diagnosis time and monitoring the progress of the disease and the effectiveness of treatment. To replace invasive diagnostic methods, biomarkers could be considered clinically useful if they comply with predetermined criteria with the sensitivity of 94% and specificity of 79%.[12,13]

Source of the samples are blood, stools, tissue, cervical, vaginal secretion and urine. Combination of markers along with clinical examination, USG, neural elements in endometrium, interleukin IL-6, IL-8, CA 125, HE4, neutrophil-lymphocytic ratio, Hs-CRP, Tumour necrosis alpha and mi RNA are currently available novel non-invasive diagnostic methods. Other biomarkers are glycoproteins, growth factors, peptides, inflammatory cytokines, angiogenic molecules and oxidative stress markers (Figure 5).

Glycoproteins are CA-125, CA-19.9, CA-15.3, CA-73, AFP and CEA. Urocortin, activin and follistatin are growth factors and VEGF, TNF-alpha, IL-6, NK cells, i-SCAM-I, MCP-1 are immunologic markers for endometriosis.

GLYCOPROTEIN CA 125

Cancer antigen 125 (CA-125) is a glycoprotein of ovarian epithelial cells origin, which is a potential marker for endometriosis. It is a well-known tumor marker with the cut off level of 35 U/ml, positive results of CA-125 in the middle of the menstrual cycle indicates very high risk of endometriosis. The sensitivity of CA-125 in endometriosis stage III and IV was 63.1%, compared to only 24.8% in

stage I and II. CA-125 remains the only marker widely used in clinical practice in the diagnosis of endometriosis and it has got a prognostic value which is useful in follow up rather than a diagnostic value.[14]

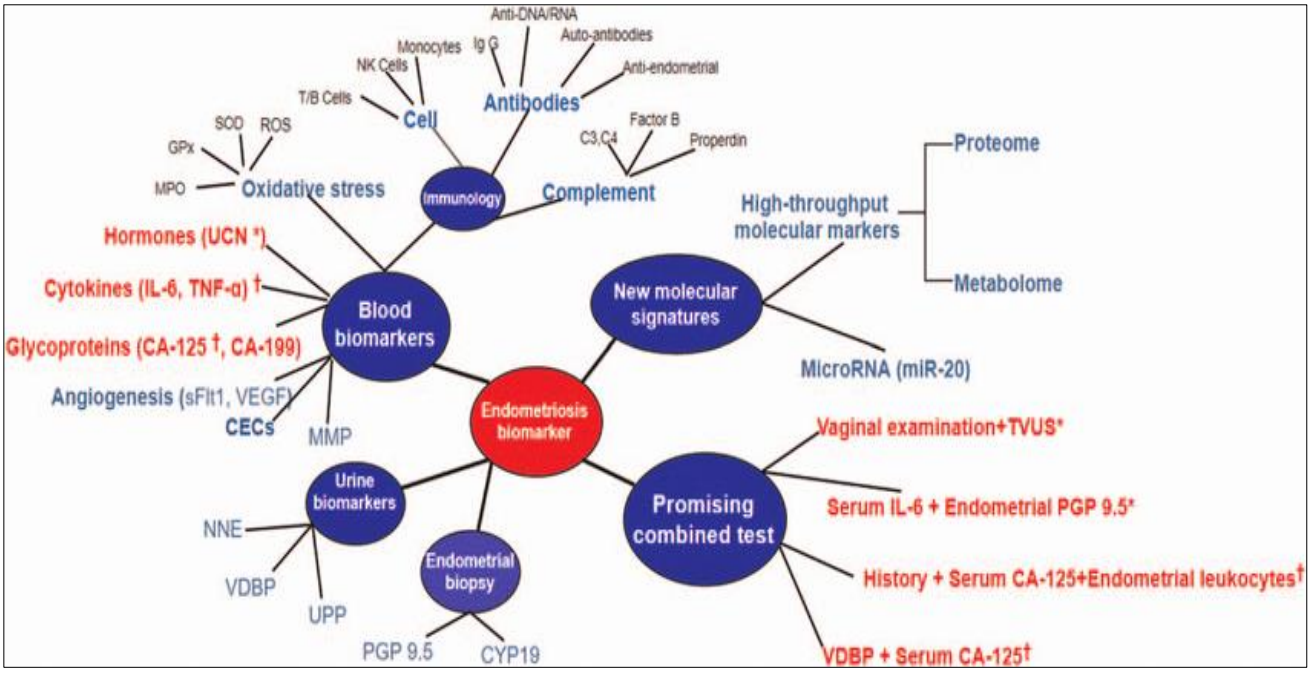

Figure 5: Biomarkers for endometriosis.

