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Role of miRNA in non-invasive diagnosis of Endometriosis – A pilot study

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Abstract: *Background:* The Background of the study is to find out whether a panel of miRNAs from the serum of patients could be used as a biomarker for non-invasive diagnosis of endometriosis. *Objective:* A panel of miRNA 125b, miRNA150-5p, miRNA 342-3p, miRNA 3613-5p and Let-7b were studied in both control group who are found to be non-endometriotic patients and in the study group proven to be endometriotic patients. The objective is to find out whether these miRNAs could be used as a biomarker for diagnosis of endometriosis. *Methodology:* This study was done at Ramakrishna Medical Centre LLP. Patients undergoing laparoscopy for infertility and pain having endometriosis served as the study group and non-endometriotic patients served as the control group. Being a pilot study, we have included only 25 patients in each group. Based on the previous studies, a panel of 5 miRNAs were analysed from the serum of the women. Fasting sample was collected, serum was separated and cryo- preserved. q-RT-PCR analysis of the miRNA was done as per the protocols by a scientific team and results were analysed. Statistical analysis was done by generation of AUC. *Results:* miRNA 125b, miRNA 150-5p, miRNA 342-3p, miRNA 3613-5p and Let-7b were studied in both control and study group. There was up regulation of miRNA125b and miRNA342-3p and down regulation of Let-7b with the significant p value of <0.001. *Conclusion:* This pilot study shows that the panel of these miRNAs in peripheral circulating serum could be used as a non-invasive diagnostic marker for endometriosis.

Keywords: Endometriosis; Non-invasive diagnosis; Biomarkers; miRNA;

Introduction

Endometriosis is a chronic inflammatory disorder which affects 10% of the women of reproductive age group. It is a condition where there is presence of endometrial tissue outside the uterine cavity. It is commonly seen within the pelvis mainly over the pelvic peritoneum, utero-sacral ligaments, ovaries etc., It can also be seen over the extra genital sites like umbilicus, urogenital tract, gastro intestinal tract, diaphragm, thorax, nose etc., Endometriosis causes mainly severe pain and infertility. Despite a higher prevalence rate, there may be a diagnostic delay of 5 – 10 years [1-2].

Early diagnosis is a challenge, as the presenting symptoms are often non-specific and the condition may be asymptomatic in early stages. By the time the patients develop symptoms, severe lesions would have occurred. The diagnosis of endometriosis is confirmed by laparoscopy which is the gold standard [3]. The direct and indirect cost for laparoscopy could be around $119 billion per annum [4]. Hence there may be further diagnostic delay which will result in further progression of the lesions leading to severe morbidity.

Psychological, social and economic impact of endometriosis upon the patients are profound. Some patients would have consulted many consultants including paediatrician, physician, surgeon, gynaecologist and even psychiatrist. This leads to negative impact upon the quality of life, education, work, social and sexual relationship, ultimately affecting their mental

and emotional well-being [5-7]. Hence, early diagnosis is essential to prevent the complications of chronic pain and infertility. But women are generally reluctant to undergo laparoscopy, which is an expensive invasive procedure that causes unnecessary delay of diagnosis and treatment [8]. Hence, the researchers are now focussing their attention towards the non-invasive methods to diagnose endometriosis at an early stage.

Imaging is a non-invasive modality for diagnosis, but early lesions cannot be picked up. Endometriosis a chronic inflammatory disease that affects the miRNA production, irrespective of the stage of the disease [9-10]. There are heterogenous combinations of non-invasive bio-markers among which serum miRNAs are found to be an important molecular biomarker for diagnosis of endometriosis.

miRNAs are short nucleotide sequence of non-coding RNAs which are involved in the regulatory pathway. They are single stranded RNA molecules of 21-25 nucleotides in length. They act as post transcriptional silencers of gene expression by degradation of their target miRNAs. These miRNAs are protected from the degradation by endogenous RNAase as they are found within the exosomes bound to protein complexes which makes them more stable and hence a better candidate marker [11-12]. Total of 2500 miRNAs have been detected in the human genome [13] and each disease has their unique expression profile [14-15]. Alteration of miRNA occurs according to the disease of multiple organ systems. Each disease has their own specific expression of miRNA. Aberrant expression of miRNAs are seen among endometriosis patients in the serum and plasma. [10, 16-17].

