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Research Article

Serum miRNA Biomarker for Endometriosis

Comparative Study of Serum miRNA as A Biomarker for Non-Invasive Diagnosis in Suspected Versus Proven Cases of Endometriosis

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Abstract: Endometriosis is a chronic systemic inflammatory disorder characterized by endometrial tissue outside the uterine cavity in females. So far, the invasive laparoscopic method is the only gold standard diagnostic option for endometriosis. The study aims to develop a non-invasive diagnosis of endometriosis in patients presenting with one or more symptoms by analyzing the peripheral circulating miRNAs from the serum of the patients. A panel of miRNA 125b-5p, 342-3p, and Let-7b was developed to diagnose endometriosis. We performed the demographic profiling in 56 patients eliciting one or more symptoms of endometriosis without imaging evidence and compared them with 40 patients with the laparoscopically established endometriotic condition. Patients presenting with one or more of the clinical symptoms of endometriosis (n=56) served as the study group, and patients who were proven to be endometriotic by laparoscopy (n=40) served as the training (control) group. The fasting peripheral blood sample was collected, serum was separated, and cryo-preserved. qRT-PCR analysis of the selected miRNAs (miR 125b-5p, miR 342-3p, and Let-7b) was studied in both training (control) and study groups. The results were analyzed using a random forest (RF) approach machine learning algorithm and 10-fold cross-validation. Results indicate significant upregulation of miRNA125b-5p and miRNA 342-3p and downregulation of Let-7b ($p < 0.001$). Further, the samples derived from the study group with one or more symptoms of endometriosis and the proven endometriotic patients exhibited similar dysregulation of selected miRNAs (miR 125b-5p, miR 342-3p, and Let-7b). Based on our study, we propose the miRNAs panel consisting of miR 125b-5p, miR 342-3p, and Let-7b to be used as an early non-invasive diagnostic marker for endometriosis efficiently without the patients being subjected to invasive laparoscopy, which will reduce the time taken for diagnosis and commence the treatment earlier and prevent morbidity.

Keywords: Endometriosis; Symptoms; Demographic profile; Non-invasive diagnosis; Biomarkers; miRNA; Management

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1. INTRODUCTION

Endometriosis affecting 10% of the women of the reproductive age group, is a chronic systemic inflammatory disorder that is typically characterized by the presence of endometrial tissue outside the uterine cavity that has the potential to spread systemically. It is commonly seen within the pelvis, mainly over the pelvic peritoneum, uterosacral ligaments, ovaries, etc.; it can also be seen over the extragenital sites like umbilicus, urogenital tract, gastrointestinal tract, diaphragm, thorax, nose, etc., Endometriosis causes mainly severe chronic pain and infertility. Clinical manifestations are dysmenorrhoea, dyspareunia, dysuria, dyschezia, diffuse abdominal pain, abnormal uterine bleeding (AUB), and difficulty in conception. Despite a high prevalence rate, there may be a diagnostic delay of 5 – 10 years.[1,2] Early diagnosis is challenging, as the presenting symptoms are often non-specific, and the condition may be asymptomatic in the early stages. By the time the patients develop symptoms, severe lesions would have occurred. Many studies have shown that even general practitioners are unaware of the symptoms of early endometriosis.[3] The diagnosis of endometriosis is confirmed by laparoscopy, the gold standard.[4] The direct and indirect costs for laparoscopy could be around $119 billion annually.[5] This may further cause diagnostic delays due to inadequate funding. This disease has a familial tendency and has seven times higher prevalence among 1st-degree relatives.[6] Despite chronic pain, many women are diagnosed with endometriosis only when they seek fertility treatment. Nearly 25 to 50% of women with endometriosis have infertility. Endometriosis's psychological, social, and economic impacts on patients are profound. Some patients would have seen many consultants, including pediatricians, physicians, surgeons, gynecologists, and even psychiatrists. It negatively impacts the quality of life, education, work, social and sexual relationships, ultimately affecting their mental and emotional well-being.[7-9] Hence, early diagnosis in a non-invasive method is essential to prevent chronic pain and infertility complications. Women are generally reluctant to undergo laparoscopy, an expensive and invasive procedure.[10] Hence, researchers are now focusing on non-invasive methods to diagnose endometriosis at an early stage. The exact pathogenesis of endometriosis is poorly understood. Proposed aetiologies are Sampson's spill theory, coelomic metaplasia theory, altered immunity, lymphovascular invasion, and stem cell theory. Sampson's spill theory (1927) is widely accepted when retro-grade menstrual blood reaches the peritoneal cavity. Peritoneal macrophages and natural killer cells clear it. In women with endometriosis, these macrophages behave abnormally and produce cytokines and other pro-inflammatory factors, like IL-1, IL-6, IL-8, TNF, RANTES, and VEGF. There is also defective NK cell activity and increased MMPs, which will lead to the attachment of the endometrium into the peritoneal layer. It gains vascularity through VEGF and develops into endometriotic lesions under the influence of estrogens. Almost every woman has retro-grade menstruation, but only 10% develop endometriosis due to altered immunity. As per coelomic metaplasia theory, the peritoneal mesothelial cells can differentiate into endometrial cells.[11] Recent research has proved that stem cells derived from bone marrow can differentiate into endometrial cells, which could explain the presence of endometriosis beyond the peritoneal cavity. Patients are in general started with NSAIDs for the management of pain. Recently all the guidelines like ESHRE (European Society of Human Reproduction and Embryology, ASRM (American Society for Reproductive Medicine), SOGC (Society of Obstetricians and Gynaecologists of Canada, WES (World Endometriosis Society) recommend starting patients on treatment without a histological diagnosis. Even imaging may not be conclusive in early lesions, but they can still be treated with drugs if they have endometriosis-associated pelvic pain. Our previous studies show that infertility, dysmenorrhea, and dyspareunia are significant among all the symptoms.[12] Imaging helps confirm the diagnosis in advanced lesions but not early ones. Medical management is the first line of treatment. Commonly used drugs are GnRH analogs, progestins like Dienogest, Depot medroxyprogesterone acetate (DMPA), and anti-angiogenic drugs. Laparoscopic surgery is indicated if pain persists despite medical management or if women are infertile, which can both be diagnostic and therapeutic. Imaging is a non-invasive modality for diagnosis, but early lesions cannot be detected. Hence, we need a diagnostic method that can accurately detect the disease earlier in a non-invasive way without laparoscopy, which will aid in starting the treatment earlier. It can also treat the disease and prevent the progression of the lesions. Over the past 10 years, a lot of light has been shed on the role of miRNAs as a potential robust bio-marker for the non-invasive diagnosis of endometriosis. Some of these dysregulated miRNAs are directly involved in the disease pathways, while others are associated with the presence of the disease. Endometriosis alters miRNA production, irrespective of the stage of the disease.[13,14] There are heterogeneous combinations of non-invasive bio-markers, among which serum miRNAs are important biomarkers for diagnosing endometriosis. Recent studies from Dr. Philippe Descamps et al. 2022[15] have shown that these miRNAs can be extracted even from the saliva of patients with symptoms. miRNAs are short nucleotide sequences of non-coding RNAs involved in the transcriptional regulatory pathway. They play an important role in fine-tuning gene expression regarding pathological conditions. Each miRNA targets multiple target RNAs across many cellular pathways. 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 degradation by endogenous RNAase as they are found within the exosomes bound to protein complexes, making them more stable and hence a better candidate marker.[16,17] Total of 2500-2600 miRNAs have been detected in the human genome[18,] and each disease has its unique expression profile. [19, 20] Alteration of miRNA occurs in many disorders, and each disease has its specific miRNA expression. Dysregulation of various miRNAs' expression is seen among endometriosis patients in the serum, plasma, and saliva.[13,21,22] This study aims to understand the application of specific peripheral circulating miRNAs in endometriosis patients, which are either up-regulated or down-regulated (2fold -10fold) and hence arrive at a diagnosis. Demographic features like age, BMI, family history, infertility, and symptoms were also studied. Earlier, the diagnosis of endometriosis can be made by non-specific inflammatory markers such as IL-6, IL-1β, hs-CRP, and CA-125[23] apart from imaging and laparoscopy. However, a sensitive, specific biomarker is thus far unavailable. A highly sensitive and specific diagnostic test based on miRNA can have great clinical significance in diagnosing endometriosis in women with chronic pelvic pain and unexplained infertility. In addition, recent studies have proved that medical management using aromatase inhibitors, GnRH analogs, or surgical treatment is found to reverse the miRNA 125b-5p and Let-7b levels which could be used for follow-up of the disease.[24] Thus, miRNAs have potential applications to serve as both early stager biomarkers and in post-treatment follow-up.

