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Non-Invasive Biomarkers for the Diagnosis of Endometriosis and Polycystic Ovary Syndrome

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One Sentence Summary: This paper involves discussing, describing, and characterizing biomarkers that have been proposed to aide in the diagnosis of benign gynecological disorders including endometriosis and polycystic ovary syndrome.

Abstract: Benign gynecological disorders can affect a high percentage of women of reproductive age, ages 15-44 years. These conditions can affect the lifestyle of the individual and can be associated with infertility. The gold standard to identify and diagnose endometriosis requires invasive surgical procedures, while the Rotterdam Criteria is used to identify and diagnose polycystic ovary syndrome. The purpose of this paper is to discuss, describe, and characterize potential non-invasive biomarkers that are present in various pathological stages of both endometriosis and polycystic ovary syndrome. These biomarkers include CA125, Serum galectin-9, hsa-miRNA-154-5p, miRNA-93, miRNA-320 and ET-1, miRNA-222, miRNA-146a, and miR-30c used in a panel[1-6].

Abbreviations: CA125= Cancer Antigen 125; PCOS= Polycystic Ovary Syndrome, MiRNA= MicroRNA; MMP= Metalloproteinases; EMT= Epithelial to Mesenchymal Transition; TGF ß-1=Transforming Growth Factor ß-1; VEGF=Vascular Endothelial Growth Factor; NGF=Nerve Growth Factor; rASRM=American Society for Reproductive Medicine; CVF=Cervicovaginal Fluid; Gal=Galectins; mRNA= Messenger RNA; GLUT4=Glucose Transporter Type 4; MCM7=Minichromosomal Maintenance Complex Component 7; ET=Endothelin; 2D-DIGE=Two dimensional Difference Gel Electrophoresis; LC-MS=Liquid Chromatography-Mass Spectrometry; TMT=Tandem Mass Tags; SDS-PAGE= Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis; CS= Eutopic tissue from asymptomatic controls scheduled for risk-reducing surgery with no evidence of disease at laparoscopy in the secretory phase; CP= Eutopic tissue from asymptomatic controls scheduled for risk-reducing surgery with no evidence of disease at laparoscopy in the proliferative phase; PS= Eutopic tissue from patients with chronic pelvic pain with no evidence of endometriosis at laparoscopy in the secretory phase; ES=Eutopic tissue from endometriosis cases in the Secretory phase and EcS= ectopic tissue from endometriosis cases in the secretory phase; EP=Eutopic tissue from endometriosis cases in the proliferative phase; LUM=Lumican; TPM2=Tropomyosin beta-chain; ECM=Extracellular Matrix; RT-PCR=Reverse Transcription-Polymerase Chain Reaction; ROC=Receiver Operating Characteristic Curve; IR=Insulin Resistance; KEGG Pathway=Kyoto Encyclopedia of Genes and Genomes; ERK=Extracellular-Signal-Regulated Kinase; ANOVA=One-Way Analysis of Variance; ELISA=Enzyme-Linked Immunoassay; CPP=Chronic Pelvic Pain;

Introduction

Benign gynecological disorders are a complex group of medical conditions in which both the symptoms and severity, are not specific to one particular disorder. A biomarker is "a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention"[7]. The idea of potential biomarkers emerged through human and animal model systems, to identify associations between biological measurements and prototypes of the disease at various levels. Clarity on these measurements and the effectiveness of the biomarkers for several contexts or uses, in the document "Biomarker, EndpointS, and other Tools" serves as an excellent resource. Biomarkers can be used to provide therapeutic interventions ranging from molecular, histologic, radiographic, or physiologic characteristics. There are various types of biomarkers characterized by their function including diagnostic, monitoring, pharmacodynamic/response, predictive, safety, susceptibility/risk, and prognostic and predictive biomarkers. This paper will focus on diagnostic biomarkers. These are biomarkers which "detect or confirm the presence of a disease or condition of interest or identifies an individual with a subtype of the disease"[7]. Generally, the validation of a biomarker starts at analytical validation. A biomarker must then be qualified using an evidentiary assessment and utilization. The biomarker can then be validated, measured reliably, precisely, and repeatably at a low cost. The diagnostic biomarker is specifically evaluated using receiver-operating characteristic (ROC) curves[8]. Classifying these biomarkers by sensitivity and specificity percentages determines the efficacy of each biomarker for potential diagnosis[9]. The sensitivity measure portrays the efficiency of a test and how accurate it can identify the presence of the disease. The specificity is a measure that states how well the test can identify the lack of a condition or disease in an individual[9].

Two common benign gynecological disorders are endometriosis and polycystic ovary syndrome (PCOS)[2]. Currently, there are no biomarkers that allow for a conclusive or a direct diagnosis for these specific benign gynecological conditions. This paper will therefore mainly characterize pre-clinical biomarkers for these conditions. A secondary objective of this paper is to determine the role miRNAs play as a pre-clinical non-invasive biomarker of PCOS and endometriosis and evaluate its possible relationship with hyperandrogenemia and insulin resistance (IR)[4,10].

Presentation, Pathogenesis and Diagnosis of Endometriosis

Although the exact cause of endometriosis is unknown, this estrogen-dependent inflammatory disease is diagnosed by the presence of endometrial glands and stroma outside of the uterine cavity. The condition may present as asymptomatic, or with varied and nonspecific presentations, creating the complexity of the condition and diagnosis. Globally, approximately 10 percent of women of reproductive age can be affected by the condition with the highest prevalence at ages between 25 to 35 years[11]. Additionally, a smaller percentage of premenarcheal girls and postmenopausal women are affected. There are various locations where the lesions can be present, including the pelvis, the bowel, diaphragm, and pleural cavity. Due to inflammation, hallmarks of the disease include dysmenorrhea, dyspareunia, chronic pain, and infertility[12].

Development of ectopic endometrial lesions

There are several theories regarding how ectopic endometrial tissue develops in locations outside of the uterus. These theories include altered immunity, imbalanced cell proliferation and apoptosis, aberrant endocrine signaling, and genetic factors[12]. The theory of retrograde menstruation is the hypothesis in which cells move through the fallopian tubes and enter the

peritoneal cavity[13]. This theory cannot explain all cases of endometriosis because retrograde menstruation can be present in up to 90 percent of all women, but only 10 percent of women present with endometriosis. Another theory explaining the presence of ectopic endometrial cells include mesothelium, stem cells, Mullerian rests, bone marrow stem cells, embryonic vestiges with lymphatic or vascular dissemination, and coelomic metaplasia[14].

Clinical presentation of endometriosis

The hallmarks of the endometriosis include dysmenorrhea, dyspareunia, chronic pain, and infertility. Pelvic pain specifically can be associated with inflammation, pain mediators, along with neurologic dysfunction. Other endometriosis-related pain is due to a combination of increased nerve fibers and an imbalance of sympathetic and sensory nerve fibers[15,16]. Specifically, pain is the result of estrogen repulsing sympathetic axons by acting as a neuromodulator while sensory innervation is preserved. Additionally, pain in the CNS can be due to the stimulation of the peripheral nerve sensitization through inflammation. A patient can present with dysmenorrhea around one to two days before, during, and after menses. Pelvic pain can be characterized from dull, throbbing, sharp and/or burning and are associated with an adnexal mass[17]. Additional symptoms include bowel and bladder dysfunction, abnormal uterine bleeding, low back pain, or chronic fatigue[12].

