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GENERAL GYNECOLOGY

PIWIL2 is overexpressed in adenomyotic lesions of women with diffuse adenomyosis

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Abstract

Purpose Adenomyosis has been studied throughout the years, however, its aetiology and physiopathology are still unknown. The aim of this study was to identify the presence of PIWI proteins in women with adenomyosis.

Methods We included 72 participants to be part of this study and were divided into two groups based on their anatomopathological diagnosis, control ($n = 36$) or adenomyosis ($n = 36$). All samples were tested for PIWIL1, PIWIL2 and PIWIL4 proteins by immunohistochemistry. The evaluation of protein expression was performed by the digital histological score (DHSCORE) and by the pathologist's analysis.

Results The participants had a mean age of 44.28 ± 5.76 years and 45.81 ± 4.86 years in the control and adenomyosis groups, respectively ($p \geq 0.05$). Other clinical characteristics of the participants showed no statistical difference as well. PIWIL2 is highly expressed in the adenomyosis in comparison to the control group ($p = 0.0001$). The PIWIL1 is downregulated in the adenomyosis ($p = 0.003$) and PIWIL4 showed no difference in its expression ($p = 0.05$).

Conclusion PIWIL2 might be involved in cellular survival and PIWIL1 may be downregulated due to the loss of tissue's function and response to the hostile environment of the myometrium. This is the first time that PIWI proteins are studied in the adenomyosis.

Keyword Adenomyosis · Hysterectomy · Immunohistochemistry · Protein expression · PIWI proteins

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Introduction

Adenomyosis is a benign disease that affects women in their reproductive years [1]. This disease is characterized by the invasion of the endometrial tissue into the myometrium [2, 3]. It is estimated that one third of the cases of adenomyosis are non-symptomatic, and therefore, they are only found during a routine imaging examination [4]. In the cases in which adenomyosis is symptomatic, it can be treated with analgesics, oral contraceptives or surgery.

In the past, adenomyosis was considered to be a pathology exclusively of women over 40 years old and with multiple gestations; however, with the advent of assisted reproduction techniques, doctors have been able to identify its presence in women throughout their reproductive years [2]. Currently, the physiopathology of adenomyosis is still the subject of study for researchers and its link to subfertility needs a more robust scientific evidence. A few studies have shown that this disease could result in embryo implantation difficulties and higher rates of spontaneous abortion [5].

For the purpose of helping to understand the mechanisms that are involved in the physiopathogenesis of adenomyosis, this work aimed to investigate the relationship between this disease and PIWI proteins. Although the search to understand how this invasion occurs is valid, there is no record in the literature of any previous study with this objective [6, 7].

The PIWI proteins have a central role in the transposons silencing process, and they are found in the germinative cell lineage. They have been reported in different types of germinative and somatic cancers, such as seminomas and multiple myelomas [8, 9]. Therefore, this study aimed to contribute to the comprehension of PIWI proteins as well as to the knowledge about adenomyosis.

Materials and methods

Ethics approval

This study was approved by the Hospital de Clínicas de Porto Alegre's (HCPA) ethics committee, and it is registered under number 16-0639 and CAAE: 63591516.0.0000.5327 at Plataforma Brasil. All methods were performed in accordance with the STROBE guideline.

Participant selection

All participants were selected from a database that included more than 300 patients that underwent a hysterectomy due to various causes since 2014. Among these, 110 patients were enrolled in a previous analysis of the viability of their formalin-fixed paraffin embedded (FFPE) tissue samples. Seventy-two hysterectomized patients were selected to participate in this study who underwent surgery from November 2014 to December 2018. The samples were divided into two groups: the control group ($n=36$), which comprised tissue samples from hysterectomized patients without adenomyosis who underwent the procedure due to leiomyomas, polyps, myomas or other benign diseases, and the study group ($n=36$), which comprised patients with a positive diagnosis of adenomyosis confirmed by a pathologist. In the adenomyosis group, we analysed the tissue from the adenomyoma and the eutopic endometrium.

