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ORIGINAL ARTICLE

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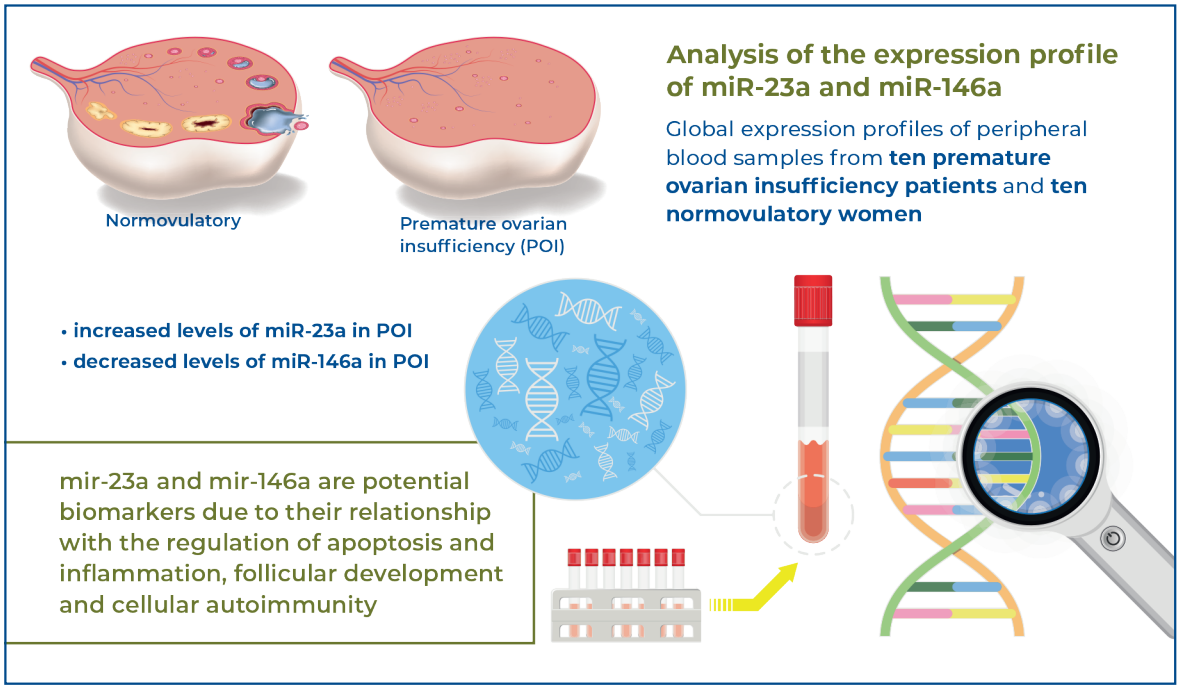

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In Brief

This study showed an increase in miR-23a levels and a decrease in miR-146a levels in patients with premature ovarian insufficiency compared with the normovulatory group. This highlights the importance of miR-23a and miR-146 as biomarkers for early diagnosis and monitoring, as well as potential therapeutic targets for premature ovarian insufficiency.

Highlights

- Higher miR-23a levels in patients with premature ovarian insufficiency vs. normovulatory women.
- Lower miR-146a levels in patients with premature ovarian insufficiency vs. normovulatory women.
- Novel biomarkers for early diagnosis and monitoring of premature ovarian insufficiency.
- Potential biomarkers to therapeutic targets for premature ovarian insufficiency.

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ORIGINAL ARTICLE

Expression profiles of miR-23a and miR-146a in the peripheral blood of women with premature ovarian insufficiency