Endometriosis can be characterized by the presence of specific symptoms, location of the lesion, along with how the lesion disrupts the organ or tissue where it is found. The most common sites of endometriosis are the ovaries, anterior and posterior cul-de-sac, posterior broad ligaments, and uterosacral ligaments, the less frequent locations include uterus, fallopian tubes, sigmoid colon and appendix[18,19]. Endometriosis can be categorized based on depth of invasion, e.g. superficial peritoneal, ovarian, and deeply infiltrating endometriosis. Deeply infiltrating

endometriosis is distinguished by a solid mass located in the peritoneum deeper than about 5 mm[20]. These endometriosis implants can be distinguished from eutopic endometrium by their fibrous tissue, blood, and cysts. During the process of inflammatory cells breaking down red blood cells, both hemosiderin-laden macrophages and pigmented histiocytes form. The increased pigment dates the older lesion[21]. Superficial peritoneal lesions can present with or without glandular or stromal components. That can be changed by cellular atypia and pigmented histiocytes, respectively[22]. Peritoneal or deeply infiltrating endometriosis can result in bladder endometriosis, bowel endometriosis, endometriosis of the abdominal wall, and thoracic endometriosis. Peritoneal or deeply infiltrating endometriosis lesions that appear on the uterosacral and cardinal ligaments and pouch of Douglas are associated with dyspareunia[20]. Various urinary symptoms can be exhibited in women with bladder endometriosis. Finally, women with bowel endometriosis can present with symptoms such as diarrhea and constipation[13]. Ovarian lesions or endometrioma formed after ectopic endometrial tissue within the ovary bleeds resulting in a hematoma surrounded by duplicated ovarian parenchyma. These endometriomas are characterized by fibrotic walls and surface adhesions[23].

Diagnosis of endometriosis

Although endometriosis can only be diagnosed through histological examination of a biopsied lesion using surgery, usually laparoscopy, information gathered through the presence of symptoms, physical examination, laboratory, and imaging findings allows for identifying both location and size of the implants that would support a presumptive diagnosis[24]. These additional findings could include the presence of adnexal masses or a tenderness on a vaginal examination. Findings via imaging using transvaginal ultrasound and MRI can show ovarian cysts and modules of the rectovaginal septum suggesting endometriosis. Identifying non-invasive

biomarkers are beneficial since there are no laboratory findings indicative of the presence of the condition[12].

The gold standard to certainly diagnose the condition is through laparoscopic surgery[13]. With laparoscopy, the condition is scored using the rASRM score from a I-IV or minimal to severe (*Figure 1*)[10]. The method involves creating small incisions to remove mild to moderate endometriosis in internal organs and remove scar tissue. This procedure, using a laparoscope, lasers, and other operative procedures functions as an alternative to open abdominal surgery. The risks to this include pelvic infection, uncontrolled bleeding, post-surgical scar tissue formation, and bowel, bladder, or uterus damage[25]. Laparoscopy can show areas of peritoneal endometriosis that present "as raised flame-like patches, whitish opacifications, yellow-brown discolorations, translucent blebs, or reddish or reddish-blue irregularly-shaped islands,"[12].

On a hormonal and cellular level, endometriosis includes abnormalities in steroid hormone production and the presence of various eutopic and ectopic receptors. Endometriosis is characterized by estrogen dependence and progesterone resistance presented with chronic inflammation. Histologically, patients with endometriosis present with abnormal levels of VCAM-1 (CD-106) and ICAM-1 (CD-54) within their serum, as well as MMP-2 and MMP-9, two types of metalloproteinases used to remodel extracellular matrix at the site of implantation[26]. This is notable when understanding the EMT since endometriosis presents with an invasive mesenchymal phenotype. Here N-cadherin is gained, and E-cadherin is lost with promoting factors such as TGF ß-1 present[13]. There are only a few studies that present a genetic cause for endometriosis showing a minor effect at multiple loci[16]. One study relayed that, somatic mutations in the genes ARID1A, PIK3CA, KRAS, and PPP2R1A, which are cancer driver genes, can lead to the onset of endometriosis lesions[27].

Currently, the lack of clinically accepted biomarkers to detect the stage of disease without invasive surgery makes this research increasingly relevant and necessary. To be an effective biomarker, which can include cells, proteins, or lipids, these biomarkers should be identified in extractable body fluids such as blood, urine, saliva, and cervicovaginal fluid (CVF) and would need to be identified at a stage of pathogenesis[13]. Being identified in CVF would be the most effective, since the biomarkers localized in that area would have increased sensitivity and specificity. The endometriosis biomarkers that will be discussed in this paper includes miRNAs, Gal, specifically Gal-9, and Endothelin-1[1,4].

Potential Non-Invasive Biomarkers for Endometriosis: CA-125 and Gal-9 and hsa-miRNA-154-5p

Identifying CA125 as a Hormonal Biomarker for Endometriosis

CA125 was determined to be the most effective marker for ovarian cancer with a clinical threshold of 35 U/mL[28]. CA125 was tested to see if it could serve as an effective diagnostic marker for endometriosis. On a broader scale, this study sought to identify numerous potential biomarkers by examining the expression of all the proteins present in eutopic and ectopic endometrial tissue specimens. These specimens, obtained from the University College London Hospital Gynecology Department, were taken from individuals who were positive for endometriosis. The samples for the controls were taken from women who lacked the onset of endometriosis but had a family history of cancer and therefore wanted to undergo exploratory surgery due to a presence of Chronic Pelvic Pain (CPP). Individuals who were part of the study consisted of women diagnosed with endometriosis using laparoscopy and further enforced histologically. The controls were divided into two groups: controls with and without pain. 21

women, part of the control with pain group, were characterized by individuals with pelvic pain stemming from a chronic pelvic inflammatory disease with no surgery or an unknown cause. The 21 individuals who were part of the controls without pain included women who were undergoing normal menstrual cycles with no identification of endometriosis post-surgery but were undergoing bilateral tubal ligation and/or prophylactic bilateral salpingoophorectomy[2].

The researchers first collected these samples prior to the proteomic profiling methods. These methods included protein extraction, quality assessment, immunodepletion, and pooling. After staining using SDS-PAGE with colloidal Coomassie Blue staining, tissues samples were further excluded from the study if the stains were excessively polluted with blood proteins. This was because the abundance of blood proteins hindered the presence of the tissue-derived proteins. Samples with very low protein expression were also excluded. The samples that were included were then divided based on clinical group and cycle phased which then resulted to six groups of 6-20 tissue samples per group labeled CS, CP, PS, ES and EcS. By proteomic profiling using 2D-DIGE, proteins identified include LUM and TPM2, which both had a higher expression in the secretory phase of endometriosis when compared to the control groups. By proteomic profiling using 3D-LC-MS/MS with TMT labelling, the proteins identified were further analyzed for functionality by using the GO biological process and the KEGG pathway which determined that the proteins played a role in metabolic pathways, ribosome, proteasome, spliceosome, and regulation of the actin cytoskeleton, focal adhesions and extracellular matrix-receptor interactions. The proteins that were highly expressed in ectopic versus eutopic tissue that had functionality associated with muscle included TPM1-4, MYLK, MYL6 and 9, PDLIM7, CNN1, CALD1, and TAGLN. Other proteins with increased expression in ectopic tissue included FN1, LUM, COL1A2, COL6A1, COL6A3, COL14A1, PRELP, OGN, DCN, BGN,

FMOD, and MFAP4. These protein groups were then compared and scores for biomarker potential were determined (*Table 1*) [2].

It is noteworthy to mention that even by using proteomic approaches described, CA125 was unable to be found due to this heterogeneously glycosylated protein's large size and low abundance[2]. It is also important to understand that in the pathogenesis of endometriosis proteins that play a role in cytoskeletal, ECM organization, and cell-matrix are vital. During implantation of ectopic endometrial cells, for the cells to adhere to the peritoneal ECM and for the retrograde-shed endometrial cells to be able to invade, these ECM proteins would be beneficial[29].

