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Circulating plasma microRNAs as potential non-invasive biomarkers in infertile women with adenomyosis: an observational study

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Abstract

Background Despite advancements in imaging techniques, the non-invasive diagnosis of adenomyosis remains particularly challenging due to the overlap of imaging features with other uterine diseases, and the lack of consistent correlation with clinical presentation, with histological confirmation after hysterectomy still necessary. In this context, circulating miRNAs emerge as promising candidates for accurate non-invasive diagnosis.

Methods To determine whether women with adenomyosis exhibit a distinct circulating plasma miRNA profile that can serve as a diagnostic marker, miRNA sequencing was performed to compare the plasma miRNA profile of women with adenomyosis with infertile and healthy fertile controls ($n = 10$/group). Adenomyosis was diagnosed by transvaginal ultrasound using Morphological Uterus Sonographic Assessment (MUSA) criteria. Plasma and endometrial biopsies were collected during the secretory phase from women undergoing hormone replacement therapy. Total RNA was extracted from plasma, libraries were prepared using NEXTFLEX® Small RNA-Seq v4 kits, and miRNAseq was performed on the NextSeq 550 NGS platform. Differentially expressed (DE) miRNAs were identified using the edgeR method (false discovery rate < 0.05). The discriminatory potential of DEmiRNAs was evaluated by receiver operating characteristic (ROC) curve analysis using the area under the curve (AUC). Validation was performed by quantitative PCR on plasma and endometrium ($n = 15$/group). The target genes of DEmiRNAs were retrieved from miRTarBase and subjected to functional enrichment analysis.

Results A total of 11 disease-specific DEmiRNAs were identified in adenomyosis compared to infertile controls (5 up- and 6 downregulated), and 23 healthy control-referenced DEmiRNAs compared to fertile controls (19 up- and 4 downregulated). Among disease-specific DEmiRNAs, miR-4433 and miR-652-5p demonstrated strong diagnostic performance for adenomyosis compared to infertile controls (AUC = 0.92 and 0.91, respectively; $p < 0.001$). For healthy control-referenced DEmiRNAs, 11 miRNAs showed statistically significant diagnostic value ($p < 0.01$), with the highest AUCs observed for miR-3200-5p, miR-193b-5p, and miR-4732-5p (AUC = 0.88, 0.88 and 0.87, respectively). Validation experiments corroborated the upregulation of miR-4433 and miR-92a-3p upregulation in plasma and endometrium.

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Conclusions This study supports the potential of circulating miRNAs as non-invasive biomarkers for adenomyosis and contributes to understanding its molecular basis. The concordance between circulating and endometrial miRNA expression suggests a possible link between systemic molecular alterations and endometrial pathology.

Keywords Adenomyosis, MicroRNAs, Non-invasive diagnosis, Plasma, Biomarkers

Background

Adenomyosis is a heterogeneous, benign gynaecological condition characterised by the presence of endometrial glands and stroma within the myometrium [1]. Heterogeneity in disease presentation across different women [2] complicates the classification and diagnosis of adenomyosis. The true prevalence of adenomyosis is unclear, with estimates ranging from 8.8% to 61.5% [3]. Since the diagnostic gold standard for adenomyosis has been histological confirmation after hysterectomy, most prevalence estimates are derived from this highly selected population, introducing potential bias, and overestimating the prevalence. Conversely, prevalence may also be underestimated, as cases that do not require hysterectomy or go unnoticed by clinicians due to the lack of severe symptoms are missed [3]. Advancements in imaging, including transvaginal ultrasound (TVUS) and magnetic resonance imaging (MRI), have facilitated the identification of adenomyosis cases [4] with high levels of sensitivity and specificity [5–7]. However, although the international Morphological Uterus Sonographic Assessment (MUSA) group proposed consensus terminology and a classification system [8–10], limitations remain regarding inter-rater agreement [11, 12], definitions, and size thresholds [13]. Therefore, the development of non-invasive and reliable biomarkers that complement imaging techniques is essential to improve adenomyosis diagnosis.

In this context, given their unique characteristics, microRNAs (miRNAs) have emerged as candidates for precise, non-invasive diagnostic applications [14]. Specifically, circulating miRNAs show specificity, accessibility, and sensitivity, remain stable in diverse biological fluids, and frequently exhibit strong tissue- or cell-type specificity [15]. In the context of adenomyosis, miRNAs have been studied in eutopic and ectopic endometrium [16, 17], in exosomes secreted by endometrial stromal cells [18], and in endometrial epithelial organoids [19]. More recently, Shao et al. used arrays to evaluate miRNA expression in exosomes from plasma, ectopic endometrium, and urine samples from women with symptoms of adenomyosis [20]. This study aimed to use miRNA sequencing to determine whether women with adenomyosis exhibit a distinct circulating plasma miRNA profile that can be used as a diagnostic marker.

Materials and methods

Study design and population

We performed a comparative analysis including three groups of women. A total of 44 women were enrolled at IVI Valencia and IVI Madrid Clinics, distributed as follows: (1) healthy fertile controls: healthy egg donors with no ultrasound evidence or clinical suspicion of gynaecological disorders ($n = 15$); (2) infertile controls: infertile women with no ultrasound evidence or clinical suspicion of gynaecological disorders ($n = 15$); and (3) women with adenomyosis: infertile women diagnosed with adenomyosis ($n = 14$). The primary analyses were conducted as two independent pairwise comparisons to evaluate adenomyosis against the two different control groups: (i) adenomyosis versus infertile controls, and (ii) adenomyosis versus healthy fertile controls. No direct comparison was performed between the two control groups.