2. MATERIALS AND METHODS

2.1. Study Population

The current prospective study was conducted at Ramakrishna Medical Centre LLP, Trichy, Tamil Nadu, India, catering mainly to obstetrics and gynecology (OBGYN) patients. Institutional ethical clearance approval was obtained from Bharathidasan University, Tiruchirappalli (IEC Ref. NO. BDU / IEC/ 2020/ 03 dated 24.6.2020). Written informed consent was obtained from the study participants at Ramakrishna Medical Centre LLP between January 2022 – December 2022. The study population was South Indian women from Tamil Nadu, India. The study group included 56 patients who reported symptoms suggestive of endometriosis-associated pelvic pain. In addition, a training (control) group of 40 patients proven to have endometriosis by laparoscopy was included. Both the groups underwent 3 specific miRNA analyses – miRNA –125b-5p, miRNA-342-3p, and Let-7b. All these data were entered into SPSS Version 20.0. Categorical data were expressed in frequency (or) percentage. Chi-squared test was performed to obtain the P-Value.

2.2. Inclusion and Exclusion criteria

All the participants from the study and training (control)groups were demographically profiled. The study group (n=56) included women aged 16-46 years presenting with one or more symptoms of endometriosis-associated pelvic pain. The training (control) group (n=40) included women aged 25-45 years with proven endometriosis confirmed by laparoscopy. Exclusion criteria consist of asymptomatic patients and patients with a history of previous surgery for endometriosis.

2.3. Sample preparation and RNA extraction.

The blood samples were collected in EDTA K2 tubes. The study population was South Indian women from Tamil Nadu, India. Around 10 ml of peripheral blood was collected from the patients in sterile tubes in fasting status during the proliferative phase. Serum was collected by centrifuging at 3000 rpm for 30 minutes. The serum was separated, transferred into new tubes, and stored in liquid nitrogen at -196°C until use (Fig -1). Serum samples were collected from both the study and training (control) group participants during the proliferative phase and quantitative RT-PCR analysis of the miRNA panel (miRNA -125b-5p, miRNA-342-3p and Let-7b) was done in both groups. This panel of miRNAs was chosen for the non-invasive diagnosis of endometriosis based on our previous study[12]