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ABSTRACT

Objective: This study aimed to analyze the expression profiles of miR-23a and miR-146a in women with premature ovarian insufficiency and to evaluate their value as biomarkers of the disease for early diagnosis and prognosis, as well as effective therapies. **Methods:** The global expression profiles of peripheral blood samples from 10 patients with premature ovarian insufficiency and 10 normovulatory women were analyzed for the targets miR-23a and miR-146a and the endogenous control miR-U6 using real-time quantitative polymerase chain reaction. Statistical analysis was performed using analysis of variance and Bonferroni tests for group analysis. Welch's *t*-test was used to adjust for unequal variances between groups, and the Friedman test was used to confirm variance similarity. **Results:** Increased miR-23a levels were observed in patients with premature ovarian insufficiency. An inverse correlation was observed for relative miR-146a expression, which showed decreased levels in the premature ovarian insufficiency group compared with the normovulatory group. The ratios of the relative expression levels of miR-23a and miR-146a significantly differed in the premature ovarian insufficiency group but not in the normovulatory group. These findings were reaffirmed by accuracy assessment, with a positive predictive value of 0.92. **Conclusion:** Expression analysis of mir-23a and mir-146a demonstrates their potential use as biomarkers for premature ovarian insufficiency, owing to their relationship with the regulation of apoptosis and inflammation, follicular development, and cellular autoimmunity. This correlation can be assessed in larger sample sizes to confirm its importance in the early diagnosis, monitoring, and identification of potential therapeutic targets for premature ovarian insufficiency.

Keywords: Primary ovarian insufficiency; Peripheral blood; Biomarkers; Autoimmunity; MicroRNAs; Inflammation

INTRODUCTION

Premature ovarian insufficiency (POI) is a clinical syndrome characterized by oligo/amenorrhea observed in women before the age of 40 years, lasting at least 4 months, with elevated follicle-stimulating hormone (FSH) levels.[1] Its diagnosis is confirmed by two serum measurements conducted at least 1 month apart that show high FSH levels (>25IU/L) and low estradiol levels.[1] The condition is caused by varying factors, including genetic, immunological, or metabolic factors; iatrogenic intervention; and environmental toxins. The reason is idiopathic in 90% of cases.[1-3] Awareness about the signs and symptoms of this condition can be useful for early diagnosis, accurate identification, and early treatment, such as hormone therapy.[1,4,5] However, besides FSH, no other biomarkers have been identified for screening this condition.

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Circulating microRNAs (miRNAs) have been shown to be potential biomarkers for detecting various diseases owing to their unique characteristics in body fluids, including the peripheral blood.[6] In addition to being minimally invasive, high-precision circulating miRNAs can complement conventional markers. The relationship between miRNAs and POI has drawn increasing interest in biomedical research, with recent studies showing correlations between circulating miRNAs and POI in different populations.[7-11]

Specifically, an increase in miR-23a expression was identified in the blood of women with POI,[12] and two studies have shown a relationship between miR-146a and POI in Korean women.[13,14] In the ovarian granulosa cells of women with POI, increased miR-23a expression leads to reduced translation of the apoptotic protein anti-Bcl-2 and, consequently, increased pro-apoptotic caspase cleavage,[15] triggering apoptosis.[16,17] Regarding miR-146a, the decreased expression in these cells in women with POI appears to be triggered by increased Bcl-2 expression, which leads to apoptosis and dysregulation of estrogen.[18,19]

Several studies have reported a correlation between miR-23a[12,15,16] and miR-146a[14,20,21] and a reduction in the inhibitory effect on apoptotic cells; however, an inversely proportional relationship between these miRNAs and POI has not been reported to date. This study aimed to analyze the expression profiles of miR-23a and miR-146a in women with POI and evaluate their value as biomarkers of the disease. These findings highlight the important roles of miR-23a and miR-146a in the pathogenesis of POI, as well as the less invasive procedures and greater reliability of these circulating miRNAs in the peripheral blood.

OBJECTIVE

To analyze the expression profiles of miR-23a and miR-146a in the peripheral blood of women with premature ovarian insufficiency in comparison to those of normovulatory Brazilian women and their importance as biomarkers of the disease for early diagnosis and prognosis, as well as effective therapies.

METHODS

Sample collection

Peripheral blood samples were collected from 20 patients, who were divided into two groups (n=10 each): POI group, composed of patients with POI aged above 40 years with normal karyotype (20 to 40 metaphases evaluated) and no thyroid alterations, presence of amenorrhea for >4 months, and associated with two FSH dosages at 25mIU/mL with an interval of more than 4 weeks and; and Control Group, composed of normovulatory patients (normal ovulation) aged between 18 and 40 years who are non-obese, without comorbidities, and had a normal karyotype and no thyroid alterations. Controls were recruited at the *Instituto Ideia Fértil*, where they were performing *in vitro* fertilization due to male infertility or obstructed uterine tubes.