The FFPE materials from women that underwent a hysterectomy were included in the study. Biological samples with a pathological diagnosis of endometrial, cervical or ovarian cancer and/or endometriosis confirmed by the pathologist were excluded from the study.

All participants signed an informed consent form to be part in the study. Additionally, all researchers signed the Hospital de Clínicas de Porto Alegre's form for utilization of biological samples which includes tissue samples.

Statistical analysis

The sample size was determined by WinPEPI (Programs for Epidemiologists for Windows) version 11.63 using the study from [10]. A total of 72 samples were necessary for a power of 80% and 5% of acceptable difference with the assumption of a proportion of 0.024 for PIWIL1 reported in endometrial samples.

Statistical analysis was calculated according to the data's characteristics. For categorical data, the Chi-square test was performed, and for continuous data, Student's *t* test or the Mann–Whitney test was performed if there was parametric or non-parametric distribution of the variables, respectively. When analysing all the groups, ANOVA or the Kruskal–Wallis test was performed, depending on the data's distribution, as determined by the Shapiro–Wilk test. Multivariable analysis was performed using a logistic regression model when necessary.

Immunohistochemistry

All 72 samples were tested for the three PIWI proteins in the study. All tissue samples that initially were embedded in paraffin were cut into 3-µm sections in a microtome (LupeTec MRP 2016, São Carlos, SP, Brazil). The tissues were positioned on previously silanized slides. After that, the slides were heated in a heater at 80 °C for 30 min, deparaffinized in xylol, and rehydrated in an ethanol gradient (100%, 90%, 80%, and 70%) over 30 s, followed by immersion in distilled water. The antigen recovery was performed in a heated-bath at 95 °C for 20 min in citrate buffer, pH 6.0, and cooled over

10 min in the same buffer. The endogenous peroxidase activity was blocked using a 5% hydrogen peroxide solution in methanol for 20 min. Non-specific protein was blocked with powdered non-fat milk at 5% in PBS for 20 min. The slides were then incubated overnight at 4 °C with primary rabbit polyclonal antibodies against the three PIWI proteins: anti-PIWIL1 (ABCAM, ab 12337; 1:50 dilution), anti-PIWIL2 (Thermo Fisher Scientific, PA5-34341; 1:600 dilution) and anti-PIWIL4 (Thermo Fisher Scientific, PA5-31448; 1:600 dilution). A peroxidase-conjugated secondary anti-rabbit polyclonal antibody (Merck Millipore, AP132P; 1:200 dilution) was then applied for 1 h 30 min at room temperature. The visualization of the reaction was obtained using Liquid Dab (Dako, K3468), as recommended by the manufacturer. After visualization, the slides were counterstained in Harris' haematoxylin for 10 s and differentiated in 2% ammonia in water for 30 s, followed by dehydration in absolute ethanol and then xylol before fixing the slides in Entellan-type resin.

Protein expression analysis

Protein presence was evaluated by a pathologist's analysis of the immunohistochemistry slides. She analysed the slide in comparison to the positive and negative controls for each PIWI protein and provided results in a qualitative matter: the expression or not of the three PIWI proteins (Fig. 1).

The quantification of protein expression was performed using the digital histological score (DHSCORE) with ImageJ (National Institute of Health—https://rsbweb.nih.gov/ij/) open software, an optical microscope (Olympus BX51, Olympus Optical Co., Tokyo, Japan) and a digital colour camera (Q-Color5, Olympus) according to Fuhrich et al. All slides were photographed using a 10 × objective lens, in which 1 mm corresponded to 1500 pixels, and were stored at 2560 × 1960 pixels size [11].