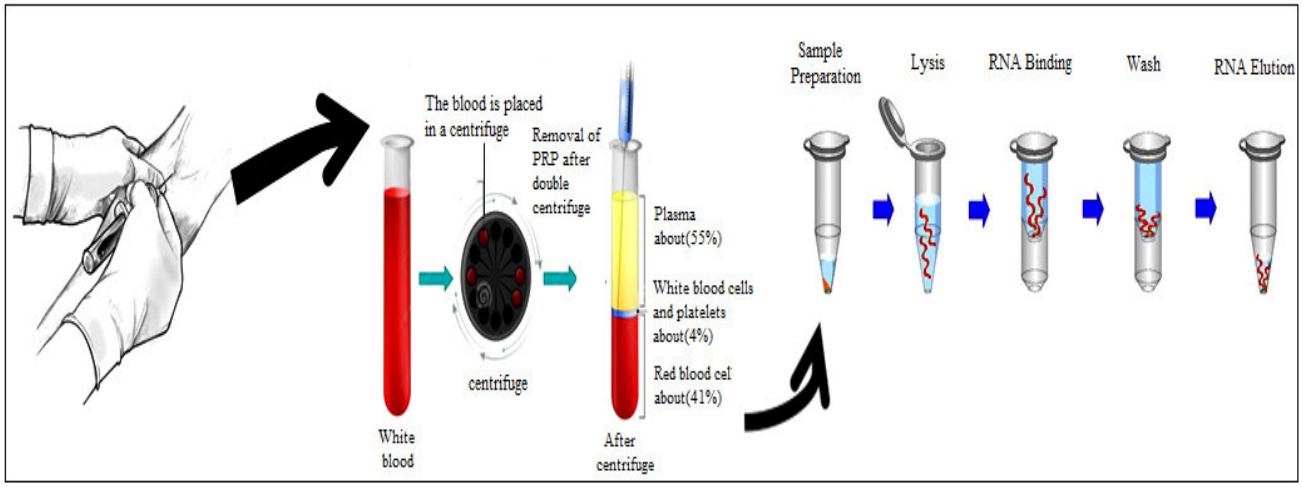


Fig 1: Sample collection and processing methodology[12]

Total miRNA was extracted using miRNeasy mini kit (Qiagen# 217084). the miRNA-enriched fraction was separated using an RNeasy MinElute Cleanup Kit (Qiagen # 74204).

2.4. cDNA synthesis and MicroRNA profiling

The extracted miRNA was reverse transcribed using Invitrogen NCode miRNA first-strand cDNA synthesis (Life Technologies # MIRC-50) according to the manufacturer's instructions.
(http://tools.thermofisher.com/content/sfs/manuals/ncode_rna_amp_man.pdf). 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 analyzed in duplicates.
Realtime quantitative polymerase chain reaction (qRT-PCR) was performed using BioRad CFX96). The number of cycles needed for the fluorescent signal to cross the threshold in qRT-PCR is known as Cycle threshold (Ct). Raw Ct values were calculated using the CFX Maestro Software 2.3. Only miRNAs whose Ct value was equal to or below 35 in at least the case or the control group were considered for further data analysis.

2.5. Real-time quantitative PCR verification.

The obtained cDNA was diluted 10 times using 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 used to prepare. 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 were considered for data analysis. Also, the NTC was negative (no Cq values) for all primers. Data analysis was performed after the 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 were 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.

2.6. Evaluation of Internal Controls for Real-Time Quantitative PCR

Previously reported retrospective studies[13] showed that a panel of serum miRNAs identified by microarray analysis were at least 10-fold increased or decreased in expression in the serum of endometriosis patients. The combination of 3 miRNAs (miRNA 125b -5p, miRNA 451-a, and miRNA 3613-5p) yielded an excellent area under the receiver operating characteristic curve (AUC) using the logistic regression model and receiver operating characteristic curve (ROC) analysis.[13] In this prospective study, we have taken a combination of miRNA 125b-5p, miRNA 342-3p, and Let-7b from the serum of patients in both study and training (control) groups. This study gave us an insight into the role of miRNA in the serum as a non-invasive diagnostic marker of endometriosis at a very early stage based on the presentation of clinical symptoms.

2.7. Validation in an Independent Sample of Endometriosis Patients and Endometriosis-free Controls

To evaluate the performance of the prior 3-markers (miRNA -125b-5p, miRNA-342-3p, and Let-7b) classifier formula developed from our previous study data set (training (control) group: n=40) in the current dataset (Study Group: n=56), we normalized and rescaled the 2 data sets to account for differences in qRT-PCR methodology. To build and test an optimal classifier using the current data set (study group) (n=56), we used machine learning with a random forest approach (RF) along with 10-fold cross-validation. This approach was chosen because of the ability to yield an AUC of 1 in the training data (control) set. The data set was split into training (control) (n=40) and testing sets (n=56) to train and assess the performance of the classifier. ROC analysis was performed to evaluate the diagnostic utility of each miRNA biomarker. As previously described, the ROC AUC was calculated using random forest machine learning classification to reduce noise and redundant information.[25]

3. RESULTS

3.12 Demographics, Symptoms, and Baseline clinical characteristics of study participants 56 patients had turned up to the outpatient department (OPD) with symptoms suggestive of endometriosis-like infertility (41 patients among which 33 had primary infertility and 8 had secondary infertility) severe, progressive dysmenorrhoea and diffuse abdominal pain and they did not have imaging evidence of endometriosis. An overview of the study design is provided in detail below (Fig - 2)