Patients with POI who had a history of chemotherapy and/or radiotherapy treatment, history of ovarian surgery, chromosomal alterations, thyroid abnormalities, altered/abnormal glycemic and lipid metabolism, autoimmune diseases, pre-mutation in the *FMR1* gene (<55 CGG repetitions), or a lack of information in their medical records to meet all the inclusion criteria were excluded. The patients had a mean age of 36.3 years (±4.5).

All patients were karyotyped as an inclusion factor. Most karyotyping was performed at the *Instituto Ideia Fértil* (Genetics Laboratory) and the rest was performed in private laboratories.

Procedure

Total peripheral blood samples (5 mL) were collected in PAXgene Blood RNA Tubes® and were stored in a freezer at -80ºC until RNA extraction. All samples were extracted using the same kit in the same experiment to avoid differences in RNA quality.

miRNA was isolated using a PAXgene Blood miRNA kit® (PreAnalytiX Company™ - QIAGEN, Valencia, CA, USA), following the manufacturer's protocol, and the concentration was measured using NanoDrop™ 2000/2000c Spectrophotometers (Thermo Fisher Scientific™, CA, USA). miRNAs (5ng/μL) were reverse transcribed to cDNA using a miRCURY® LNA® RT Kit (QIAGEN), according to the manufacturer's instructions. Subsequently, qPCR amplification of the control (U6/UniSp6) and target miRNAs (miR-23a and miR-146a) was performed in triplicate on a StepOne™ Real-Time PCR System (Applied Biosystems by Life Technologies®, Carlsbad, California, USA) using a miRCURY® PROBE PCR Kit (QIAGEN) and the polymerase enzyme coupled to the green fluorophore SybrGreen. The results are presented as the mean relative expression ($2^{-\Delta Ct}$). The primer and probe sequences used are listed in table 1.

Table 1. Primers and probes used for miRNA (microRNA) amplification

Name	Primers and probes	References and targets genes
RNA Spike-in template	UniSp6 miRCURY® LNA® miRNA Probe Assay	U6
miR-23a	hsa-miR-23a-3p	mir-23a
miR-146a	hsa-miR-146a-5p	mir-146a

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5® software (GraphPad Prism Software Inc., San Diego, CA, USA). Values are expressed as the mean and standard error with a significance level of 95% ($p \leq 0.05$). The following statistical tests were used: ANOVA and the Bonferroni auxiliary test for analysis between groups; unpaired *t*-test with Welch's correction to adjust for unequal variations between groups; and the Friedman auxiliary test to confirm variance similarity. A receiver operating characteristic (ROC) curve was constructed on a Cartesian plane, where the Y-axis represents sensitivity and the X-axis represents 1 minus specificity (1-E), both in decimal values. The area under the curve (AUC) represents the accuracy of the results. The closer the test's ability to discriminate between cases and controls, the closer the curve approaches the upper left corner, which represents the sensitivity and 1-specificity of the best cut-off value. The better the test, the closer the AUC (area under the curve/ROC CURVE) value approaches 1. Sensitivity and specificity were calculated using the Youden Index protocol to discriminate individuals using pooled blood samples.[22]