Women were eligible for inclusion if they met the following criteria: aged 18–45 years, BMI ≤ 30 kg/m^2, and had not received any systemic hormonal treatment that may affect endometrial function, including hormonal contraceptives, ovulation inducers or GnRH analogues, within the six months prior to enrolment and sample collection. A total of 10 women per group were included in the study cohort. For plasma validation, additional women were included in the healthy fertile control ($n = 5$), infertile control ($n = 5$), and adenomyosis ($n = 4$) groups.

Diagnosis of adenomyosis

All the women were examined by TVUS and/or MRI. Adenomyosis was diagnosed using MUSA criteria, which include direct (cysts, hyperechoic islands, echogenic subendometrial lines, and buds) and indirect (asymmetrical thickening, fan-shaped shadowing, translational vascularity, globular uterus, irregular junctional zone, and interrupted junctional zone) signs, with the presence of at least one direct sign required for diagnosis [10].

Sample collection and blood processing

Samples were collected from women during the endometrial secretory phase, specifically five days after the initiation of progesterone treatment in a hormonally prepared cycle (P + 5). The women were prescribed 6 mg/day of Estradiol Meriestra (Sandoz Farmacéutica, Spain) from days 1–4 of the menstrual cycle. On day 10, endometrial thickness and serum progesterone levels were checked. Once the endometrial lining was > 6.5 mm and

progesterone was < 1 ng/mL, the women received progesterone (Cyclogest, Gedeon Richter, Spain) at 400 mg/day for 5 days (P + 5). Blood samples were collected in EDTA-containing tubes and centrifuged at 3,600 rpm for 10 min at room temperature to obtain plasma. For miRNA validation, endometrial biopsies were simultaneously collected from the uterine fundus using a catheter, under sterile conditions.

RNA extraction and library construction

Total RNA, including small RNAs, was extracted from 200 µL of plasma using a miRNeasy Serum/Plasma Advanced Kit (Qiagen, Hilden, Germany). For microRNA (miRNA) sequencing, libraries were prepared from total RNA using a NEXTFLEX® Small RNA-Seq v4 Kit with Unique Dual Indices (UDIs; Revvity Inc., Massachusetts, USA). Library quality and concentration were assessed using an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) with High-Sensitivity DNA chips. Subsequently, an equimolar pool of libraries was prepared and sequenced on the NextSeq 550 platform (Illumina, San Diego, CA, USA) using single-end sequencing with 50 cycles (1 × 50 bp).

microRNA sequencing data processing

Sequencing data were processed and statistically analysed in R (version 4.5.1) using packages from Bioconductor (version 3.21). Raw FASTQ files were processed to remove adapters and clean the sequences using the following programs: FastQC [21], BBduk [22], Subread [23, 24], MultiQC [25], and edgeR [26]. On average, sequencing yielded 11.63 million reads per sample before processing and 10.85 million reads after processing. miRNAs were mapped to the human genome (GRCh38) using the Subread aligner and annotated with the miRBase database, which includes 2,562 miRNAs [27]. After read mapping and quantification with the featureCounts function from the Rsubread package (version 2.22.1), all samples had more than 1 million reads and were successfully assigned to annotated miRNAs, which was set as the minimum threshold for inclusion in the analysis. miRNAs with zero counts across all samples were removed, resulting in a total of 1,546 miRNAs. Read counts for these miRNAs were then normalised using the Trimmed Mean of M-values (TMM) method [28] and expressed as counts per million (CPM), as implemented in the edgeR package (version 4.6.3). In the exploratory analyses, after preprocessing, quality control, and data normalisation, one sample from the healthy fertile control group was identified as an outlier. Due to its high variability, it was excluded from the final analysis to ensure the robustness and reliability of the results.

Differential expression analysis and functional enrichment

Differentially expressed miRNAs (DEmiRNAs) were identified using the edgeR method, considering those with a false discovery rate (FDR) < 0.05 as significant. Two independent comparisons were performed to address complementary biological questions: adenomyosis versus infertile controls (comparison 1) and adenomyosis versus healthy fertile controls (comparison 2). The first comparison was designed to identify DEmiRNAs in association with adenomyosis within a clinically comparable infertile population (disease-specific DEmiRNAs), whereas the second comparison was intended to investigate whether miRNA alterations observed in adenomyosis were also evident when compared with a healthy fertile population to account for potential effects in the reproductive physiology (healthy control-referenced DEmiRNAs). DE miRNA target genes were retrieved from a curated human miRNA–mRNA interaction database (miRTarBase; IDs aligned to miRbase, version 21). The resulting gene lists were then subjected to enrichment analysis using the Over-Representation Analysis (ORA) method, implemented in the clusterProfiler package (version 4.16.0) [29]. Gene Ontology (GO) enrichment was assessed across the three main GO categories: biological process (BP), molecular function (MF), and cellular component (CC). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was also performed. GO terms and KEGG pathways were considered significantly enriched when the FDR was < 0.05.

Receiver operating characteristic curve analysis

CPMs from the DEmiRNAs were used to generate receiver operating characteristic (ROC) curves and calculate areas under the curve (AUC) to compare the predictive value of miRNAs for adenomyosis. To minimise the influence of outliers, data points exceeding three standard deviations from the mean were excluded. p values < 0.05 were considered statistically significant.