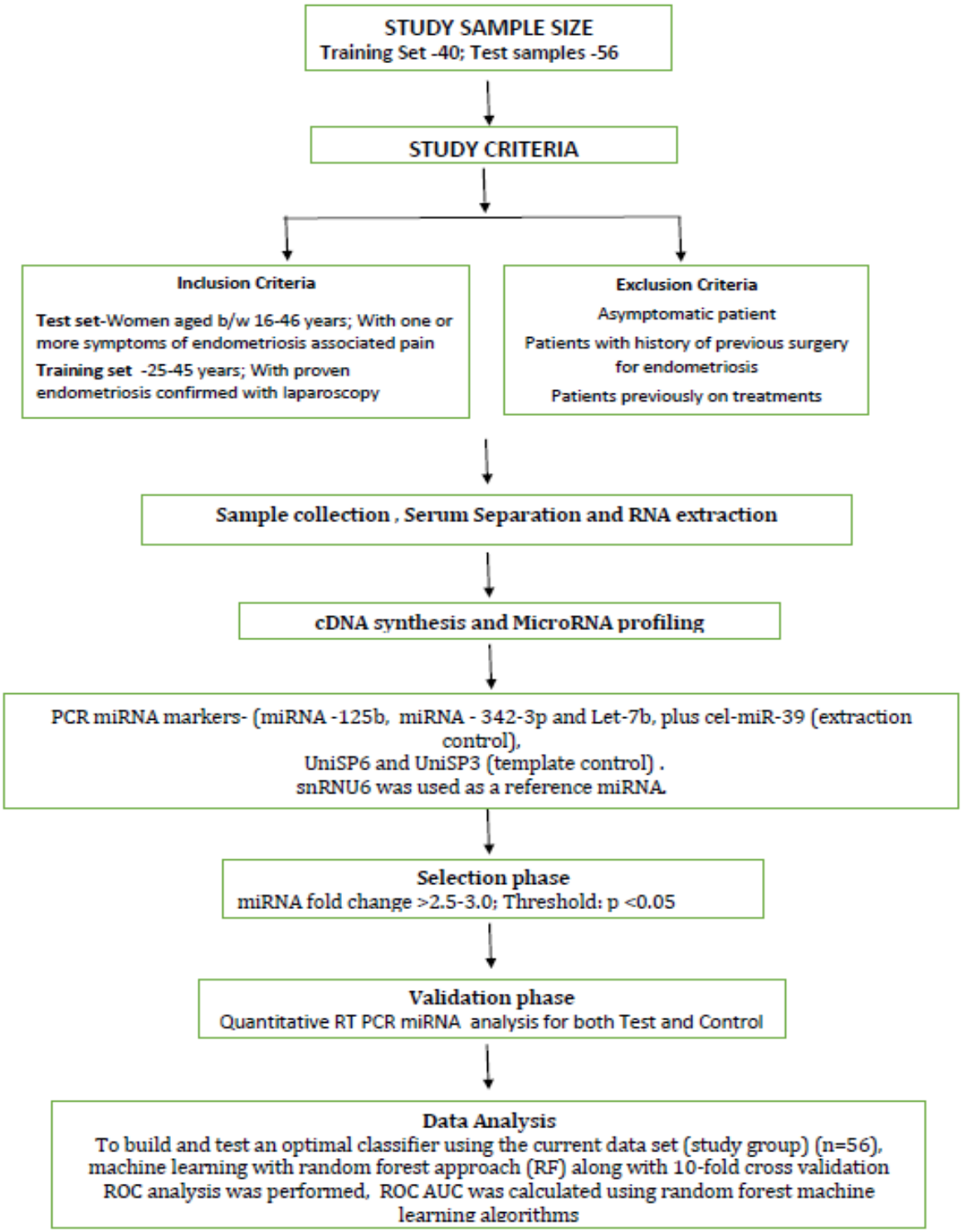


Fig 2: Overview of study design

Training (Control) group of 40 patients who underwent laparoscopy as they had stage III or stage IV endometriosis, following which they underwent fertility treatment. Among the 28 patients who had infertility, 20 had primary infertility and 8 had secondary infertility. Hence, a miRNA study was done to discover the possibility of endometriosis in these patients. All these patients had upregulation of miRNA 125b-5p, miRNA 342-3p, and downregulation of Let-7b. Therefore, it has been proved that diagnosis of endometriosis is possible by studying the miRNAs in patients with endometriosis symptoms. (Table -1). Statistical analysis was carried out using SPSS 21.0 version to statistically validate the outcomes of the Demographic profile and clinical features of the study population and also to analyze the significance of symptoms with different stages of endometriosis of both the training set and study group. The mean age of the subjects in the study group and control group were 28 and 31 years, respectively, indicating the study population represents women of reproductive age. In addition, 85% of control subjects had regular menstrual cycles as opposed to 60.7% in the study group population; however, 70% of control group subjects and 73.2% of study group subjects displayed primary and secondary infertility. The observed clinical feature was listed in detail in the table given below.

Table -1: Demographic profile and clinical features of the study population.

Demographic profile and clinical features			
Variables	Study Group (n=56)	Training (Control) Group (n=40)	p-value
Age	28.6±6.45	31.3±5.30	2.61e-4
BMI	25.6±5.18	25.0±4.37	3.85e-2
Family History	7/56 (12.5%)	3/40 (7.5%)	1.67e-2
Menstrual history			
Regular cycles	34/56 (60.71%)	34/40(85.0%)	**0.017**
Irregular cycles	22/56 (39.28%)	6/40(15.0%)	0.733
Infertility			
Primary infertility	33/56 (58.92%)	20/40(50.0%)	0.054
Secondary infertility	8/56 (14.28%)	8/40(20.0%)	1.28e-7
Symptoms			
Dysmenorrhoea	36/56(64.28%)	29/40(72.5%)	**0.019**
Dyspareunia	36/56 (64.28%)	13/40(32.5%)	**0.043**
Dyschezia	12/56(21.42%)	12/40(30.0%)	0.711
Dysuria	12/56 (21.42%)	4/40(10.0%)	0.994
Abnormal (Dysfunctional)Uterine Bleeding A(D)UB	21/56(37.50%)	17/40(42.50%)	0.651
Diffuse abdominal pain	20/56 (35.71%)	18/40(45.0%)	0.667
Difficulty in conception	41/56 (73.21%)	28/40(70%)	**0.012**
Associated findings			
PCOS	13/56(23.21%)	2/40(5.0%)	3.43e-7
Fibroids	5/56 (8.92%)	2/40(5.0%)	4.04e-5
Tubal diseases	2/56(3.57%)	3/40(7.50%)	3.15e-8
Mullerian anomalies	5/56(8.92%)	2/40(5.0%)	2.99e-1
Adenomyosis	5/56(8.92%)	7/40(17.50%)	1.87e-6
Medical complications			
Diabetes mellitus	4/56(7.14%)	3/40(7.50%)	6.11e-4
Thyroid disorder	15/56(26.78%)	7/40 (17.50%)	3.34e-7

Following variables like age, BMI, family history, menstrual history, type of infertility either primary or secondary, endometriosis-associated pain symptoms like dysmenorrhoea, dyspareunia, dyschezia, dysuria, Dysfunctional Abnormal) Uterine Bleeding A(D)UB, diffuse abdominal pain and difficulty in conception were studied in both the study and testing (control) groups. Both groups had no difference in all the variables. Following associated symptoms like PCOS, fibroids, tubal diseases, Mullerian anomalies, and adenomyosis were noted in both groups. Again, the incidence was found to be the same, with no difference in statistical significance. Diabetes mellitus and thyroid dysfunction were the common endocrine disorders seen in both groups, with no statistical significance.