The expression of CA125 and sICAM1 were compared between endometriosis and the control groups and showed to have the significantly increased expression. The results showed 40% sensitivity and 90% specificity. The area under the ROC curve for endometriosis versus pain group was 0.713. CA125 alone provided data at 40% sensitivity and 90% specificity. It was concluded that CA125 couldn't exist as a diagnostic marker alone. CA125 doesn't have the equivalent diagnostic accuracy in endometriosis as it does in ovarian cancer as its median level in the samples of endometriosis falls under that threshold[2].

In summary, CA125 was also shown to be the most effective when included in the best model for secretory phase samples along with MIF and PAEP with a sensitivity of 65% at 80% specificity (*Table 2)*[2]. It was concluded that CA125, s1CAM1, PAEP, MIF, and FST are potentially useful diagnostic markers when combined in multivariate models. These results indicate that the biomarkers are cycle dependent[2].

Gal-9 Serving as a Non-invasive Biomarker for Endometriosis

Lectins are proteins that bind to carbohydrates. Galectins are a type of lectin of that bind at the ß-galactoside and play a role in cell growth, adhesion, apoptosis, and angiogenesis[30]. Lectins have been associated with reproductive immunology, inflammation, and autoimmunity. These molecules are carbohydrate-binding proteins that associate with sugar groups causing agglutination. Previous studies have shown that a normal endometrium may contain Gal-1-4, 7-9, and 12. Out of those, Gal-1 and Gal-3 have specifically been associated with the pathogenesis of endometriosis[1].

Gal-9 has specifically been tested because of its exclusive expression in the normal endometrium by epithelial cells. This molecule, a bidirectional immunomodulator, could be a marker of endometrial receptivity pre-implantation[1]. In previous studies it was seen that there was an increased Gal-9 staining in the ectopic endometriosis lesions, which is what led to further studies to identify Gal-9 as a diagnostic potential of being a serum soluble measurement[31]. Specifically, it was found that during implantation and the early pregnant decidua, Gal-9 was only expressed by uterodomes[32]. Additionally, Gal-9 mRNA was shown to downregulate IFN-gamma production in NK cells meaning that there could be a role for Gal-9 when regulating the fetomaternal immunotolerance by navigating the cytotoxicity of NK cells[33].

The Brubel study identified 135 women of reproductive age and then divided them into 77 endometriosis patients, 28 gynecologic controls, and 30 healthy women. The endometriosis study group was divided into mild and moderate groups. 20 individuals were a part of the mild group, who had stages I-II endometriosis and 57 patients were part of the moderate to severe group, consisting of women who had stages III-IV endometriosis. This group usually had a previous surgery and had CPP[1].

A RT-PCR assay was performed to determine the gene expression of LGALS9 gene expression in the eutopic endometrium of patients with and without endometriosis along with ectopic endometriotic implants. The expression density of Gal-9 mRNA to RibS9 were then normalized to assess the expression of LGALS9 transcripts and the densitometry of Gal-9 mRNA. The results were statistically analyzed using the t-test and the Mann-Whitney *U*-test. Data was presented using the ELISA test and the ASRM score. To compare the mean or medians of Gal-9, analyses such as the nonparametric Kruskal-Wallis *H*-test with Dunn's comparison or the ANOVA test with Bonferroni's correction were used. It is notable to mention here that the results of this study suggest that other components of the peritoneal fluid have the capability of producing this lectin since an increased expression of Gal-9 was found in the peritoneal cells. Additionally, it was proven that Gal-9 was directly expressed by the ectopic implant as opposed to its surrounding areas when both locations were compared[1].

The results from the ELISA test showed increased levels of Gal-9 in minimal-mild and moderate-severe endometriosis when compared with healthy controls. The biomarker showed 94% sensitivity at 93.75% specificity (*Figure 2*)[1]. It was also concluded that benign gynecologic conditions related to CPP, or infertility might be associated with elevated serum Gal-9 levels. Through the study, Gal-9 was determined to be a new potential biomarker for non-invasive laboratory diagnosis of endometriosis. It was proved to diagnostically perform better than CA-125 and VEGF. Further studies include expanding the sample size to include a larger subgroup of gynecologic controls to address early-stage non-invasive diagnostic testing[1].

hsa-miRNA-154-5p Serving as a Non-invasive Biomarker for Endometriosis

MiRNA is a biomarker that plays a part in the detection of both endometriosis and PCOS at varied capacities. Specifically, to endometriosis, miRNA expression changes between patients that have eutopic endometrium with endometriosis and those with a healthy endometrium and ectopic lesions. These miRNAs are involved in various pathways that are part of processes such as proliferation, inflammation, and angiogenesis[34]. MiRNAs show potential as biomarkers due to these disease-related differences. These non-coding RNAs are secreted by exosomes and circulate through its associated RNA-binding proteins, the Ago2 component of the RNA-induced silencing complex (RISC), and nucleoplasmin (NPM1)[35,36].

The specific plasma miRNA signature that was found to be associated with endometriosis in this study was hsa-miRNA-154-5p which is located at the DLK1-MEG3 imprinted genomic locus[37]. This locus contains "multiple maternally expressed noncoding RNA genes and paternally expressed protein-coding genes"[38]. This specific mRNA is found in carcinosarcomas allowing for the epithelial-to-mesenchymal transition to be detected[39]. Hsa-miRNA-154-5p has also been associated with endometriomas and endometrial stroma decidualization[40].

51 patients with endometriosis were compared with 41 controls determining the changes in extracellular miRNA spectra in plasma. There were three phases of this study including biomarker screening, discovery, and validation. The expression of the miRNAs was analyzed using quantitative PCR-based microarrays. The diagnostic performance was further statistically analyzed using *in silico* bioinformatics modelling and ROC curve[3].

In this study, the 42 DE-miRNAs (differentially expressed miRNAs) were studied using a q-PCR-based profiling array to identify putative biomarkers for the disease. The parameters used from the mi-RNAs include BMI, age, and dysmenorrhea. This showed discrimination between

the controls and patients with endometriosis when comparing specificity and sensitivity values for a small sample set[3].

The main takeaway is that after the ROC curve analysis, that was performed on the cohort of 64 cases and controls, it was seen that DE hsa-miRNA-154-5p had an AUC value of 0.72 (95% confidence interval [Cl]: 0.587, 0.851, P=0.001) with the sensitivity and specificity values being 67% and 68% (*Figure 3C*) [3]. It was found that hsa-miR-154-5p has sensitivity and specificity values high enough to discriminate patient with endometriosis from controls[3]. When the clinical parameters of age and BMI were combined with that of the four DE miRNA including hsa-miR-196b-5p, hsa-miR-378a-3p, and hsa-miR-33a-5p, the prediction validity improved substantially but the AUC value for endometriosis prediction did not change (*Figure 3D*) [3].

Pathogenesis and Relevance of PCOS

PCOS is one of the most common endocrine disorders and affects 7-9% of the women who are of reproductive age[5]. The complexity of this condition is due to its multiple etiologies along with it being a diagnosis of exclusion. PCOS is characterized by menstrual irregularities, abnormal ovarian size, and hyperandrogenism. Along with the ruling out other clinical conditions, the Rotterdam Criteria is used to diagnose PCOS. The Rotterdam Criteria states that 2 of the following 3 criteria is needed for diagnosis. The criteria include oligo- and/or anovulation, clinical and/or biochemical signs of hyperandrogenism, and polycystic ovaries visualized by ultrasound. A transvaginal ultrasound is used to determine the presence of polycystic ovarian morphology (PCOM), which is 12 or more follicles in either ovary measuring 2-9 mm in diameter, and/or increased ovarian volume (>10 mL). History and physical exam can

be used to identify irregular menses and hyperandrogenic features. However, beyond that, PCOS can only be diagnosed if other conditions that mimic PCOS with oligo/anovulation and/or hyperandrogenic symptoms are excluded. The conditions include but are not limited to thyroid disease, nonclassical congenital adrenal hyperplasia (NCCAH), hyperprolactinemia, and androgen-secreting tumors[5].