Validation of microRNA expression in plasma

The expression of selected DEmiRNAs from each differential expression analysis was validated in plasma samples by quantitative PCR (qPCR). Validation was performed for each pairwise comparison, following the same contrasts used in the miRNA sequencing analysis: adenomyosis versus infertile controls (comparison 1), and adenomyosis versus healthy fertile controls (comparison 2). These analyses were performed in the samples used for miRNA sequencing and in an independent cohort of patients. RNA extraction from the independent cohort comprising healthy fertile controls (n = 5), infertile controls (n = 5), and women with adenomyosis (n = 4), was performed as described in " RNA extraction and library construction" section. Reverse transcription was

performed on both the miRNAseq cohort and the independent cohort using the miRCURY LNA RT Kit (Qiagen, Hilden, Germany). qPCR was carried out on a ViiA 7 Real-Time PCR system (Applied Biosystems; Massachusetts, USA) using a miRCURY LNA SYBR Green PCR Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. miRNA expression was quantified using miRCURY LNA miRNA PCR Assays (Qiagen) (Supplementary Methods). The qPCR validation analyses were conducted as pairwise comparisons, consistent with the analytical strategy employed in the miRNA-seq analysis. Relative miRNA expression levels were calculated using the comparative Ct (2^-ΔΔCt) method and normalised to stably expressed endogenous miRNAs used as reference controls. Briefly, Ct values of each target miRNA were first normalized to the endogenous reference control to obtain ΔCt values. The mean ΔCt of the control group was used as the calibrator, and ΔΔCt values were calculated for each sample as the difference between the sample ΔCt and the mean ΔCt of the control group. Relative expression levels (fold change) were then determined as 2^ – ΔΔCt. Using this approach, the average expression level of the control group is normalized to 1 while preserving the variability among individual samples. Since the data are represented by the median, the central value displayed for the control group does not necessarily correspond to 1. Candidate reference miRNAs were identified from the sequencing data by selecting miRNAs that showed stable expression across all comparisons of interest, defined as an absolute log fold change (logFC) < 0.1 and a log counts-per-million (logCPM) value > 10. These criteria were chosen to identify highly expressed miRNAs with negligible expression differences between groups. Further details on endogenous miRNA selection, including logFC and logCPM values, are provided in the Supplementary Methods.

Endometrial expression analysis of plasma-identified microRNAs

To assess whether plasma miRNA alterations reflect molecular changes in the affected tissue, the expression levels of the DEmiRNAs identified in plasma from each differential expression analysis were subsequently measured in endometrial samples from the same women included in miRNAseq analysis (n = 10/group). Total RNA, including microRNAs, was extracted from endometrial biopsies using a miRNeasy Mini Kit (Qiagen Hilden, Germany) according to the manufacturer's instructions. Reverse transcription, qPCR, and relative expression analyses were performed as described in the previous section.

Table 1 Characteristics of women included in the study

	Adenomyosis Group	Infertile Control Group	Fertile Control Group	ANOVA p value
Age (years)	42.2 ± 2.5**	40.6 ± 3.5**	35.1 ± 9.6	$p < 0.0001$
BMI (kg/m^2)	23.8 ± 3.2	20.9 ± 2.1	23.5 ± 3.5	$p = 0.1068$

Data are the mean ± SD. BMI, body mass index

* $p < 0.05$, ** $p < 0.01$ versus fertile controls (Tukey post hoc following one-way ANOVA)

Statistical analysis and plots

Statistical analyses were performed using GraphPad Prism 8.0. Normality of continuous variables was assessed with the Kolmogorov–Smirnov test. Group differences were analysed using ANOVA for parametric data (age and body mass index [BMI]) with Tukey's multiple comparison test. For validation and endometrial expression analyses, Mann–Whitney U tests were used owing to the small sample size and the inability to assume a normal distribution of the data. Results are presented as median ± interquartile range (IQR) for non-normally distributed variables. p values < 0.05 were considered statistically significant. Omics data were analysed using R (version 3.5.1) and visualised using the R core, gplots, and ggplot2 packages. The SRplot bioinformatics tool [30] was also used for data visualisation and graphing.

Results

Patient characteristics

As shown in Table 1, BMI values were comparable and not significantly different between groups. However, women in the healthy fertile control group were significantly younger than those in the infertile and adenomyosis groups, while no significant differences were found between the infertile control and adenomyosis groups. Despite the age difference, Pearson correlation analysis between age and miRNA expression, with false discovery rate adjusted p values, showed no significant correlations (adjusted $p > 0.05$ for all miRNAs). Therefore, age was not included as a covariate in the subsequent analysis.

Disease-specific differentially expressed miRNAs: adenomyosis versus infertile control

In order to identify disease-specific DEmiRNAs and reduce the potential cofounding effect of infertility itself and to identify for DEmiRNAs more specifically associated with adenomyosis within a clinically comparable infertile population, adenomyosis patients were compared to infertile controls. Sequencing analysis revealed a distinct miRNA expression profile in plasma from women with adenomyosis compared with infertile controls (Supplementary Table 1). Specifically, 11 DEmiRNAs were identified in adenomyosis patients versus infertile controls (disease-specific DEmiRNAs): 5 upregulated and 6 downregulated (Fig. 1A).