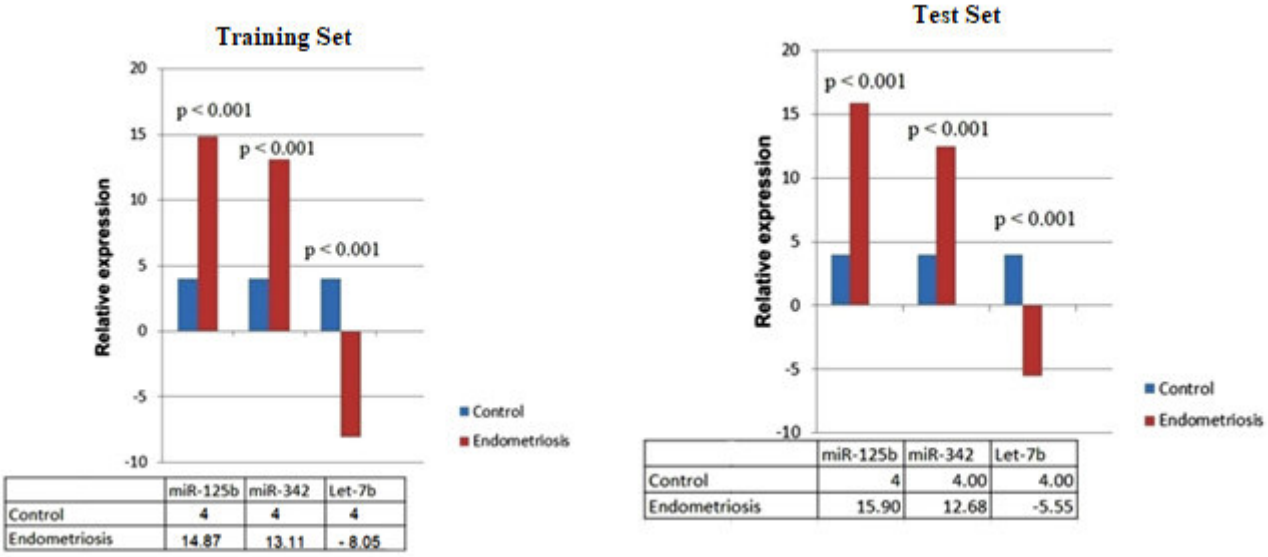
Table -2: Statistical significance between symptoms and stages.

S.no	Group	Symptoms		Total	Statistical Inference
		Dysmenorrhoea			
		Present	Not present		
1.	Study group	36	20	56	Chi-Square Value: 3.481 df-1 P >0.05 Not significant
2.	Control group	29	11	40	
	Total	65	31	96	
		Dyspareunia			
		Present	Not present		
1.	Study group	20	36	56	Chi-Square Value: 3.481 df-1 P >0.05 Not significant
2.	Control group	13	27	40	
	Total	33	63	96	
		Dyschezia			
		Present	Not present		
1.	Study group	12	44	56	Chi-Square Value: 3.481 df-1 P >0.05 Not significant
2.	Control group	12	28	40	
	Total	24	72	96	
		Dysuria			

		Present	Not present		
1.	Study group	12	44	56	Chi-Square Value: 3.481 df-1 P >0.05 Not significant
2.	Control group	4	36	49	
	Total	16	80	96	
	DUB				
		Present	Not present		
1.	Study group	21	35	56	Chi-Square Value: 3.481 df-1 P >0.05 Not significant
2.	Control group	17	23	40	
	Total	38	58	96	
	Diffuse abdominal pain				
		Present	Not present		
1.	Study group	20	36	56	Chi-Square Value: 3.481 df-1 P >0.05 Not significant
2.	Control group	18	22	40	
	Total	38	58	96	
	Difficulty in conception				
		Present	Not present		
1.	Study group	41	15	56	Chi-Square Value: 3.481 df-1 P >0.05 Not significant
2.	Control group	28	12	40	
	Total	69	27	96	

There is no statistical difference in endometriosis symptoms (Dysmenorrhoea, dyspareunia, dyschezia, dysuria, D(A)UB, diffuse abdominal pain, and difficulty in conception) concerning both stage I & II (cannot be detected by imaging) and stages III & IV (which can be diagnosed by imaging). Symptoms of stages I & II and stage III & IV are statistically insignificant in the present study, as given in Table 2. Selected miRNAs are dysregulated in all stages of endometriosis. Thus, the present study reflects that miRNA expression can be used to diagnose endometriosis based on the symptoms. Both the study and training (control) groups have similar symptoms and miRNA dysregulation (Table -2). Data indicate a lack of statistical difference, P > 0.05, in endometriosis symptoms (Dysmenorrhoea, dyspareunia, dyschezia, dysuria, AUB(D), diffuse abdominal pain, and difficulty in conception) concerning both stage I & II (stages that cannot be detected by imaging) and stages III & IV (the stage that can be diagnosed by imaging). In the present study, neither symptoms of stages I & II nor the symptoms of stages III & IV were statistically significant, calculated P is > 0.05.

3.1. miRNA expression levels in the training and test groups



	miR-125b	miR-342	Let-7b
Control	4	4	4
Endometriosis	14.87	13.11	- 8.05

	miR-125b	miR-342	Let-7b
Control	4	4.00	4.00
Endometriosis	15.90	12.68	-5.55

Fig 3: Expression levels of 3 miRNAs, normalized relative to levels of the small nuclear RNA gene U6. Data are expressed as the D cq values. Data significance, p < .001, using the Mann-Whitney U test.