CA 19-9

CA 19-9 is a tumor marker used in the diagnosis of pancreatic cancer and gastrointestinal cancers. Endometrium also produces CA19-9, researchers began to look for its application in diagnosing endometriosis. Some study reported that CA 19-9 is not related to endometriosis, while other researches have noted an increased level of these markers in women with advanced stages of endometriosis. Comparing to CA-125 its specificity and sensitivity are respectively 86-89% and 52- 61%. Other glycoproteins that were taken into consideration in the studies were CA 15-3, CA 72-4, α-fetoprotein (AFP) and Carcinoembryonic antigen (CEA), growth factors and peptides-urocortin.[15]

UROCORTIN

Urocortin is a member of CRH family and is produced by eutopic and ectopic endometrium. Evaluating plasma urocortin levels can detect asymptomatic endometriosis with high sensitivity as stated by Maria et al., 2019. Plasma urocortin >46 pg/ml is associated with endometriosis. Highest levels of urocortin were seen in women with endometriosis, who had both infertility and chronic pelvic pain. Increased levels of urocortin can be found in endometriosis patients with 88% sensitivity and 90% specificity, better than CA-125. There are controversies regarding the use of urocortin as a marker for symptomatic endometriosis.

ACTIVIN A

Activin A is a growth factor belonging to the transforming growth factor β (TGF-β) family. Normally, healthy endometrium produces activin A and during the secretory phase of endometrium peak value is achieved. It is believed to play a role in the immunological processes of cells involved in the pathogenesis of endometriosis.

There has been an elevation of activin A in both eutopic and ectopic endometrium. The highest increase was observed in ovarian endometrioma (OMA) in comparison with other types of endometriosis, but its increase in level was insufficient compared to controls to be used as a marker.[16]

FOLLISTATIN

Follistatin is an extracellular glycoprotein secreted at a constant level throughout the menstrual cycle. Endometrioma and superficial endometriosis are found to have higher levels of Follistatin compared to Deep infiltrating endometriosis (DIE) and healthy controls. The combination of activin A and follistatin as markers of endometriosis showed the highest effectiveness.[17]

OXIDATIVE STRESS

Imbalance between oxidants and anti- oxidants is called oxidative stress. The formation of ROS is a physiological process regulated by antioxidant defense mechanisms. It has a significant role in inflammatory response of endometriosis and sub fertility. There is possible use of ROS markers as diagnostic tests for endometriosis.[18]

MACROPHAGES

In response to (TNF-α) and interleukin 6 *(*IL-6*)* the macrophages start producing VEGF which leads to angiogenesis in women with endometriosis. Studies on macrophage migration inhibitory factor (MIF) have shown that it is a cytokine with strong immuno-regulatory potential, affecting angiogenesis and tissue remodeling. It has been observed to significantly increase in endometrial lesions, especially in advanced stages of the disease.[19]

NATURAL KILLER (NK) CELLS

Natural Killer (NK) cells are responsible for removal of endometrial cells that are deposited in the peritoneal cavity due to trans tubal spill. Studies have shown that NK cell activity is diminished in patients with endometriosis, as result of which there is failure of clearance of regurgitated endometrial cells.

This suggests that NK cell dysfunction allows implantation of endometrial cells and leads to endometriosis. IL-12 may the inhibit process of development of endometriosis by activation of NK cells.[20,21]

SOLUBLE INTERCELLULAR-ADHESION MOLECULE-1

Soluble intercellular-adhesion molecules are important with relevance to implantation and formation of endometrial lesions in the peritoneal cavity. The levels of sICAM -1 are found to be higher in women with infertility and endometriosis than healthy control according to the study by Matalliotakis et al.[22] Integrins as cell-adhesion molecules, are involved in the regulation of pathophysiological processes such as cell adhesion, proliferation and migration. Depolarized integrin α6 was examined as a possible marker of endometriosis, with the sensitivity of 67% and the specificity of 84%. An immune-histochemical (IHC) method to study the role of depolarized integrin α6 was used but the results could not meet the criteria of either replacement or using it as a triage test.