Some miRNAs derived from the same precursor or from closely related genomic loci (e.g., miR-122-5p and

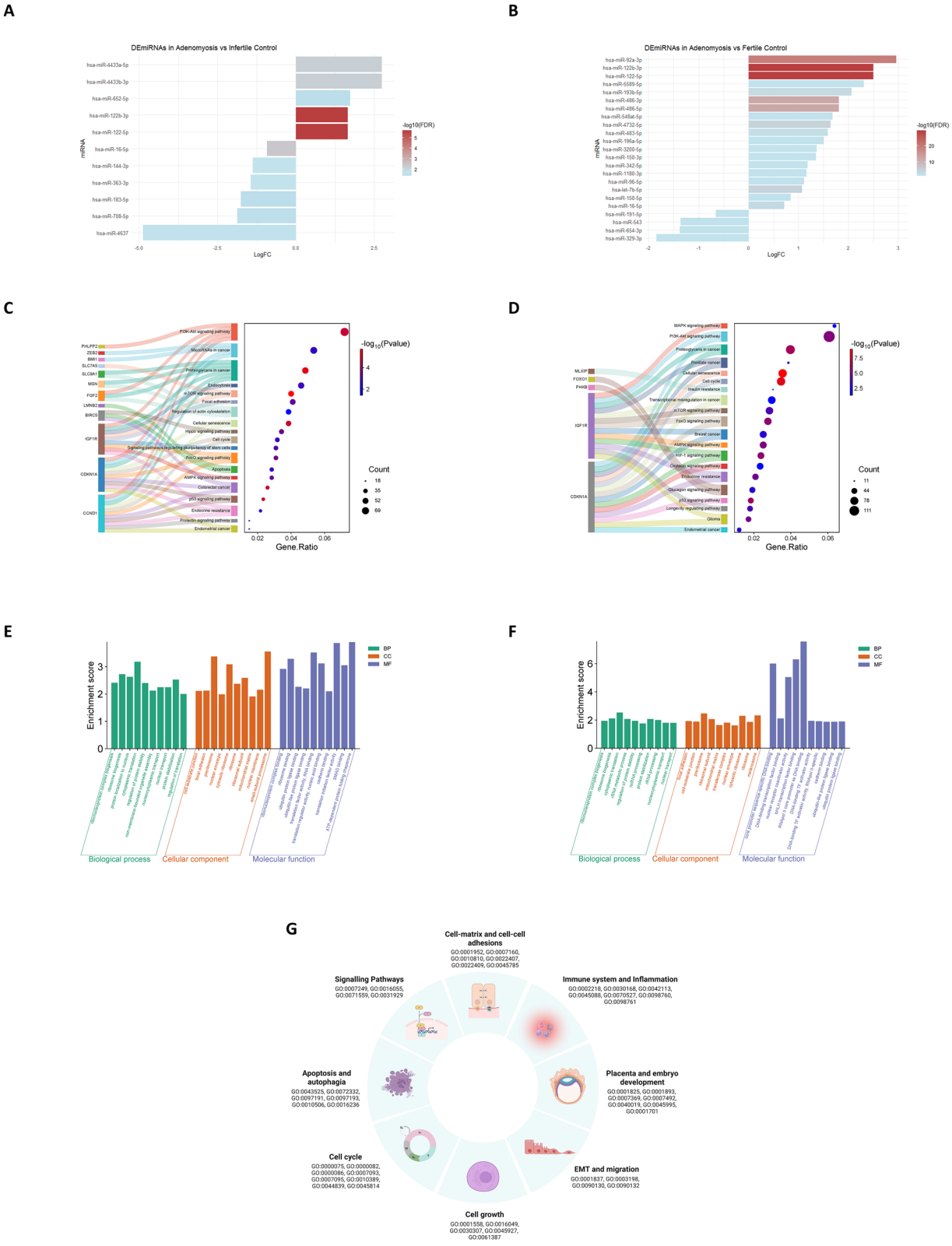


Fig. 1 (See legend on next page.)

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Fig. 1 Differentially expressed microRNAs (DEmiRNAs) in plasma of patients with adenomyosis and functional enrichment of the predicted target genes candidate diagnostic biomarkers. Differential expression analysis of microRNAs in plasma from women with adenomyosis compared with infertile controls (specific-disease DEmiRNAs) **A** or fertile controls (healthy control-referenced DEmiRNAs) **B**. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of the predicted target genes of the DEmiRNAs in plasma from women with adenomyosis compared with infertile controls **C** or fertile controls **D**. A Sankey diagram depicts the associations between target genes and their corresponding KEGG pathways. The bubble plot summarises the enriched pathways, with bubble size representing the number of genes involved and colour indicating the enrichment significance (−log10 *p*). Gene Ontology (GO) enrichment of the predicted target genes of the DEmiRNAs in plasma from women with adenomyosis compared with infertile controls **E** or fertile controls **F**. Bar plots show the enriched GO terms classified into three categories: biological process (BP, green), cellular component (CC, orange), and molecular function (MF, blue). The enrichment score reflects the significance of enrichment for each term, with higher scores indicating stronger enrichment. Functional groups of BP terms common to both comparisons with adenomyosis are also shown **G**

miR-122b-3p; and miR-486-3p and miR-486-5p) showed similar or highly similar expression patterns. Given their sequence similarity and shared origin, they were considered functionally equivalent and grouped as single entities for analytical purposes. Therefore, miR-122 and miR-486 were grouped, resulting in a total of 9 disease-specific DEmiRNAs.

Healthy control-referenced differentially expressed miRNAs: adenomyosis versus fertile control

The miRNA plasma profile of patients with adenomyosis was compared to healthy fertile patients, in order to evaluate adenomyosis-associated miRNA alterations relative to a physiological reproductive state and identify healthy control-referenced DEmiRNAs (Supplementary Table 1). The analysis revealed 23 DEmiRNAs: 19 upregulated and 4 downregulated (Fig. 1B), showing that patients with adenomyosis showed a distinct plasma miRNA expression profile compared to a healthy fertile population.