This study aims to understand the presence of specific peripheral circulating miRNA in endometriosis patients which are either up regulated or down regulated. Clinical features like age, BMI, stages of endometriosis and symptoms were also studied and compared with control group. Earlier, the diagnosis of endometriosis was detected by non-specific inflammatory markers apart from imaging and laparoscopy. Recent studies have shown that circulating miRNAs provide a disease specific signature unique to endometriosis to diagnose the disease and start the treatment before the disease progresses and leads to morbidity. It can reduce the interval from diagnosis to start of treatment, hospitalization and health care expenses. Highly sensitive and specific diagnostic test based on miRNA can have great clinical significance in diagnosing endometriosis in women having chronic pelvic pain and unexplained infertility. Further studies are required to find out, if medical or surgical treatment alters the unique marker profiles, especially combination of these five miRNAs.

Material and Methods

The current prospective case control study was carried out at Ramakrishna Medical Centre LLP, Trichy, Tamil Nādu, India catering mainly OBGYN patients. Patients who underwent diagnostic hystero-laparoscopy for infertility or pain, having endometriosis served as the study group (n=25). Control group (n=25) included women who have undergone diagnostic hystero-laparoscopy for chronic pelvic pain or infertility (PCOS, unexplained infertility and tubal disease), where there is no evidence of endometriosis.

Institutional Ethical Clearance approval was obtained from Bharathidasan University, Trichy, Tamil Nadu (IEC Ref.NO.BDU/ IEC/ 2020/03 dated 24.6.2020) dated 12.01.2022 and written consent for participation in this study was obtained from the patients who were posted for diagnostic hystero-laparoscopy at Ramakrishna Medical Centre LLP between July 2021 – November 2021.

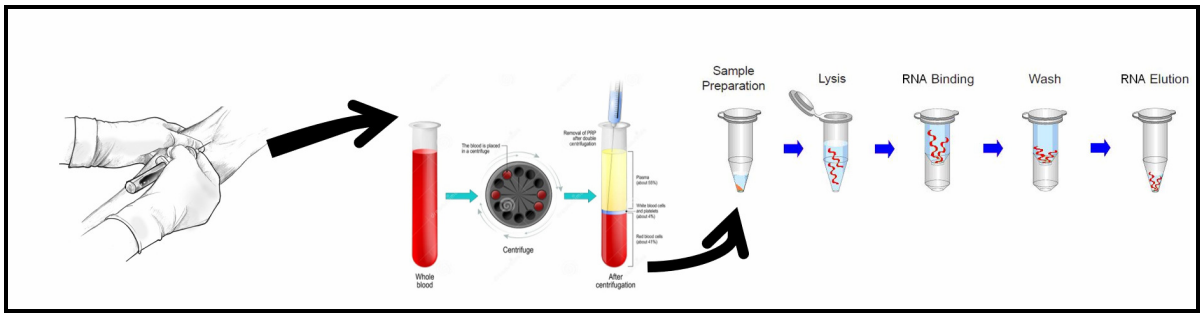
Inclusion criteria: Women who underwent diagnostic hystero-laparoscopy for infertility and pain were found to have endometriosis of any stage were included as study group. Control group included women who did not have endometriosis where diagnostic hystero-laparoscopy was done for chronic pelvic pain, unexplained infertility, tubal disease and PCOS. Age group of women included in both study and control group were ranging from 20 to 45 years.

Exclusion criteria: Post-menopausal women, pregnant women and women having malignancy were excluded from the study.

Revised ASRM classification (2012) was applied to stage endometriosis [18]. Pathology reports were obtained to confirm the disease in advanced stage. Early lesions were picked up by visual examination during laparoscopy. The selection of miRNA to be studied was collected from previously published data by various authors [10, 18]. In a study by Cosar E et al., 2016 [10] 24 patients were included as control group and 24 patients were included in endometriosis group. In a study by Canis M, et al., 1996 [18] 41 endometriosis patients and 59 control patients were included. Six miRNAs were studied: miRNA 125b, 150-5p, 342-3p, 451a, 3613-5p and Let-7b. In this current pilot study, we had taken 25 subjects in control group and 25 subjects in study group. miRNA 125b, miRNA 150-5p, miRNA 342-3p, miRNA 3613-5p and Let-7b were analysed.