The clinical hallmarks of PCOS include oligomenorrhea and hyperandrogenism along with hirsutism and polycystic ovaries[41]. The complexity of this condition is because these features are not necessarily always present or can be evident in varying degrees. This complexity results in a slew of evaluation tests based on symptoms. A transvaginal ultrasound is not necessary when a patient presents with both oligomenorrhea and hyperandrogenism along with when other differential conditions are ruled out. It must be noted that a transvaginal ultrasound with polycystic ovaries alone does not confirm the diagnosis of PCOS[41]. Conditions that need to be ruled out include hyperprolactinemia, thyroid disease, and pituitary tumors inhibiting gonadotropic secretion. The evaluation that can be used specifically to exclude similar disorders include thyroid-stimulating hormone level, prolactin level, 17-hydroxyprogesterone, oral GTT, lipid panel, and 24-hr urine free cortisol[42]. PCOS may also result in increased risk for associated conditions such as obesity, risk for diabetes mellitus, hypertension, lipid abnormalities, sleep disorders, depression, and metabolic syndrome[42]. About 60-70% of PCOS patients demonstrate insulin resistance[5].

Hyperandrogenism in PCOS

As stated previously, one of the hallmarks of PCOS is hyperandrogenism, which is due to increased gonadotrophin secretion of luteinizing hormone (LH) from the pituitary. Specifically, ovarian theca cells secrete high levels of androgens, such as testosterone, from the LH secretion

leading to hyperandrogenism. Symptoms of hyperandrogenism include acne, hirsutism, and male-pattern balding or hair loss. It is advised that patients who present with hyperandrogenism alongside with oligo-ovulation undergo biochemical testing to serum androgens. Hirsutism is characterized by the presence of terminal hair longer than 5 mm with pigment and shape. This presentation is graded by using the modified Ferriman-Gallwey (mFG) score in nine areas of the body with scores from 0 to 4 and a total score of 36. A mFG score greater than 6 is defined as hirsutism. Differentials that need to be ruled out regarding hyperandrogenism include non-classical congenital adrenal hyperplasia (NCCAH), androgen-secreting tumor, and ovarian hyperthecosis[41]. Patients with both hyperandrogenic symptoms and oligomenorrhea are advised to undergo testing for serum total testosterone and an early morning 17-hydroxyprogesterone. Serum total testosterone is determined and identified using liquid chromatography-tandem mass spectroscopy (LC-MS/MS). Severe hyperandrogenism has serum testosterone levels at >150 ng/dL, with normal levels at 45-60 ng/dL[41].

Oligo-ovulation in PCOS

Clinically, women with PCOS, may present with oligo-ovulation or anovulation in coordination with polycystic ovarian morphology (PCOM) and/or increased ovarian size. Oligo-ovulation is defined as menstrual cycles that stray from the normal cycle length of 21-35 days, with menstrual bleeding less frequent than once a month. Oligomenorrhea or amenorrhea, which is the lack of menstrual bleeding, is present in about 70%-80% of women with PCOS. An abnormal ovarian size has an increased volume of 10 mL^3 and/or follicles that are between 2 and 9 mm in size in 1 ovary[42]. Another hallmark of PCOS is the ovary with follicles that have increased resistance to follicle stimulating hormone (FSH). Finally, the presence of increased

anti-Mullerian hormones secreted by preantral follicles in the granulosa cells is associated with this condition[42].

Potential Non-Invasive Biomarkers for PCOS: miRNAs

MiRNAs are the most prominent type of biomarkers used for gynecological disorders. MiRNAs serve multiple critical regulatory functions at the post-transcriptional level including cell proliferation, differentiation, survival and apoptosis, and the stress response. These non-coding molecules are characterized by single strands of RNA that are 18-24 nucleotides in length. These molecules induce degradation or inhibit protein translation after the miRNAs bind to the 3' un-translated regions of mRNAs. MiRNAs regulate the expression of various downstream target genes and through feedback regulation mechanisms, the products of miRNA and their target genes can amplify or inhibit a signal[43]. Therefore, an alteration of any capacity can regulate physiologic cellular functions. During some early studies, it was found that there was a higher expression of miRNA-21, miRNA-27b, miRNA-103, and miRNA-155 in obesity and PCOS[44]. There are about 6 miRNAs, miRNA-93, miRNA-223, miRNA-320, miR-222, miR-146a, and miR-30c that have been associated with PCOS either individually or as part of a panel[4-6].

A recent study found that there was a down regulation of the insulin sensitive-glucose transporter GLUT4 gene expression by miRNA-93 in adipose tissue. MiR-93 is intronic, meaning that it doesn't code for amino acids. Through multiple studies it was hypothesized that miR-93 is transcribed with its host gene, MCM7. It is integrated into the 13th intron of MCM7 and since it is intronic, it could be transcribed, or it could have its own promoter[45]. A different

study concluded that PCOS patients with insulin resistance showed an overexpression of miRNA-93 in adipose tissue, but it was discordant for the expression of its host gene MCM7[45].

MiR-320 has been associated with PCOS and has been shown to be involved in growth, proliferation, and the cell cycle. The miR-320 functions to regulate molecules, especially ET-1, its target gene. ET-1, a molecule produced by endothelial cells, is a bioactive peptide that promotes cell mitosis, participates in tumor growth, and induces mitosis within tumor growth. ET-1 is the most potent biomolecule, but there are two other types of ET including ET-2 and ET-3[4].

Another study focused on miR-222, miR-146a, and miR-30c used in a panel. These were chosen due to miR-222 association with insulin sensitivity along with its marked expression in Type 2 Diabetes[46]. MiR-146a was shown to suppress the release of progesterone, androgens, and estrogens along with general secretion[47]. Finally, all three MiRNAs have shown involvement in signaling pathways such as Wnt, MAPK, and Jak-STAT, apoptosis and endocrine, suggesting a role the pathogenesis of PCOS.

Increased Expression of miRNA-93 in Women with PCOS

Studies have compared miRNA-93 and miRNA-223 expression in women PCOS[48]. MiRNA-93 was studied in comparison with miRNA-223 to determine their correlation to the metabolic indices found in individuals with PCOS compared to the normal controls that were age and BMI matched. In the study, 25 medication naïve women aged 18-45 years with PCOS and biochemical hyperandrogenemia were recruited from the local PCOS biobank. The control consisted of 20 normal women who were also recruited from the PCOS bank aged 20-44 years of whom were also age and BMI matched. The subjects were diagnosed with PCOS using the Rotterdam criteria including evidence of hyperandrogenemia, specifically with a Ferriman-

Gallwey score greater than 8 and free androgen index greater than 4. Additionally, the presence of oligomenorrhea and polycystic ovaries on the transvaginal ultrasound were identified. Conditions such as diabetes mellitus, non-classical 21-hydroxylase deficiency, hyperprolactinemia, Cushing's disease and androgen-secreting tumors were excluded[5].

Both miRNA-93 and mRNA-223 were determined by total RNA reverse transcription and qPCR. Both an unpaired t-test and a ROC curve were used to determine their discriminating effects. The results showed that insulin, HOMA-beta, and testosterone were significantly increased in the PCOS group and HOMA-IR did not differ when looking at all the parameters *(Table 3)*[5]. Both the expression of miRNA-93 and miRNA-223 were increased in PCOS when compared to the control group. The sensitivity, specificity, and AUC of miR-223 and miR-93 were 0.56, 0.72 and 0.60 (95% Cl: 0.5-0.82) and 0.64, 0.76 and 0.72 (95% Cl: 0.58-0.86) respectively[5]. With these results, miR-93 is more efficient than miR-223 as a biomarker for diagnosing PCOS. After the data was analyzed, it was determined that the area under the ROC curve for miRNA-223 and miRNA-93 were 0.66 and 0.72 respectively (*Figure 4*)[5].