Some miRNAs derived from the same precursor or from closely related genomic loci (miR-122-5p and miR-122b-3p; and miR-4433a-5p and miR-4433b-3p) were grouped (miR-122 and miR-4433) resulting in a total of 21 healthy control-referenced DEmiRNAs.

Functional implications of the presence of microRNA target genes in plasma from women with adenomyosis compared with infertile women

When compared with infertile controls, the specific-disease DEmiRNAs found in plasma from women with adenomyosis targeted 423 genes (Supplementary Table 2), of which 52 were targeted by three or more miRNAs and were considered highly relevant (Supplementary Table 3). Functional enrichment analysis identified 83 KEGG pathways (Supplementary Table 4), including PI3K–AKT, mTOR, Hippo, FoxO, AMPK, p53, and prolactin signalling, proteoglycans in cancer, focal adhesion, cellular senescence, cell cycle, apoptosis, and endometrial cancer (Fig. 2A). Notably, *CCND1* (cyclin D1), *CDKN1A* (cyclin dependent kinase inhibitor 1 A), and *IGF1R* (insulin-like growth factor 1 receptor) were involved in many of the enriched pathways, suggesting a potential role in the pathogenesis of adenomyosis (Fig. 1C).

Gene Ontology (GO) analysis revealed a total of 1,153 enriched terms, 880 biological process (BP), 166 cellular component (CC), and 107 molecular function (MF) terms (Supplementary Table 5). The top 10 GO terms for each category (BP, CC, and MF) were primarily associated with translation and ribosome biogenesis, nuclear transport, protein stability regulation, and cellular structural organisation, highlighting their potential involvement in both intracellular signalling and structural remodelling, which are relevant to the pathophysiology of adenomyosis (Fig. 1E).

Functional implications of microRNA target genes in plasma from women with adenomyosis compared with fertile women

The healthy control-referenced DEmiRNAs present in plasma from women with adenomyosis compared with healthy fertile controls targeted 1,800 genes (Supplementary Table 6), of which 19 were targeted by six or more and were considered highly relevant (Supplementary Table 7). Functional enrichment analysis identified 186 KEGG pathways (Supplementary Table 8), including MAPK, PI3K–AKT, mTOR, AMPK, HIF-1, and p53 signalling, proteoglycans in cancer, cellular senescence, cell cycle, insulin resistance, and endometrial cancer (Fig. 1D). Among the highly relevant genes, *CDKN1A* and *IGF1R* were involved in many of the enriched pathways. Interestingly, most of the enriched pathways, and genes primarily associated with them, overlapped with those identified in the comparison between adenomyosis and infertile controls (Figs. 1C and D).

GO analysis revealed a total of 1,632 enriched terms, 1,221 BP, 223 CC, and 188 MF (Supplementary Table 9). Remarkably, most of the top 10 GO terms for each category (Fig. 1F), including ribonucleoprotein complex and ribosome biogenesis, protein stabilisation, focal adhesion, mitochondrial matrix, cadherin binding, and ubiquitin protein ligase binding, overlapped with those identified in the first comparison (Figs. 1E and F).

A deeper analysis of the BP overlapping between both comparisons ($n = 688$) revealed enrichment of terms related to adenomyosis pathogenesis, including cell–matrix and cell–cell adhesions, immune system and inflammation, placenta and embryo development,