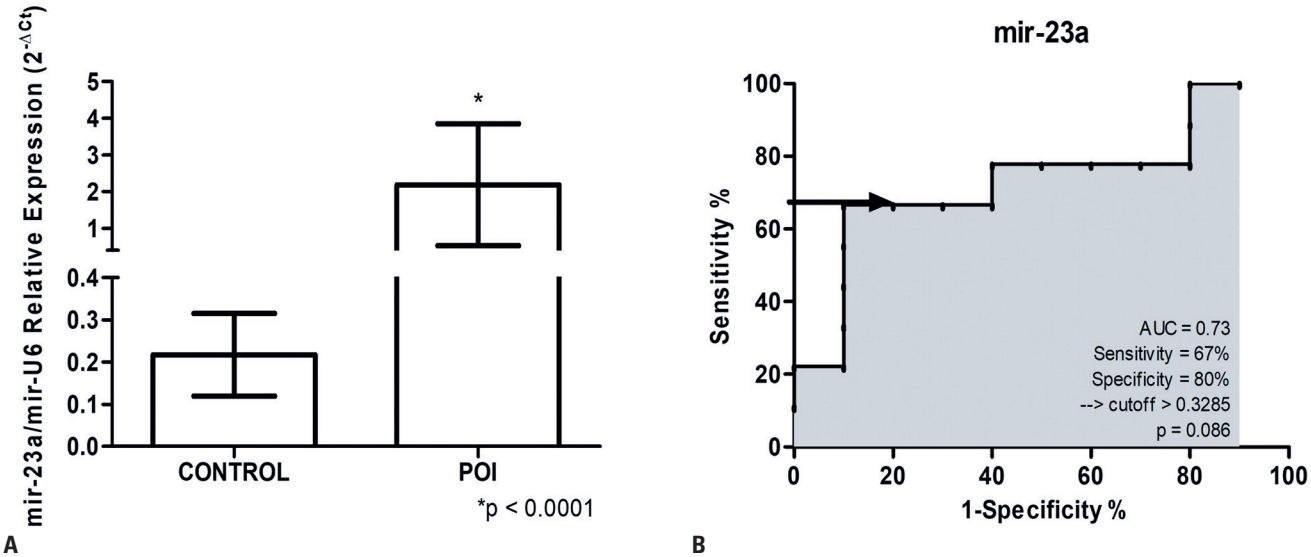
This study was approved by the Ethics Committee Process *Centro Universitário FMABC* (CAAE: 31029520.2.0000.0082 5.592.449; #5.592.449).

RESULTS

Relative miR-23a expression levels in the peripheral blood were higher in women with POI than in normovulatory women (control) ($p<0.0001$; Figure 1A). The accuracy of the results was assessed using an ROC curve, which had an AUC value of 0.73 (Figure 1B).

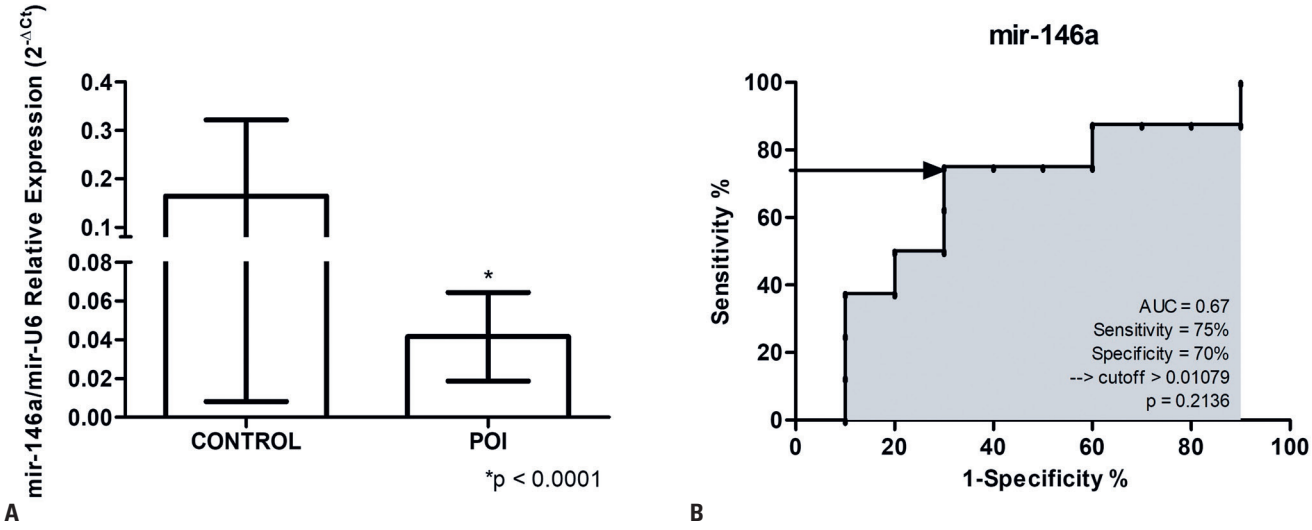
Meanwhile, relative miR-146a expression levels in the peripheral blood were lower in women with POI than in normovulatory women (control) ($p<0.0001$; Figure 2A). The accuracy of the results was assessed using an ROC curve, which had an AUC value of 0.67 (Figure 2B).