INTEGRIN AS CELL ADHESION MOLECULE

The expression of integrin β1 is seen in ectopic endometrial tissues, which are involved in the occurrence of endometriosis. Integrin β1 expressions are seen in glandular and stromal epithelium with a sensitivity of 18% and specificity 87% in the glandular epithelium and sensitivity of 76% and specificity of 0% in the stromal epithelium. Researchers attempted to determine the expression of α3β1 and α4β1 integrins in endometrial biopsy samples from women with endometriosis. In glandular epithelium, α3β1 integrin demonstrated a sensitivity of 100% and a specificity of 27%, and in stromal epithelium, it had a sensitivity of 53% and a specificity of 27%. In turn, α4β1 integrin in glandular epithelium had a sensitivity of 65% and specificity of 40%, and in stromal epithelium it demonstrated a sensitivity of 59% and a specificity of 20%.[23,24]

IMMUNOLOGICAL MARKERS

Immune system dysfunction is one of the main reasons for development of endometriosis. Hence, various studies are conducted in different population to identify whether these immune cells can be used as a non-invasive diagnostic module for endometriosis. Macrophages which are present in the significant amount in the peritoneal fluid are the reason of endometrial cell adhesion, implantation and growth. Macrophages are supposed to secrete numerous pro-inflammatory substances which again influence the development of endometriosis.

INTERLEUKIN 6 AND TNF-A

The past few decades IL-6 has been studied extensively with a sensitivity of 0.70 (95% CI 0.57-0.80) and a high specificity of 1.00 (95% CI 0.88-1.00) with a cutoff value of >12.20 pg/ml.Tumor necrosis factor alpha with a cutoff >12.45 pg/ml, found to be inconsistent with diagnosis of endometriosis. Therefore, it is better that future researchers focus on the diagnostic efficacy of IL-6 combined with other cytokines instead of IL-6 alone.[25]

CIRCULATING ENDOMETRIAL CELLS (CEC)

Although endometriosis is a benign disease, it has many malignant features. Presence of endometrial cells in peripheral blood of patients with endometriosis, referred as circulating endometrial cells. Assay of circulating endometrial cells had 89.5% sensitivity and 87.5% specificity in differentiating endometriosis from benign ovarian masses. We need to investigate many aspects to find out, if CECs can be used as a non-invasive biomarker for diagnosis of endometriosis.[26,27]

NEUTROPHIL/LYMPHOCYTE RATIO

Neutrophil/lymphocyte ratio in the peripheral blood can be used as a marker for diagnosis of endometriosis. They have shown that women with endometriosis may have neutrophilia coexisting with lymphocytopenia. The combined use of neutrophil/lymphocyte ratio and CA-125 concentration demonstrated high sensitivity for endometriosis detection with sensitivity of 69.3% and specificity of 83.9%.[28]

PROTEOMICS-SPECIFIC PLASMA BIOMARKER FOR EARLY DIAGNOSIS

Proteomics-specific plasma biomarker obtained during menses identifies the protein finger prints which are markers of the disease and this can be either up or down regulated. Proteomic technologies along with genetic profiling are newer modalities of non-invasive diagnosis. Saliva based diagnosis of genetic marker may replace surgical diagnosis.

ENDOMETRIAL NERVE FIBRE ASSAY

Sensory C nerve fibers are only detected in the functional layer of endometrium of women with endometriosis and never in women without endometriosis. The density of nerve fibers stained with PGP9.5 in the basal layer of endometrium and in myometrium in women with endometriosis (mean density±SD, 18±8/mm^2), was found to be 14 fold increased than non-endometriotic l group (3.3±1.2/mm^2). The specificity and sensitivity were 83% and 98%, respectively, positive predictive value was 91% and negative predictive value was 96%. Women with endometriosis and pain symptoms had significantly higher nerve fiber density in comparison with women with infertility without pain (2.3 and 0.8 nerve fiber per mm2 respectively p<0.005.[29]