Sample collection: Study population were South Indian women from Tamil Nadu, India. Sample blood was collected from the women prior to surgery. Around 10 ml of sample blood was collected from the patients in sterile tubes in fasting status and in the proliferative phase. Serum was collected by centrifuging at 3000 rpm for 30 minutes. The serum was separated and stored in liquid nitrogen at -196°C.

Fig-1: Methodology



Methodology:

Serum samples were collected from women prior to undergoing laparoscopy for suspected benign gynaecological conditions: The samples were classified as, study group if visual or pathology findings from surgery confirmed the presence of endometriosis and control group if surgery revealed other benign conditions except endometriosis.

In this study all the samples were taken only during the proliferative phase irrespective of the prior hormonal treatment. Based on a power of 0.8, an alpha of 5%, an incidence of 50% the effect size observed in previous published studies (100+) samples are needed to assure adequate sample size and this work is being further continued. Being a pilot study, we have included only 25 patients in the control group and 25 in the endometriosis group. In this study, we had taken a combination of miRNA 125-b, miRNA 150-5p, miRNA 342-3p, miRNA 3613-5p and Let-7b from serum of patients with endometriosis of any stage.

Quantitative RT-PCR analysis of miRNA [19]: MiRcury LNA miRNA custom PCR panels containing specific forward and reverse primers for the miRNAs: miR-candidate internal reference miRNA (miRNA -125b, miRNA - 150-5p, miRNA - 342-3p, miRNA - 3613-5p and Let-7b, plus cel-miR-39 (extraction control), and UniSP6 and UniSP3 (template control) were included on the panel. snRNU6 was used as a reference miRNA. Each primer was analysed in duplicate.

cDNA was diluted 10 times with the use of nuclease free water, and a mixture of 5 ml of miRcury LNA SYBR Green, 4 mL diluted cDNA and 1 mL nuclease-free water per reaction was prepared. qPCR was performed with the use of BioRad CFX96, according to the miRcury LNA miRNA Custom PCR Panels handbook and the recommended qPCR program for BioRad CFX96. Calculated mean Cq values and melt curves for each target were obtained from instrument software, and those Cq values were used for further analysis.

We included one negative template control (NTC) sample. Cq values < 35 are considered for data analysis. Also, the NTC was negative (no Cq values) for all primers. Data analysis was performed after calibration of each plate to eliminate all possible qPCR efficiency differences between plates using inter-plate calibrator (IPC) UniSP3 and UniSP6. Cq values for each miRNA was normalized to average Cq values of reference miRNA using instrument software. The 2DCq value was calculated for each miRNA in each sample. Mean expression levels of serum miRNAs between the groups were compared using the Mann-Whitney U test.

Previously reported retrospective studies reported that a panel of serum miRNAs are identified by micro array analysis which is at least 10-fold increased or decreased in expression in the serum of endometriosis patients [10]. The combination of 3 miRNA's (miRNA 125b -5p, miRNA– 451a and miRNA– 3613-5p) yielded an excellent area under the receiver operating characteristic curve (AUC) using logistic regression model and receiver operating characteristic and receiver operating characteristic curve (ROC) analysis.

In this study, we had taken a combination of miRNA 125b, miRNA150-5p, miRNA 342-3p, miRNA 3613-5p and Let-7b from serum of patients with endometriosis of any stage. Pre-operative blood sample was taken from the patients posted for diagnostic hystero-laparoscopy for infertility or pain. After surgery, samples were separated into endometriosis group and control group which included benign gynaecological lesions like fibroids, PCOS, tubal disease or unexplained infertile patients. Staging of endometriosis was done as per rASRM classification. The analyst did not know the group to which the patients belonged. This study gave us an insight regarding the role of miRNA in circulating plasma in differentiating endometriosis from benign gynaecological diseases among the South Indian population and probably the first study as far as we know among this population.

Statistical Analyses, generation of AUC and cut-off values: Each miRNA with a P value <0.05 was considered to be statistically significant. For differentially expressed miRNAs between endometriosis and control groups, receiver operating characteristic (ROC) curves were prepared and the areas under the curve (AUCs) calculated. Areas under the curve (AUCs) of the receiver operating characteristic (ROC) curves for miRNAs and logistic regression were generated using custom R scripts run on Python, identified at the threshold indicated by the highest calculated Youden index. Odds ratio were assigned using Random Forest classifiers run on Python based Machine Learning algorithms. Multiple regression analysis was performed with assigned variables to generate the cut-off values and the related specificity and sensitivity scores.