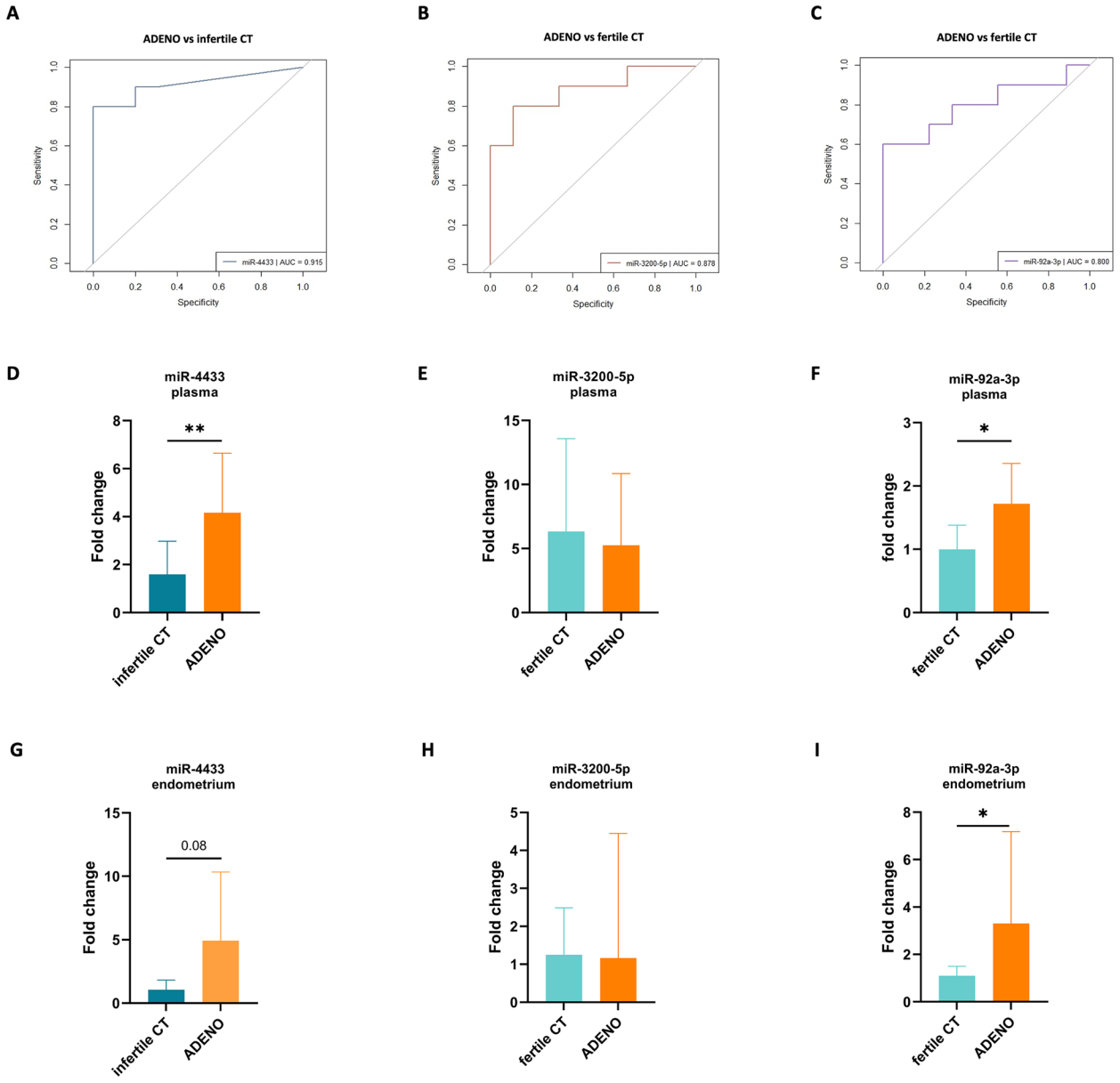


Fig. 2 Diagnostic performance evaluation of candidate biomarkers. Receiver operating characteristic (ROC) curves illustrating the classification performance of miR-4433 to identify women with adenomyosis compared with infertile controls **A**; and miR-3200-5p **B** and miR-92a-3p **C** to identify women with adenomyosis compared with fertile controls. The area under the curve (AUC) with its 95% confidence interval (CI) is shown as a measure of overall discriminative ability. Expression levels of candidate miRNAs in plasma **D-F** and endometrial tissue **G-I**. Data are represented as median ± interquartile range. * $p < 0.05$, ** $p < 0.01$

epithelial-to-mesenchymal transition (EMT) and migration, cell growth, cell cycle, apoptosis and autophagy, and the WNT, TGF, and TOR signalling pathways (Fig. 1G).

Predictive diagnostic values of circulating microRNAs in plasma from women with adenomyosis

The predictive diagnostic values of the miRNAs identified as dysregulated in plasma from women with adenomyosis were compared via ROC curve analysis. From the nine specific-disease DEmiRNAs identified in adenomyosis compared with the infertile controls, two exhibited excellent power to distinguish adenomyosis from infertile controls (Table 2). Specifically, the AUC of miR-4433 and miR-652-5p was 0.92 and 0.91, respectively ($p < 0.001$), indicating strong diagnostic performance.

For the healthy control-referenced DEmiRNAs (adenomyosis versus healthy fertile control group), the AUC was significant for 11 miRNAs (Table 2). Finally, miR-3200-5p (AUC = 0.88, $p < 0.001$), miR-193b-5p (AUC = 0.88,

Table 2 Receiver operating characteristic curves for individual microRNAs

miRNA	AUC	95% Confidence Interval	*p* value
Disease-specific DEmiRNAs (Adenomyosis vs. Infertile Control Group)			
miR-4433	0.92	0.78–1.00	$p < 0.001$
miR-652-5p	0.91	0.79–1.00	$p < 0.001$
Healthy control-referenced DEmiRNAs (Adenomyosis vs. Fertile Control Group)			
miR-3200-5p	0.88	0.72–1.00	$p < 0.001$
miR-193b-5p	0.88	0.69–1.00	$p = 0.01$
miR-4732-5p	0.87	0.67–1.00	$p = 0.01$
miR-191-5p	0.86	0.61–1.00	$p = 0.01$
miR-548at-5p	0.84	0.64–1.00	$p = 0.01$
miR-1180-3p	0.84	0.64–1.00	$p = 0.01$
miR-342-5p	0.83	0.60–1.00	$p = 0.02$
miR-654-3p	0.82	0.62–1.00	$p = 0.02$
miR-329-3p	0.81	0.61–1.00	$p = 0.03$
miR-543	0.81	0.58–1.00	$p = 0.03$
miR-92a-3p	0.80	0.59–1.00	$p = 0.03$

AUC, Area Under the Curve

$p = 0.01$), and miR-4732-5p (AUC = 0.87, $p = 0.01$) showed the highest discriminative abilities.

Validation of specific-disease DEmiRNAs in plasma

The expression of miR-4433 was evaluated by qPCR in plasma samples from the study and validation cohorts, since it is the miRNA with the highest predictive value among the specific-disease DEmiRNAs (adenomyosis vs. infertile controls) (Fig. 2A). There was a significant increase in miR-4433 in plasma from women with adenomyosis compared with infertile controls (median ± IQR = 2.9 ± 1.3 vs. 0.65 ± 1.5, $p = 0.0028$; Fig. 2D).