Analysis of the ratio of miR-23a and miR-146a expression in the peripheral blood of patients with POI showed that these two miRNAs were inversely proportional to the presence of POI, as shown in figure 3A. The ratios of the relative expression levels of miR-23a and miR-46a did not significantly differ in the Control Group ($p>0.05$). In contrast, in the POI group, when the expression of miR-23a increased, that of miR-146a decreased ($p<0.0001$), and an inversely proportional relationship to the presence of POI was identified (Figure 3A). Assessment of the accuracy (AUC) reaffirmed this condition with a positive predictive value of 0.92, sensitivity of 100%, and specificity of 67% ($p<0.05$; Figure 3B).



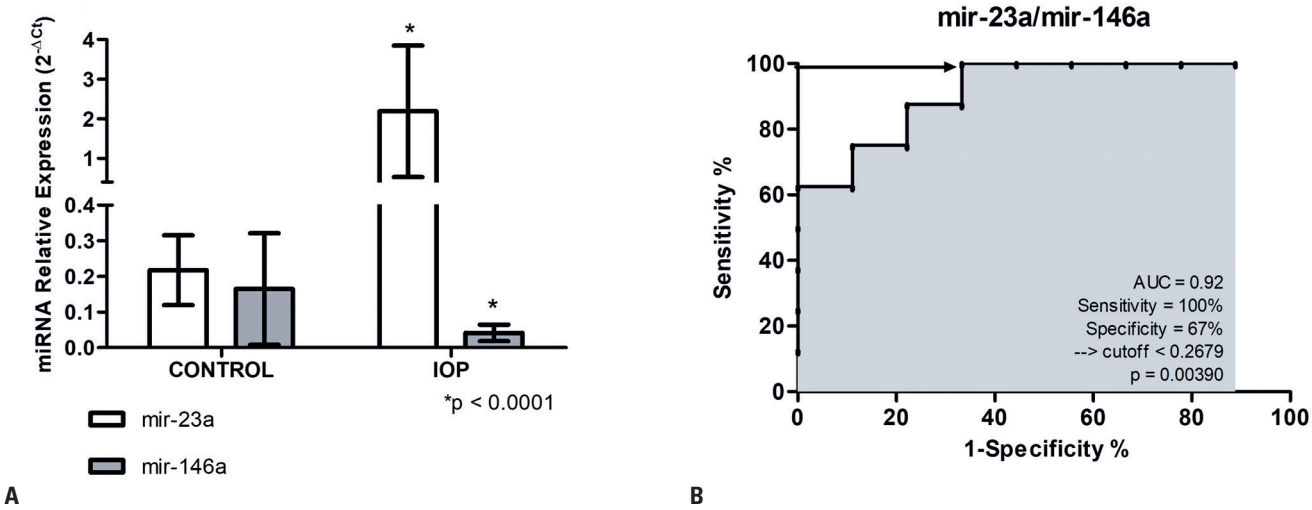
Welch's *t*-test was used to adjust for unequal variances between groups and the auxiliary F test to verify the variance. Significance was set at $p \leq 0.05$. ROC curve is represented by % sensitivity on the X-axis *versus* 1 % specificity on the Y-axis. The area under the curve (AUC) represents accuracy; the better the test, the closer the AUC value approaches 1.

Figure 1. Relative expression profile of miR-23a in the peripheral blood of women with premature ovarian insufficiency. Values were obtained via quantitative RT-PCR (RT-qPCR). (A) Relative miR-23a expression in patients with POI *versus* normovulatory women (control); (B) ROC curve for miR-23a expression in the peripheral blood of patients with premature ovarian insufficiency *versus* normovulatory women (control). Expression levels were calculated using the $2^{-\Delta Ct}$ method, with miR-U6 as the reference. Values represent the mean and standard error in triplicate



Welch's *t*-test was used to adjust for unequal variances between groups, and the auxiliary F test to verify the variance. Significance was set at p≤0.05. ROC curve is represented by % sensitivity on the X-axis *versus* 1 % specificity on the Y-axis. The area under the curve (AUC) represents accuracy; the better the test, the closer the AUC value approaches 1.