Due to the sensitivity and specificity values from the ROC curves being too low, miRNA-93 may not be able to serve as a single diagnostic test, rather it can serve as a positive diagnosis of PCOS rather than being one by exclusion. The authors concluded that there was a correlation between increased circulating levels of miR-93 and miR-233 and elevated insulin and testosterone levels. No association between elevated miRNA levels and insulin resistance or hyperandrogenemia was found. It was strongly suggested that elevated miR-93 expression in the plasma may represent a non-invasive biomarker for the diagnosis of PCOS[5].

Associating MiRNA-320 and ET-1 with the Pathogenesis of PCOS

This particular study involved 60 patients with PCOS and 40 individuals who were part of the control. The PCOS subjects were then divided based on the HOMA-IR or homeostasis model assessment of insulin resistance depending on the presence or absence of IR. ELISA was used to measure ET-1 levels and PCR or the polymerase chain reaction was used to determine MiRNA-320 expression levels[4].

Studies showed that expression levels of miRNA-320 in PCOS patients were significantly lower compared to the control group[4]. It was seen that there was a significant negative correlation between serum miRNA-320 expression levels and ET-1 present (*Figure 5, Figure 6*)[4]. Additionally, PCOS and insulin resistance patients had significantly higher serum levels of ET-1 compared to PCOS women without insulin resistance. It was concluded that miRNA-320 acts through its target ET-1 and inhibits IR in patients with PCOS. MiRNA-320 uses IRS-1 regulating the ERK ½ signaling pathway and miRNA-320 controls pathways involved in follicular maturation[4].

Increased expression of miRNA-222, miRNA-146a and miR-30c with the Pathogenesis of PCOS

In this study, 68 patients who were diagnosed with PCOS were obtained from the Nanjing Maternity and Child Health Hospital ages 23.8 to 29.4 years. 68 healthy controls were also age matched and recruited from the same hospital and were between the ages of 24.5 to 31.3 years. Both hyperandrogenism and chronic anovulation were excluded. During phase I of the study, biomarkers were discovered using TLDA chip assays. During phase II, the quantitative reverse transcription-polymerase chain reaction was used on all subjects to validate the miRNAs

that were screened. Both the Mann-Whitney *U*-test and determining the area under the ROC curve (AUC) and 95% confidence interval (CI) were used for statistical analyses[6].

During phase I of the study, 8 up-regulated mRNAs were found including miR-222, miR-16, miR-19a, miR-106a, miR-30c, miR-146a, miR-24 and miR-186 along with one-down regulated miRNA-320. During phase II, three miRNAs were found to be significantly up regulated in women with PCOS compared to those without. These include miR-222, miR-146a, and miR-30c, with p-values of 0.014, 0.024, and 0.031 respectively. The results showed AUC values of 0.799, 0.706, and 0.688 for miR-222, miR-146a, and miR-30c, respectively. It was also seen that the largest area under the ROC curve was present during the combination of the three miRNAs *(Figure 7)*[6].

Conclusion

It must be noted that there are no biomarkers that are currently being used in a clinical setting. The biomarkers discussed and characterized above for both endometriosis and PCOS are currently pre-clinical studies but based on the studies present as strong suggestive tools for potential diagnosis of the conditions. As stated previously, these non-invasive biomarkers would be particularly helpful in diagnosing benign gynecological conditions early on. The complexity of the diagnosis of endometriosis lies in the fact that invasive surgery or a laparoscopic procedure is needed. On the other hand, the complexity of the diagnosis of PCOS lies in fact that it is a diagnosed based on multiple criteria and particularly exclusion. Overall, it was seen that MiRNAs play a big role in potentially diagnosing both conditions. It would be difficult to rank these biomarkers from least effective to most effective since AUC values can change when the biomarkers are used in as a single diagnostic test as opposed to when the biomarkers are part of a panel.

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Supplemental Materials

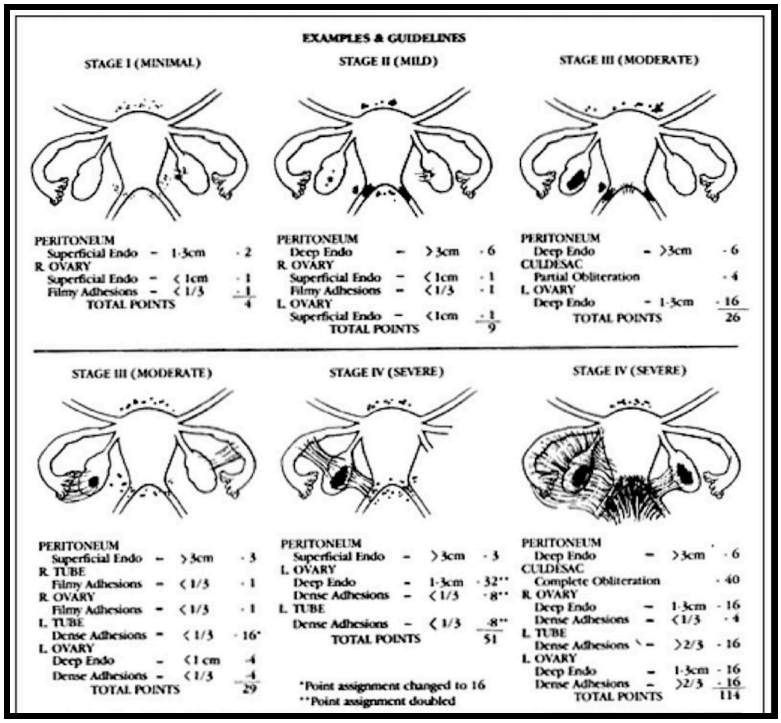

Figure 1. Revised American Society for Repro. Medicine Classification of Endometriosis[10]