Validation of healthy control-referenced DEmiRNAs in plasma

For DEmiRNAs identify when comparing adenomyosis versus fertile controls (healthy control-referenced DEmiRNAs), the expression of miR-3200-5p and miR-9a-3p (Fig. 2C) was evaluated by qPCR in plasma samples from the study and validation cohorts. miR-3200-5p (Fig. 2B) showed the highest predictive value, and miR-92a-3p was included based on its diagnostic performance and previously described involvement in adenomyosis, miR-92a-3p (Fig. 2C) [31]. While no significant differences were observed in the expression of miR-3200-5p (Fig. 2E), miR-92a-3p expression was significantly higher in the adenomyosis versus infertile control groups (median ± IQR = = 1.25 ± 1.7 vs. 0.88 ± 0.52, $p = 0.01$; Fig. 2F).

Endometrial expression mirrored circulating microRNA alterations

Based on their diagnostic relevance in plasma, DEmiRNAs from each plasma-based comparison were further evaluated in endometrial samples from the study cohort to determine whether circulating alterations were mirrored in the affected tissue. For DEmiRNAs with good diagnostic performance identified in comparison 1, the results showed an overexpression of miR-4433 in endometrial tissue from women with adenomyosis compared with the infertile controls (median ± IQR = 2.2 ± 4.5 vs. 0.8 ± 1.4, $p = 0.08$; Fig. 2G). For DEmiRNAs identified in comparison 2, no differences were found for miR-3200-5p expression (Fig. 2H), while miR-92a-3p expression was significantly higher in the endometrium of women with adenomyosis compared with healthy fertile controls (median ± IQR = 4.12 ± 3.02 vs. 1.07 ± 0.6, $p = 0.01$) (Fig. 2I).

Discussion

The principal finding of this study was that infertile women with adenomyosis have a distinct circulating plasma miRNA expression profile compared with both fertile and infertile controls. The inclusion of both control groups aimed to distinguish between disease-associated changes from those potentially related to reproductive status. Infertile controls were used to account for potential confounding effects related to infertility status, which may independently influence circulating miRNA expression profiles. In parallel, healthy fertile controls were included to provide a physiological baseline representing normal reproductive conditions, thereby allowing assessment of the extent to which adenomyosis-associated alterations deviate from a healthy state. A greater separation was observed between adenomyosis and healthy fertile control groups than between adenomyosis and infertile control groups. Several identified miRNAs demonstrated a strong ability to discriminate adenomyosis from control groups and displayed expression patterns consistent with those observed in the endometrium, suggesting they are potential biomarkers for adenomyosis.

Among the specific-disease DEmiRNAs, miR-4433a-5p and miR-4433b-5p (grouped as miR-4433) and miR-652-5p showed strong discriminatory power to distinguish women with adenomyosis from infertile controls, consistent with the aim of identifying molecular alterations associated with adenomyosis rather than infertility alone. miR-4433b-5p upregulation in plasma from women with implantation failure after IVF embryo transfer compared with those who achieved clinical pregnancy has been previously reported [32]. Because women with adenomyosis showed lower clinical pregnancy rates than infertile women without the disease [33, 34], miR-4433 upregulation may represent a link between adenomyosis

and impaired endometrial receptivity. In addition, miR-4433a-5p has been proposed as a marker and potential therapeutic target for chronic obstructive pulmonary disease, highlighting its involvement in PI3K–AKT signalling pathway regulation [35], itself implicated in the progression of adenomyosis [36]. Moreover, miR-652-5p upregulation in cervical cancer [37] and downregulation in the ectopic endometrium of women with endometriosis compared with endometriosis-free women [38], has been reported.

The miRNA plasma profile of women with adenomyosis also differed from that of fertile women, with miR-92a-3p (previously associated with adenomyosis) showing especially strong diagnostic potential, which contextualized adenomyosis-associated circulating miRNA alterations relative to a physiological fertile reproductive state. miR-92a-3p was upregulated in exosomes secreted by ectopic endometrium from women with adenomyosis and urinary exosomal miR-92a-3p levels and adenomyosis-related symptoms were correlated [20]. miR-92a-3p was also found in extracellular vesicles secreted by endometrial organoids generated from eutopic endometrium from women with adenomyosis [19]. miR-92a-3p overexpression in both plasma and eutopic endometrium from women with adenomyosis highlights its potential as a diagnostic biomarker.

Moreover, miR-342-5p upregulation in plasma from women with adenomyosis is consistent with reports in serum [39], uterine cavity exosomes [40], and ectopic endometrium [41] from women with endometriosis. This latter study also demonstrated that miR-342-5p activates the PI3K–AKT–mTOR signalling pathway, thereby promoting the malignant-like phenotype of endometrial stromal cells [41] and supporting a role for this miRNA in the pathogenesis of adenomyosis. Similarly, miR-193b-5p was upregulated in endometrioma compared with paired eutopic endometrium and is involved in cell migration control [42]. The expression of miR-543, which is downregulated in plasma from women with adenomyosis, was decreased in eutopic endometrium from women with endometriosis-associated infertility during the window of implantation [43], suggesting a role in fertility and its potential as a non-invasive biomarker of adenomyosis.

Among the DEmiRNAs with non-significant ROC performance, most have been reported in adenomyosis (miR-144-3p and miR-196a-5p) [18] or endometriosis (miR-183-5p, miR-96-5p, miR-654-3p, and miR-486-5p) [42, 44–48], with the same direction of change as in our plasma data. This consistency supports their potential involvement in the pathogenesis of adenomyosis or associated infertility, despite their limited diagnostic performance in our analyses.