Figure 2. Relative expression profile of miR-146a in the peripheral blood of women with premature ovarian insufficiency. Values were obtained via quantitative RT-PCR (RT-qPCR). (A) Relative miR-146a expression in patients with premature ovarian insufficiency *versus* normovulatory women (control); (B) ROC curve for miR-146a expression in the peripheral blood of patients with premature ovarian insufficiency *versus* normovulatory women (control). Expression levels were calculated using the $2^{-\Delta Ct}$ method, with miR-U6 as the reference. Values represent the mean and standard error in triplicate



Welch's *t*-test was used to adjust for unequal variances between groups and the auxiliary F test to verify the variance. Significance was set at p≤0.05. ROC curve is represented by % sensitivity on the X-axis *versus* 1 % specificity on the Y-axis. The area under the curve (AUC) represents accuracy; the better the test, the closer the AUC value approaches 1.

Figure 3. The ratios of the relative expression of miR-23a and miR-146a in the peripheral blood of women with premature ovarian insufficiency. Values were obtained via quantitative RT-PCR (RT-qPCR). A) Ratio of relative expression of miR-23a/miR-146a in patients with premature ovarian insufficiency *versus* normovulatory women (control); B) ROC curve as a representation of the ratio between the expression levels of miR-23a/miR-146a in the peripheral blood of patients with premature ovarian insufficiency. Relative expression levels were calculated, with mir-U6 as the reference gene. Values represent the mean and standard error in triplicate

DISCUSSION

Premature ovarian insufficiency is a complex condition requiring a multidisciplinary approach for effective management. Early diagnosis and appropriate treatment are essential for improving the quality of life of affected women. miRNAs are small noncoding RNA molecules that play crucial roles in the regulation of gene expression. They have emerged as important biomarkers for various medical conditions owing to their stability in body fluids, specificity, and sensitivity. In the context

of POI, miR-23a and miR-146a have been reported to be potential biomarkers. Although clinical trials with miRNAs for the treatment of gynecological diseases are still in their early stages, an increasing number of studies are being conducted in this field, and miRNAs may eventually become a new cell-free therapeutic strategy for gynecological diseases.[23] Luo et al. showed that miR-23a overexpression in ovarian granulosa cells can induce the downregulation of the anti-apoptotic protein Bcl-2 and upregulate the pro-apoptotic cleaved form of caspase-3, triggering apoptosis of these cells.[15] Furthermore, miR-23a overexpression significantly increases the levels of p-ERK protein and leads to the appearance of specific cleavage fragments of caspase-3 in ovarian granulosa cells, consequently leading to apoptosis of these cells.[15-17] Studies have shown an increase in miR-23a expression in the plasma[12] and granulosa cells of rats following upregulation of the apoptosis inhibitor protein XIAP (X-linked inhibitor of apoptosis protein).[24] Some plasma miRNAs are differentially expressed between patients with POI and women with a normal cycle. miR-23a, which is significantly upregulated in the plasma/serum of patients with POI, is essential for inducing apoptosis in human granulosa cells by targeting XIAP and the caspase signaling cascade.[17,19,22]

Thus, the results of the present study corroborate those in the literature, showing an increase in the relative expression level of miR-23a in total peripheral blood samples from patients with POI compared with those from normovulatory women (Control Group). This highlights the relationship of this circulating miRNA with the pathological conditions of POI well defined in the literature. It is worth noting that circulating miR-23a expression in the total peripheral blood of humans with an established POI has not yet been analyzed. Our results showed that miR-146a expression was inversely proportional to miR-23a expression. A decrease in mir-146a expression in the peripheral blood was observed in patients with POI compared with controls. Similar results have been reported for the peripheral blood,[13] plasma,[14,25] serum, and follicular fluid[26] of patients with POI.[17] The use of mir-146a mimetics (molecules that mimic the function of miRNAs) has been explored as a therapeutic strategy to reduce inflammation and protect ovarian cells in women with POI.[13,27] miR-146 has been shown to be associated with the regulation of the innate immune system, including inflammation and oxidative stress.[20] *In vivo* studies conducted in rats showed that the decrease in miR-146 expression can regulate the TLR4 and NF-κB signaling pathways in the inflammatory response by binding target genes to inflammatory factors such as TNF-α and IL-6.[18,28] Other studies also showed that the increase in miR-146 expression can lead to a decrease in the levels of IL-6 and TNF-α in granulosa cells, suggesting that the reduction in miR-146 expression and elevation in IL-6 and TNF-α expression are involved in the occurrence and development of POI as a low-grade chronic inflammation.[18,29] Furthermore, the increased expression of miR-146 has anti-inflammatory and antioxidant effects in POI,[18] likely by inhibiting TNF-α and IL-6 expression in septic vascular endothelial cells.[30] Overexpression of Bcl-2 can present resistance at the level of Bax and promote a decrease in apoptosis of granulosa cells through the metabolism regulated by estrogen hormones [19] and increase the expression of miR-146 inhibitors.[18] Recent studies have demonstrated the use of miR-146 as an immunomodulatory factor and as a new target for the treatment of POI, which corroborates the results of the present study which showed the use of miR-146a as a biomarker of POI in total circulating peripheral blood.[18-20]