Acc. No.	Description	Biomarker score	Protein score	Unique peptides	PSMs	Ratio ES/CS	Ratio ES/PS	Ratio EP/CP	Ratio EcS/ES	Ratio PS/CS
P14384	*Carboxypeptidase M CPM*	26	154	2	6	1.619	2.525	2.451	0.319	0.633
P26599	Polypyrimidine tract-binding protein 1 PTBP1	24.6	264	7	11	2.646	2.771	0.953	0.377	0.787
Q14764	Major vault protein MVP	23	151	3	7	0.143	0.158	0.425	10.978	0.894
P29373	Cellular retinoic acid-binding protein 2 CRABP2	23	83	2	3	2.078	1.612	2.544	1.436	1.382
Q01995	*Transgelin TAGLN*	22.2	2119	17	95	1.105	1.324	2.171	18.832	1.240
Q01105	Protein SET	22.2	937	6	29	0.914	0.919	1.084	0.330	0.881
P00915	Carbonic anhydrase 1 CA1	22	5011	14	263	2.086	2.902	0.933	1.285	0.627
O94788	Retinal dehydrogenase 2 ALDH1A2	21.4	301	9	12	0.838	0.898	1.542	0.456	0.903
P16949	Stathmin STMN1	21.2	293	7	16	1.044	0.926	1.411	0.471	0.994
Q13308	Tyrosine-protein kinase 7 PTK7	21.2	362	3	13	0.510	0.677	1.497	0.293	0.807
P06703	Protein S100A6	21	762	5	47	1.379	1.653	0.795	3.364	0.774
P09466	*Glycodelin PAEP*	20.6	698	2	18	0.622	0.863	0.950	0.210	0.705
P02751	*Fibronectin FN1*	20.4	251	8	9	2.706	1.288	0.630	2.104	2.005
Q96KP4	Cytosolic non-specific dipeptidase CNDP2	20.2	1519	16	43	0.724	1.078	0.535	0.333	0.710
P00167	Cytochrome b5 CYB5A	20	692	7	29	0.835	0.880	0.205	0.979	0.948
P06401	*Progesterone receptor PGR*	20	37	1	1	1.415	1.762	2.420	0.321	0.792
P59665	Neutrophil defensin 1 DEFA1	19.6	137	3	11	0.325	2.476	1.315	4.989	0.133
P17661	Desmin DES	19.6	4279	24	216	0.904	0.930	1.381	3.056	1.063
Q05682	Caldesmon CALD1	19.4	677	6	25	1.162	0.950	1.260	7.685	1.223
Q7KZ85	Transcription elongation factor SPT6 SUPT6H	19.4	53	1	6	2.143	3.101	0.960	0.843	0.682
Q9HC84	Mucin-5B MUC5B	19.2	471	12	18	0.193	3.853	0.386	0.650	0.088
P12111	*Collagen alpha-3(VI) chain COL6A3*	19	687	12	27	1.933	1.254	1.209	18.478	1.597
P51884	*Lumican LUM*	19	1340	13	51	1.021	0.952	1.216	14.892	1.033
P60660	Myosin light polypeptide 6 MYL6	19	2726	12	92	1.378	0.878	1.148	5.003	1.382
P20774	Mimecan OGN	18.6	731	9	28	0.831	0.798	1.212	20.121	0.905
P09493	*Tropomyosin alpha-1 chain TPM1*	18.6	4361	8	186	1.004	1.007	1.245	10.119	0.964
P07951	*Tropomyosin beta chain TPM2*	18.6	4277	6	178	0.988	1.032	1.239	9.162	0.913
P06702	Protein S100A9	18.6	286	3	7	0.406	7.751	2.632	7.255	0.061
P67936	Tropomyosin alpha-4 chain TPM4	18.4	4288	12	201	1.014	1.023	1.230	7.301	0.918
P24821	*Tenascin TNC*	18.4	83	5	5	1.817	1.682	1.502	1.151	1.022
O00264	*PGRMC1*	18.4	191	4	8	1.013	1.035	1.561	0.609	1.228
P52907	F-actin-capping protein subunit a1 CAPZA1	18.2	145	4	7	3.031	1.078	0.885	1.459	1.280
P51888	Prolargin PRELP	18	84	3	5	0.504	0.471	1.466	33.466	1.145
Q05707	*Collagen alpha-1(XIV) chain COL14A1*	18	1210	18	49	1.294	0.834	0.898	10.120	1.426
P21333	Filamin-A FLNA	18	2103	41	77	1.227	0.967	1.509	2.216	1.357

Table 1. High-scoring protein of interest identified by TMT 3D-LC-MS/MS profiling[2].

Models	AUC	Sensitivity	Specificity
E versus C (all phases)			
CA125, sICAM1, CPM	0.768	0.667	0.8
CA125, sICAM1, VEGF	0.777	0.644	0.8
CA125, sICAM1, FST	0.77	0.644	0.8
CA125, sICAM1	0.778	0.6	0.9
CA125, sICAM1, IL1R2	0.758	0.6	0.9
CA125, sICAM1, MCP1	0.757	0.6	0.9
E versus P (all phases)			
sICAM1, FST, TNC	0.679	0.667	0.8
sICAM1, TNC	0.708	0.622	0.8
sICAM1, TNC, Oestradiol	0.68	0.622	0.8
sICAM1, PAEP, TNC	0.695	0.622	0.8
sICAM1, MIF, PAEP	0.697	0.622	0.8
sICAM1, LUM	0.665	0.444	0.9
E versus C + P (all phases)			
CA125, sICAM1, FST, CPM	0.706	0.578	0.8
CA125, sICAM1, VEGF, PAEP	0.71	0.578	0.8
CA125, sICAM1, PAEP	0.719	0.578	0.8
CA125, sICAM1, MIF, PAEP	0.704	0.578	0.8
CA125, MIF	0.621	0.467	0.9
E versus C + P (Proliferative)			
sICAM1, FST, Oestradiol	0.769	0.769	0.8
sICAM1, MIF, FST	0.781	0.692	0.8
sICAM1, FST	0.802	0.692	0.8
CRP, sICAM1, FST	0.802	0.692	0.8
CA125, sICAM1, FST	0.814	0.692	0.8
sICAM1, MIF, FST	0.781	0.615	0.9
E versus C + P (Secretory)			
CA125, MIF, PAEP	0.705	0.654	0.8
CA125, sICAM1, MIF	0.725	0.615	0.8
CA125, MIF, TNC	0.683	0.615	0.8
CA125, MIF, PAEP	0.705	0.538	0.9
CA125, MIF, TNC	0.683	0.577	0.9

Table 2. Performance of cross-validated multi-marker models for discriminating Endometriosis from control groups[2].

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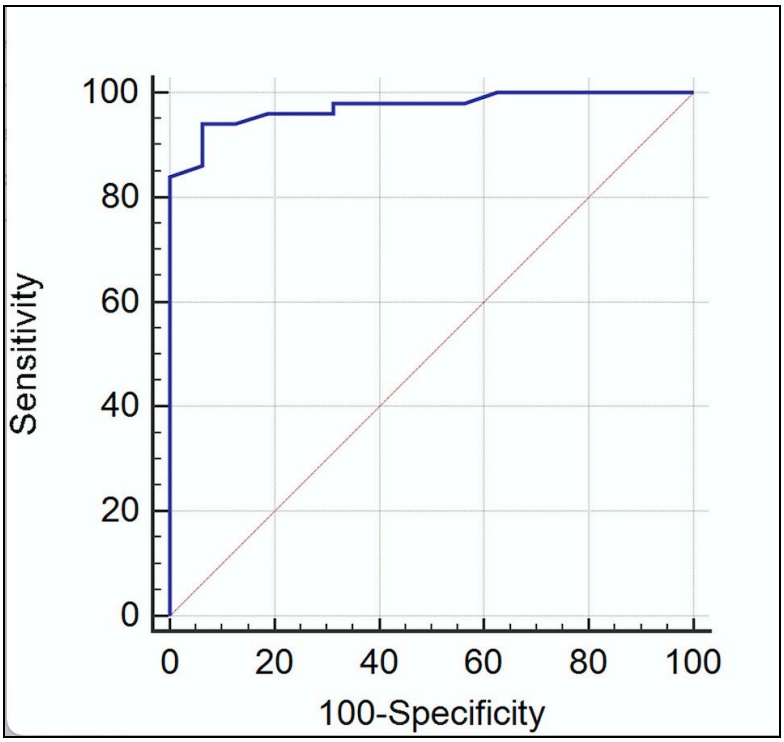

Figure 2. ROC curve and AUC of the serum Gal-9 ELISA[1]