ENDOMETRIOSIS AND VAGINAL MICROBIOME

Endometriosis appears to be associated with an increased presence of *Proteobacteria*, *Enterobacteriaceae*, *Streptococcus suppurata* and *Escherichia coli* across various microbiome sites. The phylum Firmicutes and the genus Gardnerella also appear to have an association; however, this remains unclear. Laboratory and clinical studies demonstrate that there are indeed differences in the microbiome composition of hosts with or without endometriosis.[30]

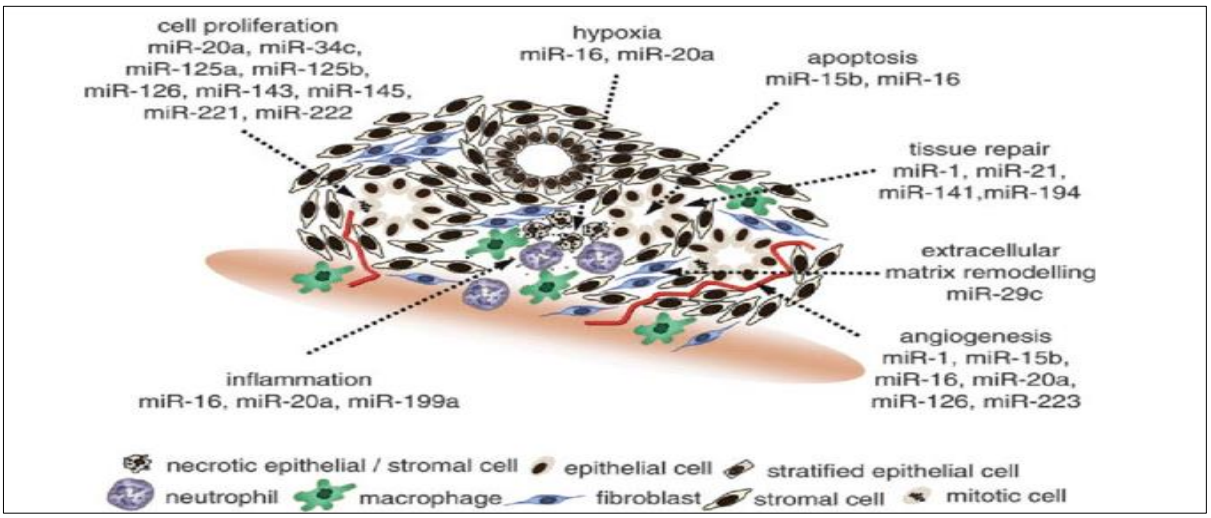

Figure 6: Types of miRNA.

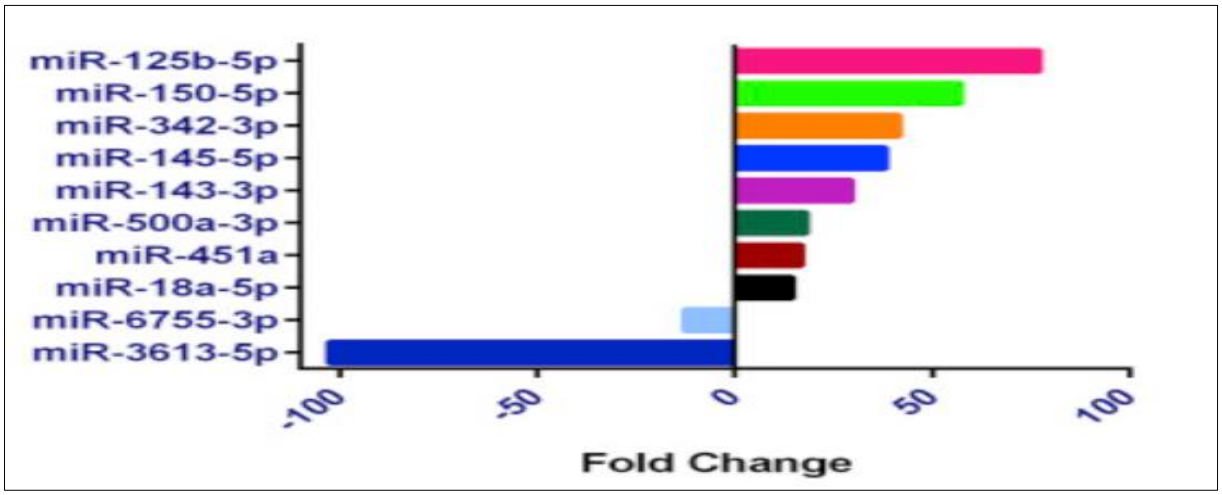

Figure 7: Expression of varying miRNA.