Figure 3 shows the expression levels of 3 miRNAs that were assessed prospectively. Among the 3 miRNAs, miR-125b and miR-342-3p were significantly upregulated, whereas Let-7b was significantly downregulated in both the endometriosis

training and test groups compared to the control group, which included subjects without endometriosis.

3.2. *Diagnostic utility of a 3-miRNA biomarker*

Using machine learning with a random forest approach, a classifier algorithm was developed for the 3-marker (miRNA 125b-5p, miRNA 342-3p, and Let-7b). This algorithm was validated in 2 ways: by random subsampling dividing the total data set into training and testing subsets and by testing against our previous data set, which was not used for model development (n=40). The AUC scores for the model performance in the training and testing data sets are shown in Fig-3. An AUC of 0.976 for the machine learning classifier algorithm was attained in the test data set (n=56). An AUC value of 0.973 is indicative of the robust diagnostic utility of the miRNA-based non-invasive screening for endometriosis (Fig -4).

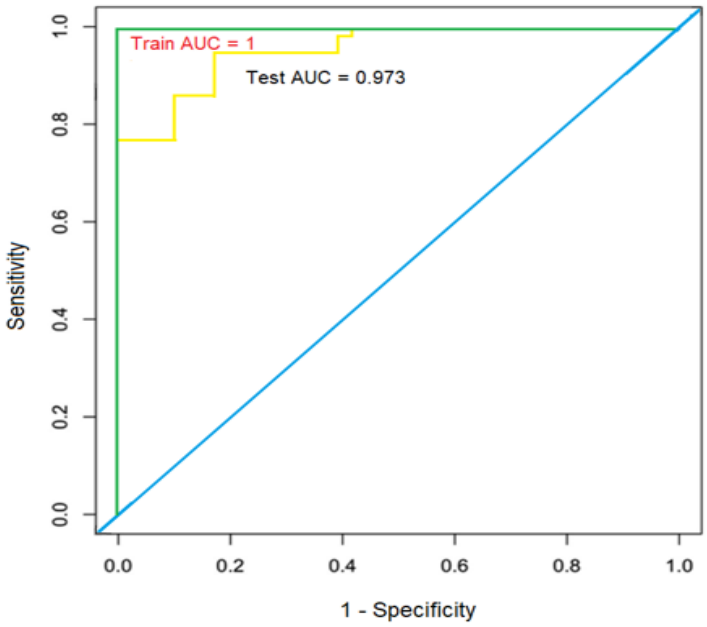


Fig 4: Diagnostic accuracy of the 3 miRNA biomarkers: ROC curve of the training and test groups.

Diagnostic accuracy of forest plot depicting robust diagnostic utility of the miRNA-based non-invasive screening for endometriosis using 3-miRNA marker panel that includes miR125b-5p, miR 342-3p, and Let-7b. Random Forest(RF) approach and 10-fold cross-validation were used. The algorithm was validated in 2 ways: by random subsampling dividing the total data set into training and testing subsets and by testing against our previous data set, which was not used for model development (n=40). The AUC for the machine learning classifier algorithm in the test data set (n=56) was found to be 0.976

4. DISCUSSION

Endometriosis is a debilitating chronic systemic inflammatory disease affecting the quality of life. The disease is associated with infertility and chronic pelvic pain. This disease is a public health concern, as it impacts physical and psychological health and socio-economic profiles, affecting the economy of the individual and the nation. Moreover, endometriosis needs a life-long management plan, mostly medical and avoiding repetitive surgeries[4] (ASRM). Currently, the diagnosis of endometriosis is based on laparoscopy and histopathological examination. A non-invasive diagnosis like imaging modalities is useful only in the advanced stages of the disease. Researchers have tried to develop and standardize a non-invasive diagnostic methodology based on relevant biomarkers in the last three decades. This prospective study was conducted at Ramakrishna Medical Centre LLP, Trichy, Tamil Nadu. We included samples from 56 patients in the study group and 40 patients in the training (control) group collected between July 2021 – July 2022. The mean age in the study group (n=56) was 28.6 ±6.45 years, and the testing (control) group (n=40) was 31.3±5.30 years. There is not much of a difference between both groups. (Statistically not significant p=2.61e-4). In a study by Mishra V et al., 2015[26] the mean age was 28.5 ± 4.2 years, which correlates with our study. Another study by Tomar *et al*., 2017[27] also reported a similar mean incidence age of 27 ± 3.6 years. Our previous study showed the mean age group of the endometriosis group was 31.0±4.35years.[12] The incidence of primary infertility is more between the age of 26 and 30 years than that of secondary infertility. It is similar to the study by Vercellini*et al*., 2014.[28] BMI was calculated in both groups. BMI was 25.6±5.18 in a study group, and among the testing (control) group, it was 25.0 ±4.37. (p=3.85e-2 statistically not significant).[29,30] All the patients in both groups were not in the obese category. They were in the normal and overweight category. A study by Saha et al., 2013[30] showed that there was an inverse correlation between endometriosis and body weight. Nurses' health study II which included a cohort of 116, 430 female nurses showed 5504 (4.72%) had a proven incidence of endometriosis. Amongst these, there was a gradual reduction in the incidence of endometriosis as the BMI increased. The prevalence of endometriosis was very low in women with severe obesity, which could be explained due to the increasing incidence of anovulation; there are few cycles and hence reduced retro-grade spill. In the study group, 12.5% had a family history of endometriosis, and the testing (control) group had 7.5%. Which is again statistically not significant(p=1.67e-2). Our previous study showed a family history among 16% of the subjects with endometriosis.[12] The incidence of primary infertility, according to Tsuji *et al*., 2009 was 63%.[31] In this study, primary infertility was found in 33/56 (58.92%) and secondary infertility in 8/56 (14.28%) in the study group. The remaining 15 patients had pain as the chief complaint and did