Results

Of the 50 patients included in this study, 25 patients were in control group. 25 patients were in endometriosis group, including 4 patients who turned up for OPD with symptoms suggestive of endometriosis like severe, progressive dysmenorrhoea and diffuse abdominal pain and they did not have imaging evidence of endometriosis. Hence, miRNA study was done to find out the possibility of the presence of endometriosis in these patients. All these 4 patients had up regulation of miRNA 125b, miRNA 150-5pand miRNA 342-3p and down regulation of miRNA 3613-5p and Let-7b and hence included in the endometriosis group. This could be helpful to diagnose endometriosis by means of non-invasive method. There was no difference in the miRNA expression in various stages of endometriosis. [10]All the patients had the miRNA expression done during the proliferative phase.

Few of these endometriosis patients could have had, hormonal treatment, prior to participation, but this was not recorded in our study. All were patients who reached us for either fertility treatment or pain and laparoscopy was carried out. Patients who had moderate and severe endometriosis were diagnosed by imaging mostly by USG. Patients who had minimal and mild endometriosis did not have imaging evidences, but were picked up only through laparoscopy. Control group patients did not have endometriosis but were having PCOS, fibroids, tubal disease etc.

Table-1: Demographic profile and clinical features		
Variables	**Endometriosis group (n=25)**	**Control group (n=25)**
Age	31.0± 4.35	29.8±5.03
BMI	25.2±4.29	27.1±4.5
rASRM Endometriosis stage		
Minimal & mild endometriosis (Stage I & II)	12/25 (48%)	-
Moderate endometriosis (Stage III)	7/25(28%)	-
Severe endometriosis (Stage IV)	6/25(24%)	-
Menstrual history		
Regular cycles	20/25(80%)	14/25(56%)
Irregular cycles	5/25(20%)	11/25(44%)
Infertility		
Primary infertility	13/25(52%)	13/25(52%)
Secondary infertility	6/25(24%)	3/25(12%)
Symptoms		
Dysmenorrhoea	18/25 (72%)	14/25(56%)
Dyspareunia	12/25(48%)	4/25(16%)
Dyschezia	11/25(44%)	1/25(4%)
Dysuria	6/25(24%)	1/25(4%)
A(D)UB	12/25(48%)	6/25(24%)
Diffuse abdominal pain	11/25(44%)	5/25(20%)
Difficulty in conception	19/25(76%)	16/25(64%)
Associated findings		
PCOS	4/25(16%)	11/25(44%)
Fibroids	1/25(4%)	8/25(32%)
Tubal diseases	1/25(4%)	1/25(4%)
Mullerian anomalies	2/25(8%)	1/25(4%)
Medical complications		
Diabetes mellitus	2/25(8%)	-
Hypertension	-	2/25(8%)
Thyroid disorder	4/25(16%)	4/25(16%)

The mean age group of endometriosis group was 31.04 ±4.35years and control group was 29.80± 5.03 years. BMI was categorised in both groups. BMI was either normal or over weight in endometriosis group and most of the patients among control group were in overweight or obese category [20-21]. Stages of endometriosis were categorized as rASRM classification [22]. Stage I, II (mild), III (moderate) and IV (severe) whereas control group had normal findings or benign pathology like PCOS, fibroids etc., There was family history among 16% of the subjects with endometriosis [23]. As far as the family history was concerned either the mothers (2/4) or sisters (2/4) who had endometriosis. Regularity of the cycle was more in endometriosis group and irregular cycles in control group probably due to anovulation or PCOS [24].

19/25(76%) cases of diseased group only wanted fertility treatment. Rest of the patients

wanted treatment only for pain. Similarly, 16/25 (64%) cases of control group wanted fertility treatment. Rest of the patients underwent laparoscopy or laparotomy for other reasons. Endometriosis associated pelvic pain were found to be numerically higher in endometriosis group, though dysmenorrhoea was found in both groups. Dyspareunia, dyschezia and dysuria was found to be more common in endometriosis patients.