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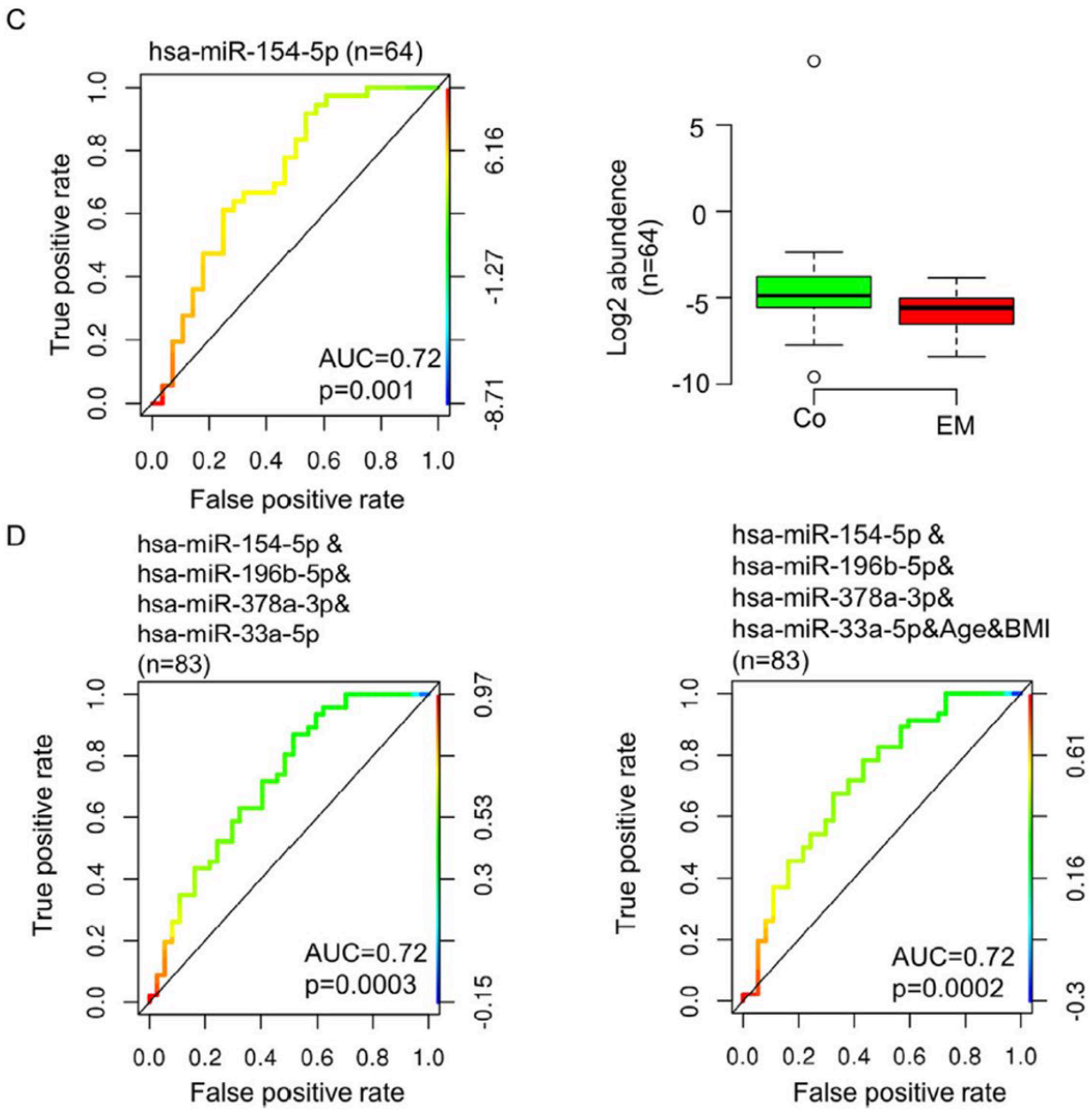

Figure 3. (C) Diagnostic value of hsa-miRNA-154-5p for endometriosis expressed by ROC curve analysis (left) of the *log2* transformed expression data (right) obtained in a cohort of n=64 cases and controls. The AUC and P-values are indicated in the graph. (D) Diagnostic performance of the combined four DE miRNA in a cohort of n=83 when analyzed together (left) or in combination with the clinical parameters age and BMI (right) is graphically presented. The AUC and corresponding P-values for each test are indicated in the graphs[3].

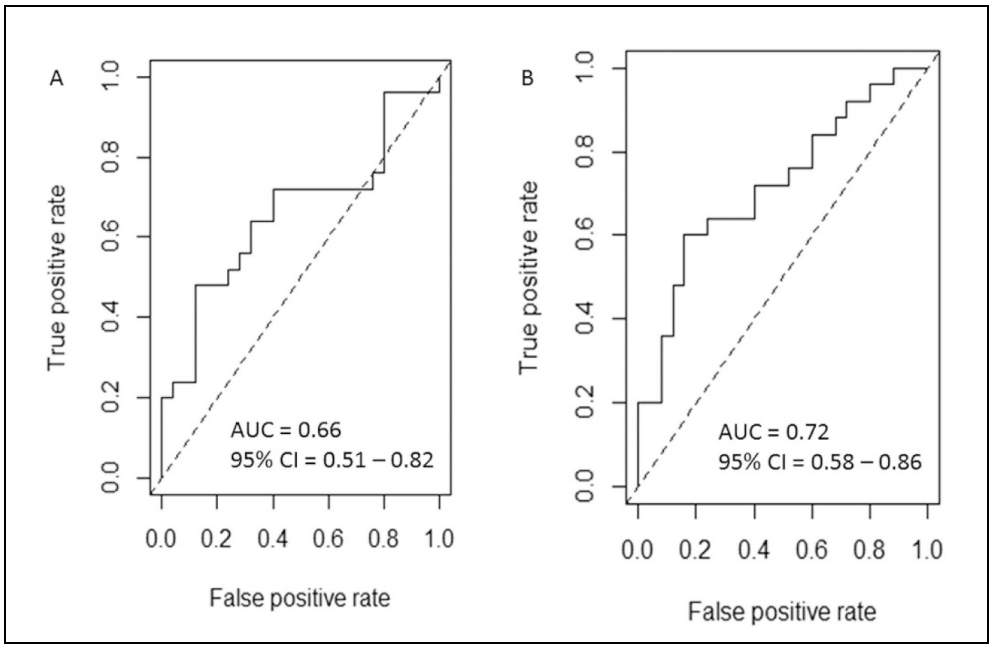

Figure 4. ROC curve analysis of (A) miR-233 and (B) miR-93 to discriminate women with PCOS from healthy controls[5].

	Normal n = 25	PCOS n = 25	P value
	Mean (SD)	Mean (SD)	
Age (years)	32.2(7.7)	32.1(9.0)	0.97
Weight (kg)	76.0(18.8)	77.4(16.3)	0.79
BMI (kg/m^2)	27.1(5.8)	28.8(5.4)	0.31
Fasting glucose (mmol/l)	4.7(0.4)	4.8(0.6)	0.56
2 Hour glucose (mmol/l)	4.9(1.2)	5.7(1.3)	0.06
Androstenedione (nmol/l)	8.4(5.1)	10.7(6.7)	0.25
ALT (IU/L)	24.3(14.4)	23.3(12.7)	0.79
Insulin (μU/ml)	6.8(3.7)	10.2(6.4)	0.03
HOMA-IR	1.5(0.9)	2.3(1.7)	0.07
HOMA-β	38.4(66.1)	158.8(89.7)	0.001
Testosterone (nmol/L)	1.2(0.7)	2.3(1.6)	0.03
SHBG (mmol/L)	81.8(105.8)	52.6(53.1)	0.29
FAI	2.6(1.7)	11.1(18.9	0.13
hsCRP (mg/l)	2.3(3.8)	2.8(3.9)	0.65
miR-93 expression (relative to control group)	1.0(0.6)	2.0(1.6)	0.009
miR-223 expression (relative to control group)	1.0(0.7)	2.01(2.2)	0.029

Table 3. **Demographics, biochemical and clinical markers for the PCOS and control group (unpaired t test)** (BMI, body mass index; FAI, free androgen index; SHBG, sex hormone binding globulin; HOMA-IR, homeostatic model assessment-insulin resistance; HOMA-beta, homeostatic model assessment-insulin beta cell sensitivity; hsCRP, high sensitivity C-reactive protein.)[5].