not want fertility treatment. Among the testing (control) group, 20/40 (50.0%) had primary infertility,8/40 (20.0%) had secondary infertility, and the rest of the 12 patients did not want fertility treatment. Both the data were not statistically significant. (Primary infertility p=0.054 and secondary infertility p=1.28e-7). In Mishra *et al*., 2015[26] study 85.14% of patients had primary infertility and 14.85% had secondary infertility. In our previous study in 2015, primary infertility was seen in 84.5% and secondary infertility in 15.5%.[32] Women with endometriosis had a 2-fold increased risk of infertility. The incidence of primary infertility was 70%.[33,34] A study by Tomare*t al*., 2017[27] also reported a 70% incidence of primary and 30% of secondary infertility. There is a high variation in the incidence of endometriosis among infertile women because of the lack of facilities for laparoscopy. The present study reports a higher incidence of primary infertility. According to Chapron *et al*., 2019,[35] the diagnosis of endometriosis could be by mere symptoms and clinical findings, which helps in starting the treatment empirically with progestins. The management plan depends upon the need of the patient, and the treatment should be based on the symptoms. Pain can be managed only through medical management (COCs, Progestins, GnRH analogs, LNG-IUS, and Aromatase inhibitors), failing which, surgery can be undertaken. With this aim only, we analyzed the miRNA for the symptomatic patients. Accordingly, we started the treatment with Dienogest for patients who did not want fertility treatment, and the rest were directed toward fertility treatment.

4.1. Relationship between miRNAs and clinicopathological features of endometriosis patients

USG may not pick up patients with infertility in the early stages of endometriosis, and most often these patients are treated as unexplained infertility. We had 41/56 patients who had early stages of endometriosis treated for infertility. Management of these patients could be either by surgery or direct referral to ART if they have associated male factor or tubal factor anomalies. Surgery for endometriosis improves the spontaneous conception rate and reduces pain to almost 60–70%. Still, the recurrence rate in the form of pain or lesion may be up to 20% at the end of 2 years and 40–50% at the end of 5 years.[36] Symptoms provide valuable information leading to the diagnosis of endometriosis. Dysmenorrhoea was the most important presentation seen among 64.28% of the study group and 72.5% in the testing (control) group (p=0.019); Dyspareunia was seen in 64.28% of the study group and 32.5% in the testing (control) group (p=0.043), dyschezia was seen in 21.42% in a study group, and 30.0% in testing (control) group (p=0.711), dysuria was seen in 21.42% in a study group, and 10% in testing (control) group (p=0.994), AUB was seen 37.50% is study group and 42.50% in testing (Control) group (p=0.652), diffuse abdominal pain was seen in 35.71% in a study group, and 45.0% in testing (control) group (p=0.667) and difficulty in conception was seen in 73.21% in a study group and 70% in testing (control) group (p=0.012). There was no statistical significance in the study or the testing (control) group. Associated findings like PCOS, fibroid, tubal factors, and Mullerian anomalies were almost equally seen in both groups. There was a numerical increase in the associated adenomyosis in the testing (control) group which shows moderate and severe endometriosis this association is more. There was no difference in the medical complications in both groups. All the symptoms of endometriosis pertaining to the study and testing(control) group are not statistically significant. The development of the next generation of sequencing technology has characterized many genes in the process of expression disorders.[37-39] Circulating non-coding RNAs are regulators of gene expression and play an important role in the pathology of endometriosis. These miRNAs are present in the body fluids like serum, plasma, saliva, and urine. They are found to be stable and hence can be used as a non-invasive marker for the diagnosis of endometriosis.[40-44] Earlier meta-analysis showed little attention was paid to the circulating non-coding miRNAs. Few other studies failed to provide comprehensive diagnostic data (overall sensitivity, specificity, AUC, etc.). A Cochrane review published by Nisenblat et al., 2016[45] showed that the accuracy of biomarkers, including CA-125, was not profound regarding the diagnosis of endometriosis. So far, glycoprotein CA-125 has been used as an important biomarker in the endometriosis diagnosis, elevated only in advanced stages of endometriosis. Hence this can be used only for follow-up. CA -125 is elevated in malignancies and inflammatory conditions such as tuberculosis and is not specific for endometriosis alone. Hence, as an alternate, circulating miRNAs can be used as a candidate marker for the non-invasive diagnosis of endometriosis. These miRNA expressions are variable in several diseases, including oncologic, inflammatory, cardiovascular, metabolic, and reproductive disorders. In 46 studies, miRNAs were found to be dysregulated in endometriosis. Among the dysregulated miRNAs in endometriosis, 30 are in whole blood, 27 in serum, and 18 in plasma. Altered expression of miRNAs is also detected in the peritoneal fluid of patients with endometriosis. The endometriotic lesions produce some miRNAs, while others are altered because of the effects of endometriosis upon other tissues. There is no variation in the miRNA expression based on the cycle phase and hormonal treatment use, as reported in previous studies. We have taken all the samples during the proliferative phase without considering the previous hormonal intake. In contrast to our study, Sarah Moustafa et al., 2020[46] didn't know about the menstrual cycle phase in more than 50% of the patients, but a history of hormone intake was noted in all patients which could be the reason for not knowing the phase of the cycle. These variable expressions of miRNAs were not affected by prior hormonal treatment, and no variation was seen based on the stages of endometriosis. Our study also reflects the same, as few of our patients had hormonal treatment. However, this panel of miRNAs can identify early stages of endometriosis, which imaging studies cannot pick up. In a study by Sarah Moustafa et al., 2020[46] following 6 miRNAs, miRNA 125b, miRNA150-5p, miRNA 342-3p, miRNA 451 a, miRNA 3613-5p, and Let-7b were studied. They included 41 patients in the endometriosis group and 59 in the control group. miRNAs, miRNA 125b, miRNA150-5p, miRNA 342-3p, miRNA - 451a were upregulated and miRNA 3613-5p and Let 7b were down regulated. In our previous pilot study, miRNA125b, miRNA150-5p, miRNA 342-3p, miRNA 3613-5p, and Let -7b were studied in both the control and endometriosis groups. There was upregulation of miRNA 125b and miRNA 342-3p and downregulation of Let -7b with a significant p-value of <0.001. But, miRNA 150-5p was up-regulated, and miRNA 3613-5p was down-regulated with a significant p-value of <0 .01 in the endometriosis group Vs the control group.[12] Hence, in this study, we have narrowed down to these 3 miRNAs - miRNA125b-5p, miRNA 342-3p, and Let -7b. Both in the study and testing (control) group, which was found to be dysregulated. In a study by Darya et al., 2021[47] they compared Let 7b with CA-125 biomarkers for its specificity. In our study,