PCOS was seen more in (11/25) control group and (4/25) in endometriosis group, fibroids were seen, (8/25) in control group and (1/25) in endometriosis group, tubal diseases were found in 1 in each group. Mullerian anomalies were seen in both groups. One subject in control group had bicornuate uterus and two subjects in endometriosis group had septate uterus. Majority of them did not have any medical complications in both groups. Six subjects in each group had diabetes mellitus, hypertension and thyroid dysfunction [25]. The present study, was carried out as a single blinded study. The blood samples were given appropriate labelling's as codes and sent for analysis. In our study all samples were taken only during proliferative phase in fasting status.

Expression of five miRNAs, miRNA 125b, miRNA 150-5p, miRNA 342-3p, miRNA 3613-5p and Let-7b were analysed. According to Canis M, et al., 1996 [18] showed prior hormone treatment did not alter the expression of various miRNAs and hence the history of prior hormone treatment was not taken into consideration in our study.

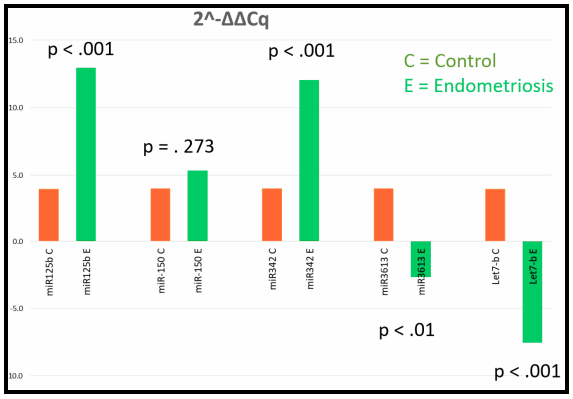
ROC analysis of individual miRNAs:

Table-2: ROC analysis of individual miRNAs

miRNA	AUC (95% CI)	Cut-off value	Sensitivity (95% CI)	Specificity (95% CI)	Odds Ratio (95% CI)
miR-125b	0.92 (0.84-0.96)	5.817[a]	0.84 (0.77-0.90)	0.81 (0.74.0.87)	24.72 (9.63-39.81)
miR-150	0.71 (0.64-0.78)	3.461[a]	0.49 (0.42-0.56)	0.28 (0.21-0.34)	6.15 (3.52-8.78)
miR342	0.81 (0.75-0.86)	4.011[a]	0.78 (0.70-0.86)	0.76 (0.66-0.87)	15.79 (6.77 -24.81)
miR-3613	0.86 (0.81-0.92)	3.674[b]	0.72 (0.68-0.76)	0.58 (0.51-0.64)	11.4 1 (5.53-17.28)
Let-7b	0.87 (0.81-0.92)	4.638[b]	0.82 (0.73-0.89)	0.83 (0.71-0.91)	44.83 (16.41-73.26)

[a]Values below this cut-off indicates the miRNA is significantly increased in Endometriosis samples as compared to healthy controls. [b] Values above this cut-off indicates the miRNA is significantly decreased in Endometriosis samples as compared to healthy controls.