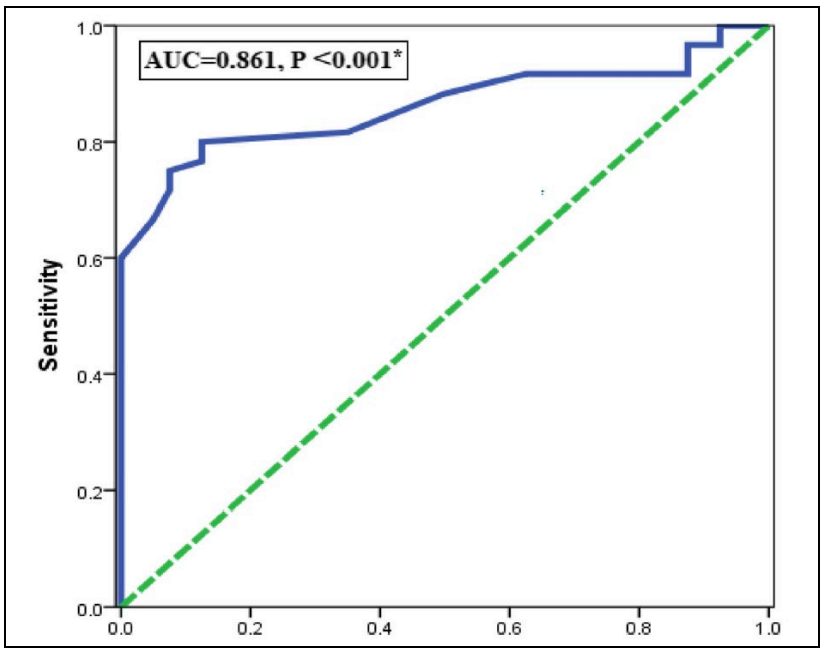

Figure 5. ROC curve for serum miRNA-320 expression level or prediction of PCOS[4].

5

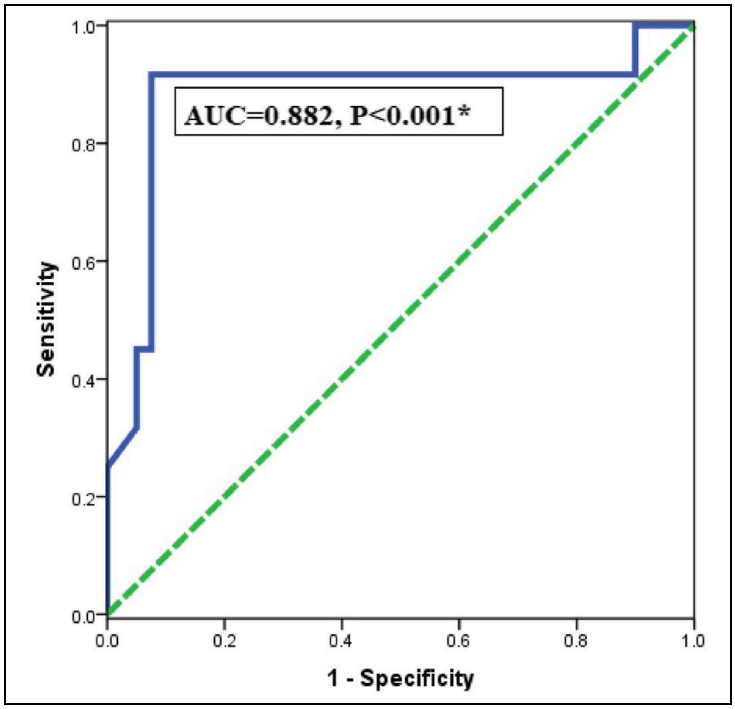

Figure 6. ROC curve for serum ET-1 expression level or prediction of PCOS[4].

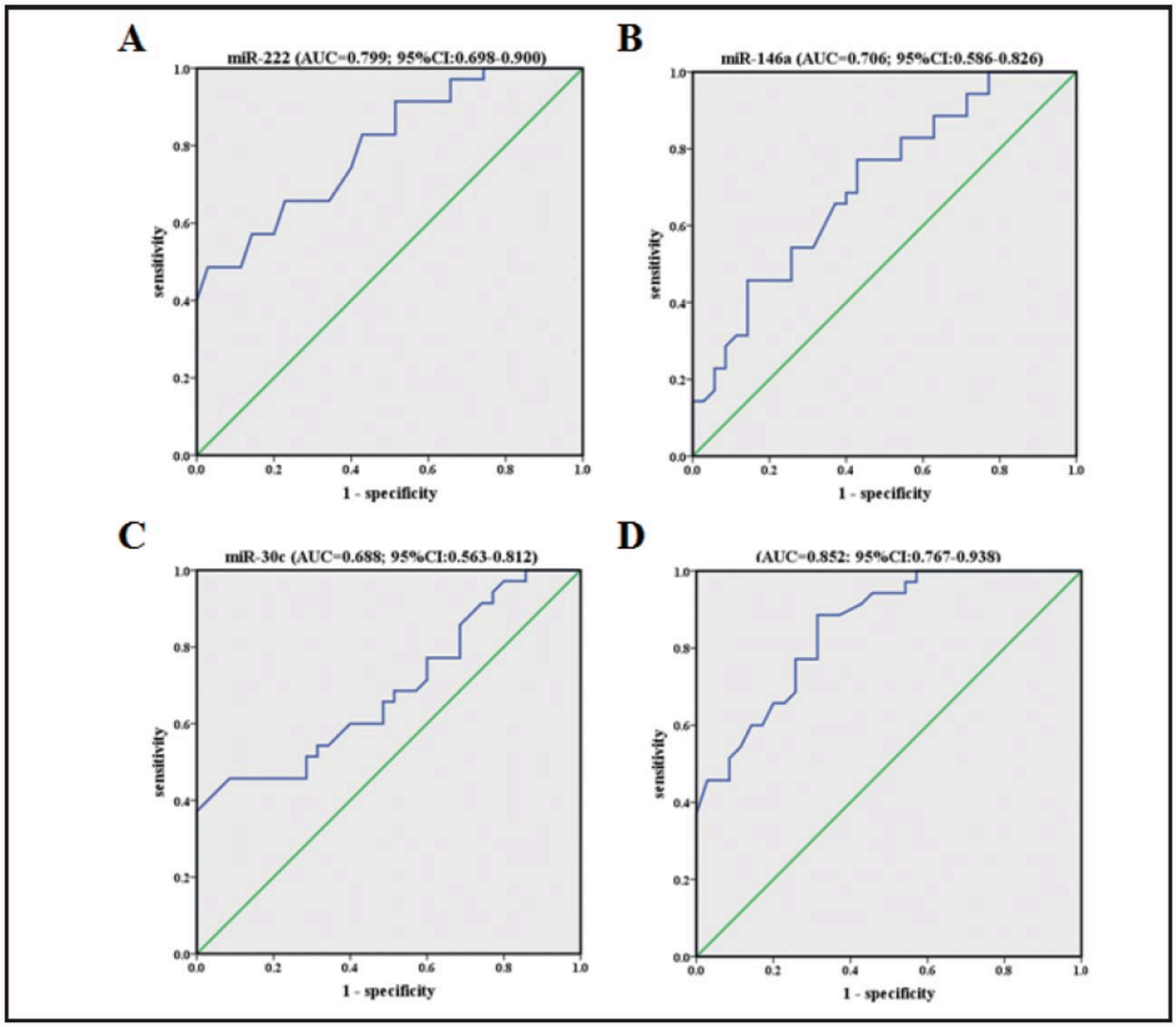

Figure 7. ROC curve analysis using three miRNAs to discriminate women with PCOS from healthy controls. (A) miR-222, (B) miR-146a, (C) miR-30c, (D) ROC curve of the combination of the 3 miRNAs using multiple logistic regression analysis. The combination of the three miRNAs (miR-222, miR146-a and miR-30c) yielded the largest area under the ROC curve (AUC)[6].