Samples from endometrium, DIE lesions and vaginal fluid were taken. DNA was extracted and the samples were analysed to identify the microbiome by DNA sequencing of the 16S rRNA marker gene which was done using next generation sequencing. Amplicon sequencing showed DIE lesions seems to have different bacterial composition, less predominant of Lactobacillus spp and with more abundant *Alishewanella spp, Enterococcus spp* and *Pseudomonas spp than the control group. There is significant increase in the presence of Acinetobacter spp, Pseudomonas spp, Streptococcus spp,* and *Enhydrobacter spp.*

There is significant decrease in *Propionibacterium spp, Actinomyces spp, and Rothia spp* in the endometriosis group compared to the control group ($p<0.05$). These finding strongly suggest that microbiome composition is altered in the peritoneal environment in women with endometriosis.

miRNA IN ENDOMETRIOSIS AS A POTENTIAL BIO-MARKER

These are the numerous miRNA involved in endometriosis (Figure 6). miRNAs are short nucleotide sequence of non-coding RNA involved in regulatory pathways. miRNA expression profiles are gaining appreciation as diagnostic measures in wide variety of diseases.[31] Many studies have shown differences in up-regulation and down-regulation of miRNAs in endometriosis verses control group (Figure 7). There has been increased validity of panels of candidate miRNAs over a single miRNA. They can be used as a non-invasive bio-marker for diagnosis of endometriosis. Also, they can be used to find out the treatment response to hormones. Moustafa et al has recently shown that a set of 6 miRNAs are able to distinguish endometriosis from other gynaecological diseases, regardless of hormone treatment or phase of menstrual cycle.[32] miRNA panel used are miRNA 125b, miRNA150, miRNA342, miRNA451a, miRNA3613 and Let-7b. They resulted in 90% sensitivity and specificity. Hence patients presenting with symptoms of endometriosis if subjected to miRNA study, diagnosis can be established and treatment can be started earlier. More over various phenotypes of endometriosis are likely to have various miRNA expression.[33]

MITOCHONDRIAL MUTATIONS AS POTENTIAL BIOMARKERS?

There are multiple mitochondria per cell and each mitochondrion contains multiple copies of mitochondrial genome. 16,569 bp containing 37 genes encoding 13 proteins, 22 tRNAs, 2 rRNAs are present. Mutation rate is about 100-fold higher than in nuclear genome. Study by Suresh Govatati et al confirmed the strong association between mitochondrial DNA variations and endometriosis risk.[13] Haplogroup M5 exhibited an increased risk of endometriosis incidence. A recent study provides cumulative evidence that mitochondrial DNA is a rational biomarker for the detection of endometriosis. Further investigation is warranted as to the functional implications of identified mutations in endometriosis.[34]

CONCLUSION

Endometriosis is an enigmatic disease and it takes long time for diagnosis. So far, laparoscopy was found to be the gold standard in diagnosis. Currently imaging studies like USG &MRI are gaining popularity in diagnosis. Early diagnosis by imaging has certain limitations. We need to have novel methods for early diagnosis which can reduce morbidity. Early diagnosis of endometriosis is possible with development of diagnostic bio-markers which is crucial. Cancer Antigen 125 (CA-125), other glycoproteins, growth factors and immune markers, genomics and endometrial nerve fiber assay are important bio-markers as far as non-invasive in diagnosis of endometriosis is concerned. Of late, microbiomes and miRNAs are gaining significance in the diagnosis of endometriosis. However, the search for an ideal bio-marker for endometriosis continues. Lot of researches are going on, to identify the potential bio-markers in diagnosis of endometriosis which will improve and speed up the non-invasive diagnosis of endometriosis so as to reduce the morbidity of the disease. There is urgent unmet clinical need for non-invasive biomarkers in endometriosis. No clinically proven biomarker is available and hence large, collaborative studies are essential to have an ideal biomarker for diagnosis of endometriosis.

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