Let 7b was found to be a promising non-invasive biomarker for the diagnosis of endometriosis, and it was not compared with CA 125. Interestingly, the miRNAs identified in our study are known to be critical regulators of pro-inflammatory mediators such as IL-6, IL-1β, IL-17A, etc.,[48-50] that are known to regulate the expression of angiogenic factors implicated in endometriosis such as Vascular Endothelial Growth Factor (VEGF)[51]and matrix metalloproteinases such as MMP-1, MMP-3, and MMP-9[49]. Therefore, the miRNAs identified in our study have a diagnostic value. The therapeutic targeting of these miRNAs and their regulatory pathways has a huge translational potential in the future theranostics of endometriosis.

4.2. Highlights of the study

Endometriosis is a chronic systemic inflammatory disease with a systemic signature consisting of serum miRNA biomarkers has immense translational diagnostic potential. We have demonstrated that a combination of serum miRNA biomarkers and demographic profiling can accurately diagnose endometriosis in a non-invasive way. Specifically, a 3-miRNA panel consisting of miR 125b, miRNA 342, and Let 7b; when patients have 2 out of 7 endometriosis-related symptoms, they could accurately be diagnosed as having endometriosis. The study was a prospective study allowing a direct correlation between the levels of various miRNAs and their association in the presence of endometriosis. It will help in the diagnosis of concurrent pathology also. We have included 56 subjects in the study group having minimal to mild endometriosis who are symptomatic and 38 subjects in the study group who underwent laparoscopy. The remaining 18 patients who did not undergo laparoscopy were started on medical management with Dienogest and had symptomatic improvement. It was compared to 40 patients in the control group with moderate to severe endometriosis, and all were subjected to laparoscopy and planned for fertility treatment. It proves dysregulation of specific miRNAs correlates well with the diagnosis of endometriosis. Furthermore, it was possible to diagnose endometriosis based on altered miRNA expression in patients with symptoms suggestive of endometriosis but without any image-based evidence. This variation is independent of the stage of the disease.

4.3. Strengths and limitations of the study

The strength of our study is that it was a prospective study that shows the dysregulation of miRNA in symptomatic patients and patients with early lesions, which is at par with the dysregulation of miRNAs in moderate to severe disease. Hence, the 3 miRNAs considered in the current study can help in early diagnosis in a non-invasive way. The limitation of the study is using selected miR 125b-5p, miR 342-3p, and Let-7b, which are found to be associated with ovarian cancer, breast cancer, and brucellosis. But we have compared these miRNAs in our previous study with proven and control groups and also in the current study. All three miRNAs were studies that were exclusively proven endometriosis patients by laparoscopy and those who are negative for endometriosis. None of the patients had ovarian cancer, breast cancer, and brucellosis. So instead of a single marker, these three markers can be ideally used to diagnose endometriosis. A study by Cosar et al. in 2017 using these miRNAs gives clear evidence that they can be used for non-invasive diagnosis of endometriosis. Recent studies done by Dr. Phillipes Descamps have used the same miRNA from saliva as a marker for non-invasive diagnosis of endometriosis. We probably need to have more samples so that we will get better accuracy. The study's weakness is that we need to have a higher number of sample populations. In addition, further studies are required to determine if medical or surgical treatment could alter the unique marker profiles, especially the combination of these three miRNAs (miR -125b-5p, miR-342-3p, and Let-7b).

5. CONCLUSION

Early diagnosis of endometriosis can reduce hospitalization, economic loss, and morbidity. This study used a panel of 3 miRNAs (miR -125b-5p, miR-342-3p, and Let-7b) as non-invasive biomarkers to diagnose endometriosis. Our findings revealed the up-regulation of miRNA 125b-5p, miRNA 342-3p, and down-regulation of Let -7b. In the study group, the dysregulation of miRNA was highly significant, and the training (control) group also showed the dysregulation of the 3 miRNAs as per our previous study. In our study, the dysregulation of miRNAs was robust among the patients having symptoms suggestive of endometriosis and proven cases of endometriosis. By using this, miRNA study as a non-invasive diagnosis of endometriosis, clinicians can start the treatment without the laparoscopy, which helps to reduce the time to diagnosis from the onset of symptoms. It can ultimately reduce the pain and prevent the progression of the lesion. Fertility is also not affected. Large databases of this miRNA study may play a key role in using this as a biomarker in future studies. Future studies can be extended towards the response of the disease the hormonal treatment. We need larger prospective studies to evaluate the accuracy of diagnosis, treatment, and disease outcomes. This study has yielded an excellent diagnostic potential that miRNAs can be useful as a diagnostic biomarker for endometriosis.

6. AUTHORS CONTRIBUTION STATEMENT

Dr. T. Ramani Devi conceptualized and designed the whole study, including sample collection, processing, and clinically correlating the impact of miRNA on stages of endometriosis. Dr. C. Anchana Devi contributed to the statistical analysis and editing of the manuscript. Dr. B. Kadalmani contributed to the overall guidance and better study outcome. All the authors read and approved the final version of the manuscript. I acknowledge the technical assistance in the analysis of miRNA provided by Dr. V. Hari Balaji of VIVAGEN DX Labs. I acknowledge Mrs. S. Kalpana for the secretarial help.

7. CONFLICT OF INTEREST

Conflict of interest declared none.

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