Fig-2: Statistical analysis



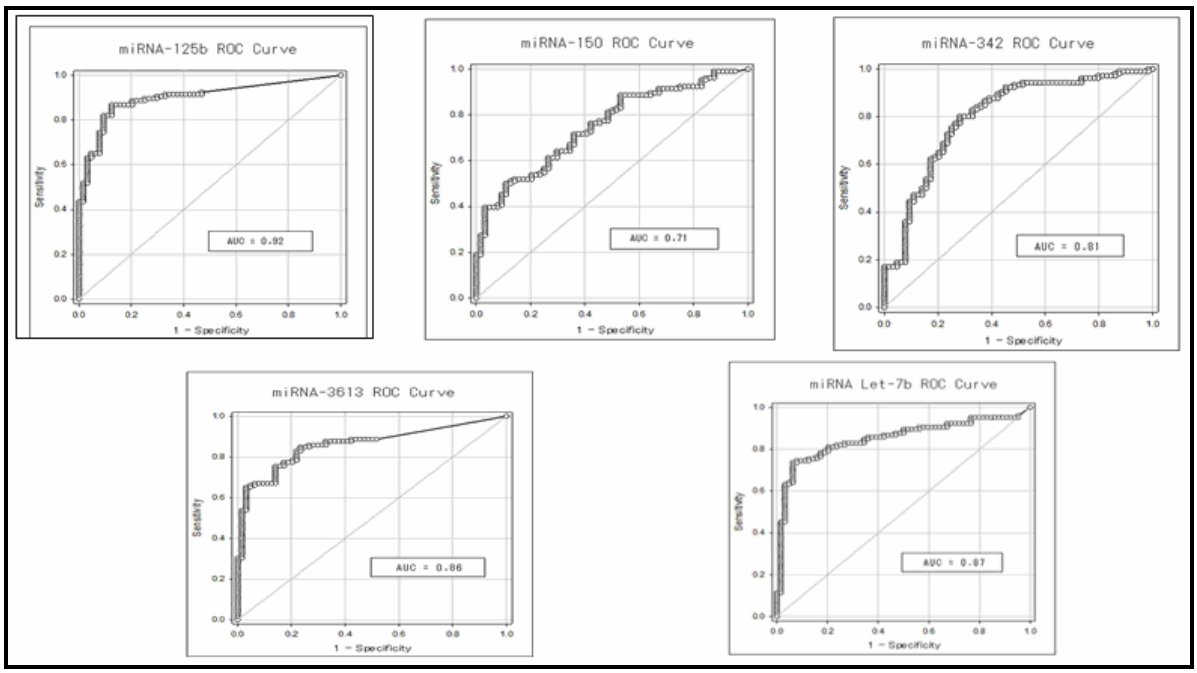
miRNA 125b, miRNA150-5p, miRNA 342-3p, miRNA 3613-5p and Let -7b were studied in both control and endometriosis group. There was up regulation of miRNA 125b and miRNA 342-3p and down regulation of Let -7b with the significant p value of <0.001.But, miRNA150-5p was not altered and miRNA 3613-5p was down regulated with the p value of <0 .01.

ROC analysis demonstrated the significant diagnostic value of combinations of miRNAs. Women belonging to various stages of endometriosis irrespective of previous hormonal treatment were included. In this study single serum miRNA biomarker with the most reproductive diagnostic performance was miRNA 125b.

This miRNA had an AUC of 0.92 in this study and miRNA 342-3p had an AUC of 0.81, whereas miRNA 150- 5p had an AUC of 0.71

in the ROC curve. miRNA 125b and miRNA 342-3p were significantly upregulated. miRNA 3613-5p had AUC of 0.86 in the ROC curve and miRNA Let - 7b had an AUC of 0.87 in the ROC curve. These two miRNAs were significantly down regulated. Among the combinations of 5 miRNAs selected, 4 miRNAs were significantly altered in expression. In a study by Canis M et al., 1996 [18] showed miRNA 125b and miRNA 3613 -5p had an AUC of 0.8 similar to our study. Current model yielded the following results; miRNA 125b has a sensitivity of 0.84% and 81% specificity; miRNA 342-3p has a sensitivity of 78% and 76% specificity. Let - 7b has a sensitivity of 82% and specificity 83.9%. miRNA 125b and Let-7b panel of biomarkers are having a higher sensitivity and specificity which can be used as a screening test. Previous studies have observed that there was no difference in the expression of these miRNAs irrespective of the phase of the cycle. [10].

Fig-3: ROC Curves of miRNA 125-b, miRNA 150-5p, miRNA 342 – 3p, miRNA 3613-5p and Let -7b



Discussion

Endometriosis is a chronic debilitating disease that affects the quality of life. The disease is associated with infertility and chronic pelvic pain. This disease has got a public health concern, as it has an impact on physical, psychological health and socio-economic profiles, which affects the economy of the individual as well as the nation.

More over endometriosis needs life-long treatment plan. Currently the diagnosis of endometriosis is based upon laparoscopy and HPE. Non-invasive diagnosis like imaging modalities is useful only in advanced stages of the disease. In the last three decades, researchers are trying to develop and standardize a non-invasive diagnostic methodology based upon the biomarkers.

Cochrane review published in 2016, [26] Nisenblat et al., showed that the accuracy of biomarkers including CA-125 are not profound as far as the diagnosis of endometriosis. So far glycoprotein, CA-125 has been used an important biomarker in diagnosis of endometriosis. CA- 125 is only elevated in advanced stages of endometriosis. Hence, it can only be used for follow up.

Hence, as an alternate, circulating miRNAs can be used as a candidate marker for non-invasive diagnosis for endometriosis. These miRNAs expressions are found to be variable in several disease including oncologic, inflammatory, cardiovascular, metabolic and reproductive disorders. In 46 studies, miRNAs found to be dysregulated in endometriosis. Among the dysregulated miRNAs in

endometriosis, 30 are in blood, 27 in serum and 18 in plasma. Altered expression of miRNAs are also detected in the peritoneal fluid of patients with endometriosis [27].