Target gene prediction and functional enrichment analysis allowed us to explore the pathways these dysregulated miRNAs may influence. *CDKN1A*, which encodes p21, a cell cycle inhibitor, senescence inducer, and tumour suppressor, was among the most targeted genes [49]. Similarly, *CCND1*, whose dysregulation in adenomyosis [50] and potential implication in prolactin signalling [51] has been reported, was enriched in both comparisons and identified as a key pathological driver of adenomyosis [50, 52]. The prolactin signalling pathway (hsa04917) interacts with MAPK and PI3K–AKT signalling pathways, which are both involved in adenomyosis pathogenesis [53, 54], and were enriched for both comparisons. *IGF1R*, which contributes to endometrial decidualisation [55, 56], activates PI3K–AKT and mTOR signalling [57], and is linked with adenomyosis pathogenesis [58], was enriched and strongly targeted by DEmiRNAs. Similarly, predicted targets were enriched for apoptosis/autophagy, cell growth, EMT/migration, and inflammation [59]. These results suggest a role for miRNAs in regulating pathways and biological processes involved in adenomyosis pathogenesis. These findings highlight the potential clinical relevance of circulating miRNAs as non-invasive adenomyosis markers, although further validation is required before clinical use.

Since miRNAs were analysed in plasma, their tissue of origin cannot be established. To partially address this limitation, miRNAs with strong discriminatory power were assessed in eutopic endometrial tissue of women with adenomyosis and observed that it mirrored the alterations detected in plasma according to the original pairwise comparison (miR-4433 from adenomyosis vs infertile and miR-92a-3p from adenomyosis vs healthy fertile). However, these tissue-based results are preliminary and should be interpreted with caution, as further studies assessing miRNA expression in adenomyosis tissue and integrating tissue and circulating profiles are needed to confirm an endometrial contribution to the circulating miRNA profile and their role in adenomyosis.

This study is the first to use miRNAseq to characterise plasma miRNAs in women with adenomyosis. However, this exploratory study was limited by its small sample size, underscoring the need for larger validation studies. Because miRNA expression was assessed in plasma and not endometrial tissue, target prediction and pathway enrichment analyses should be interpreted cautiously.

Conclusions

Dysregulated miRNAs identified in plasma from women with adenomyosis have previously been linked to adenomyosis and endometriosis, supporting their involvement in the pathogenesis of this disease. Notably, miR-4433 and miR-92a-3p emerged as novel candidates with strong discriminatory power, suggesting new avenues for diagnosis. These findings underscore the role of miRNAs in

adenomyosis and associated infertility and provide a foundation for studies to improve non-invasive diagnosis.

Abbreviations

AUC	Area under the curve
BP	Biological process
BMI	Body mass index
CC	Cellular component
DEmiRNAs	Differentially expressed miRNAs
EMT	Epithelial-to-mesenchymal transition
FDR	False discovery rate
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes pathways
MRI	Magnetic resonance imaging
miRNAs	MicroRNAs
MF	Molecular function
MUSA	Morphological Uterus Sonographic Assessment
ORA	Over-Representation Analysis
ROC	Receiver operating characteristic
TVUS	Transvaginal ultrasound

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12958-026-01598-x.

Supplementary Material 1: Supplementary Table 1. Differentially expressed microRNAs in plasma from women with adenomyosis.

Supplementary Material 2: Supplementary Table 2. Predicted target genes of the differentially expressed microRNAs in plasma from women with adenomyosis compared with infertile controls.

Supplementary Material 3: Supplementary Table 3. Genes most commonly targeted by differentially expressed microRNAs in the adenomyosis group compared with the infertile control group.

Supplementary Material 4: Supplementary Table 4. Kyoto Encyclopedia of Genes and Genomes pathway enrichment of predicted target genes of differentially expressed plasma microRNAs in adenomyosis compared with infertile controls.

Supplementary Material 5: Supplementary Table 5. Gene Ontology enrichment analysis of predicted target genes of differentially expressed plasma microRNAs in adenomyosis compared with infertile controls.

Supplementary Material 6: Supplementary Table 6. Predicted target genes of the differentially expressed microRNAs present in plasma from women with adenomyosis compared with fertile controls.

Supplementary Material 7: Supplementary Table 7. Genes most commonly targeted by differentially expressed microRNAs in the adenomyosis group compared with the fertile control group.

Supplementary Material 8: Supplementary Table 8. Kyoto Encyclopedia of Genes and Genomes pathway enrichment of predicted target genes of differentially expressed plasma microRNAs in adenomyosis compared with fertile controls.

Supplementary Material 9: Supplementary Table 9. Gene Ontology enrichment analysis of predicted target genes of differentially expressed plasma microRNAs in adenomyosis compared with fertile controls.

Supplementary Material 10.

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Authors' contributions

AC was involved in the study conceptualisation, methodology, investigation, data curation, formal analysis, and writing-original draft. MPF was involved in investigation, data curation and formal analysis. AF was involved in investigation and validation. PA, CV, and JG were involved in investigation. AP and JAGV were involved in study supervision and writing-review and editing. HF was involved in study conceptualisation, methodology, formal analysis, project administration, writing-review and editing.

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Data availability

All raw sequencing data underlying this article are available through the Gene Expression Omnibus (GEO) under accession number GSE317995.

Declarations

Ethics approval and consent to participate

The collection and use of human samples was approved by the Clinical Research Ethics Committee at Hospital La Fe (2301-FIVI-008-HF) and informed written consent was obtained from all participants.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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