miR-23a and miR-146a expression has been the subject of study in various pathological conditions, including POI, as this condition is related to apoptosis regulation (regulation of Bcl2 and XIAP),[13-15,18,25] inflammation and autoimmunity (modulation of inflammatory responses),[8,18,21] and follicular development (ovarian reserve and reproductive function).[18,21]

Specifically, the expression and roles of miR-23a and miR-146a in POI have been studied.[31,32] The relationship of the two miRNAs was found to be inversely proportional, with an accuracy level of 0.92 (AUC), sensitivity of 100%, and specificity of 67% based on the generated ROC curve. Therefore, the presence of POI can be detected and/or monitored through the presence of miR-23a and miR-146a in the peripheral blood, with a higher miR-23a expression and lower miR-146a expression suggesting a greater possibility of POI.

The limitations of existing studies on POI are related to differences in the study design of miRNA profiling (*e.g.*, miRNA analysis method, sample type, diagnostic criteria for POI, and definition of differential expression [fold change]), which makes direct comparison of the results difficult. Another limitation is that although a variety of miRNAs are differentially expressed in POI, most of their biological roles remain unknown. Additional analyses, including functional studies focused on cell lines or human tissues, ROC curve analysis, and larger cohorts, are needed to explore the underlying mechanisms and reveal the diagnostic and therapeutic value of biomarkers in ovarian insufficiency.[31,32]

In terms of sample size, the present study included 20 individuals (10 cases and 10 controls) who were selected using extremely strict and reliable inclusion and exclusion criteria. Such meticulous aspects were not reported in any of the reviewed articles in the literature. The global prevalence of the condition should also be considered for the sample composition. The prevalence of POI in the Hispanic population is 1% in women aged < 40 years and 0.1% (1:1000) in women aged < 30 years.[33] The frequency appears to vary with ethnicity, being more frequent in women of Hispanic and African-American origin (1.4%) and less frequent in Japanese women (0.5%).[34]

This study is the first work conducted in Latin America to investigate miRNAs, particularly miR-23a and miR-146a, as biomarkers for POI. Other studies investigating this association that used a methodology similar to that of this study were conducted in Asian populations. Thus, by corroborating the results in Asian populations and considering different ethnicities that comprise the population of São Paulo (Brazil), the obtained results may reinforce the role of these miRNAs as universal biomarkers for POI and contribute to the early diagnosis of and/or more efficient therapies for POI.

Despite being a pilot study, limitations such as the relatively small sample size and the choice of specific targets instead of a transcriptome should be addressed. A larger sample size is needed to ensure replicability of the association observed in this study. In addition, transcriptome analysis of a larger sample size could reveal other targets that are differentially expressed between cases and controls.

Given the therapeutic potential of miRNAs, their application in treatment is expected. Furthermore, alteration of miRNA functionality and the development of new in vivo delivery systems to achieve the targeted modulation of specific miRNA functions are being actively pursued as novel therapeutic interventions for many gynecological diseases.[17,23]

CONCLUSION

The expression of miR-23a and miR-146a is a potentially significant biomarker for premature ovarian insufficiency because of their relationship with the regulation of apoptosis, inflammation, follicular development, and cellular autoimmunity. Therefore, they can be utilized for early diagnosis and monitoring and as potential therapeutic targets for premature ovarian insufficiency.

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AUTHORS' CONTRIBUTION

Thérèse Rachell Theodoro: sample processing, results analysis, statistical analysis, bibliographic review, technical review, and article preparation. Rafael Bitelman Barreiro and Isabella Verdi Cunha: case survey, sample processing, laboratory analysis, and statistical analysis. Bianca Bianco and Caio Parente Barbosa: technical review. Denise Maria Christofolini: planning, financing, and technical review.

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