Some miRNAs are produced by the endometriotic lesions while others are altered because of the effects of endometriosis upon other tissues. There was no variation in the miRNA expression based on the phase of the cycle and the use of hormonal treatment as reported in the previous studies.

In a study by Sarah Moustafa et al., in 2020 [27], following 6 miRNAs, miRNA 125b, miRNA150-5p, miRNA 342-3p, miRNA 451-a, miRNA 3613-5p and Let-7b were studied. It included 41 patients in the endometriosis group and 59 in the control group. miRNAs, miRNA 125-b, miRNA 150-5p, miRNA 342-3p, miRNA – 451-a were up regulated and miRNA 3613-5p and Let-7b were down regulated. We have included only 5 miRNAs excluding miRNA 451-a. our results were almost similar to the study.

We had significant up regulation of miRNA 125b and miRNA 342-3p and Let-7b was significantly down regulated in our study with the p value of 0.001. miRNA 150-5p was not altered and miRNA 3613-5p was also down regulated only with the p value of 0.01. In our study, miRNA 125b had the highest performance with AUC of 0.92 and Let-7b had an AUC of 0.87 in the ROC curve. In a study by Darya et al., 2021[28] have compared Let-7b with CA-125 biomarkers for its specificity, whereas in our study Let-7b was found to be a promising non-invasive biomarker for diagnosis of endometriosis and it was not been compared with CA-125.

We have taken all the samples during proliferative phase without considering the previous hormonal intake. In contrast to our study, Sarah Moustafa et al., study in 2020, [27] they didn't know about the phase of the menstrual cycle in more than 50%of the patients, but history of hormone intake was noted in all patients which could be the reason for not knowing the phase of the cycle. This variable of expression of miRNA was not affected by prior hormonal treatment and no variation was seen based on the stages of endometriosis. Our study also reflects the same. There may be difficulty in identifying the stage and phenotypes of the disease as this is a pilot study with 50 samples only and future research work may be directed towards this. However early stages of the condition which cannot be picked up by imaging studies, can be identified using this panel of miRNAs.

Strength and limitation of the study: Study was a prospective study allowing direct correlation between the levels of various miRNAs and its association in the presence of endometriosis. This study also includes women with varying diseases as control group which has varying expressions of 5 miRNAs. This will help in diagnosis of concurrent pathology also.

Weakness of the study is the limited sample size. Being a pilot study, we have included only 50 samples. Here association of miRNA with varying subgroups of endometriosis and other features like stage of endometriosis, phenotype of endometriosis and its relation to miRNA expression could not be studied in detail due to small sample size.

Conclusion

miRNA 125b, miRNA 150-5p, miRNA 342-3p, miRNA 3613-5p and Let-7b are promising non-invasive biomarkers for diagnosis of endometriosis. miRNA 125b, miRNA 342-3pwere up regulated and Let -7b and miRNA 3613-5p were down regulated in this study. Based on the assay of these miRNAs and clinical findings, it was possible to diagnose endometriosis at an early stage. In our study, there was no variation in the expression of miRNAs with the stages of endometriosis and phenotypes of endometriosis.

Among patients who had symptoms suggestive of endometriosis, like dysmenorrhoea and dyspareunia, but no imaging evidence of disease, serum miRNA assay can aid in diagnose of endometriosis. Non-invasive diagnosis of endometriosis will help the clinician to start the treatment much earlier without invasive laparoscopy. This can definitely reduce the time to diagnose and initiate appropriate treatment which will optimize the overall results, thus helps to reduce the pain and promote fertility among women with endometriosis.

This early diagnosis could reduce the surgical risk, years of suffering, hospitalization health care expenses, economic losses and finally reduce the morbidity, progression of the disease and associated complications. The large database from samples of endometriosis patients may play a key role as a biomarker investigation in future studies. We can also extend our study towards the response of the disease following hormonal treatment. We need larger prospective studies to evaluate the accuracy of diagnosis, treatment, and outcome of the disease. This study has yielded an excellent diagnostic potential to be useful as a diagnostic biomarker for endometriosis.

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Conflicts of interest: There are no conflicts of interest.

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