ImageJ analysis

The global settings for the images were configured as 1 mm = 1500 pixels, and the measurement settings were configured to deliver data from the area, standard deviation, minimum, and maximum grey values, mean and modal grey values and area fraction. After establishing this setting, every image was set for its own background removal by selecting a blank area and applying the command Process > Subtract Background. Following this step, every image was separated in three files that corresponded to red, green and blue (RGB) using the Colour Deconvolution Plugin for HDAB (Fig. 2). Once the images were separated in those three files, one

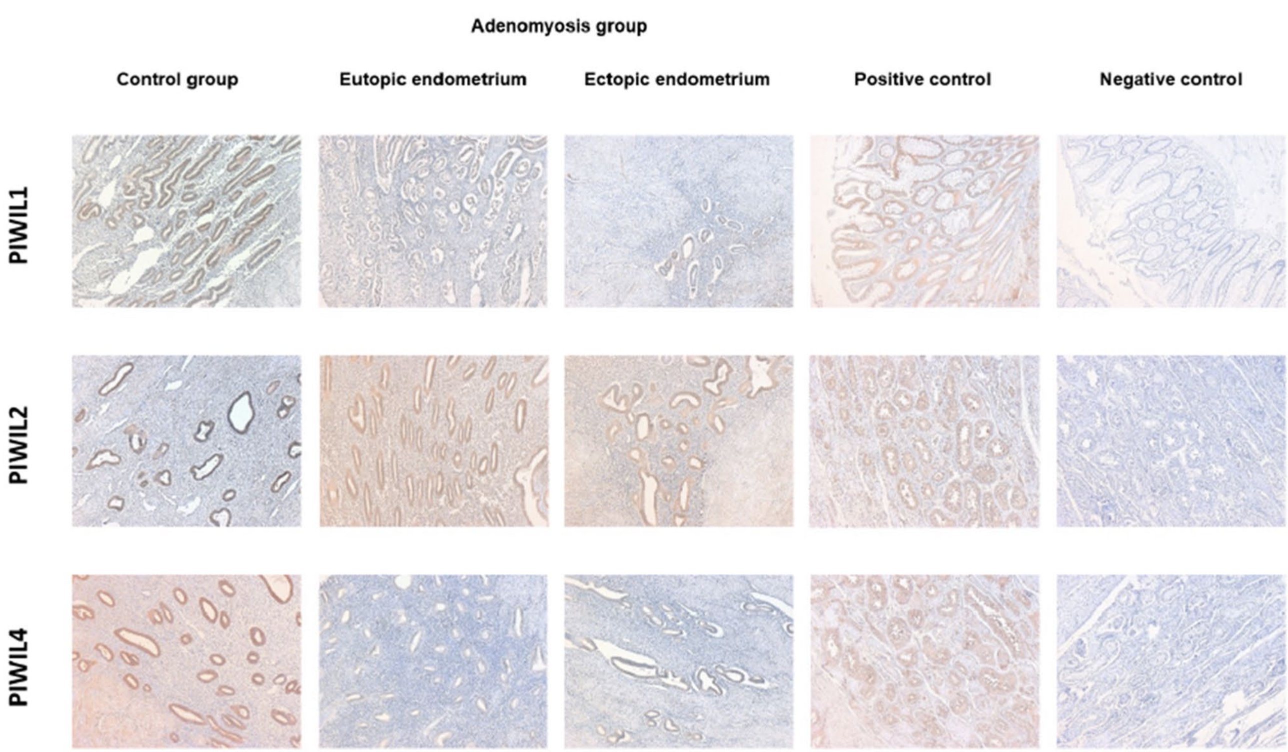

Fig. 1 Protein expression analysis by the pathologist's evaluation. Qualitative analysis of the expression of PIWIL1, PIWIL2 and PIWIL4 in the immunohistochemistry slides by the pathologist. The presence or not for each PIWI protein was always in comparison to the positive and negative controls. For PIWIL1 the control was colon cancer and for PIWIL2 and PIWIL4 the control was seminoma

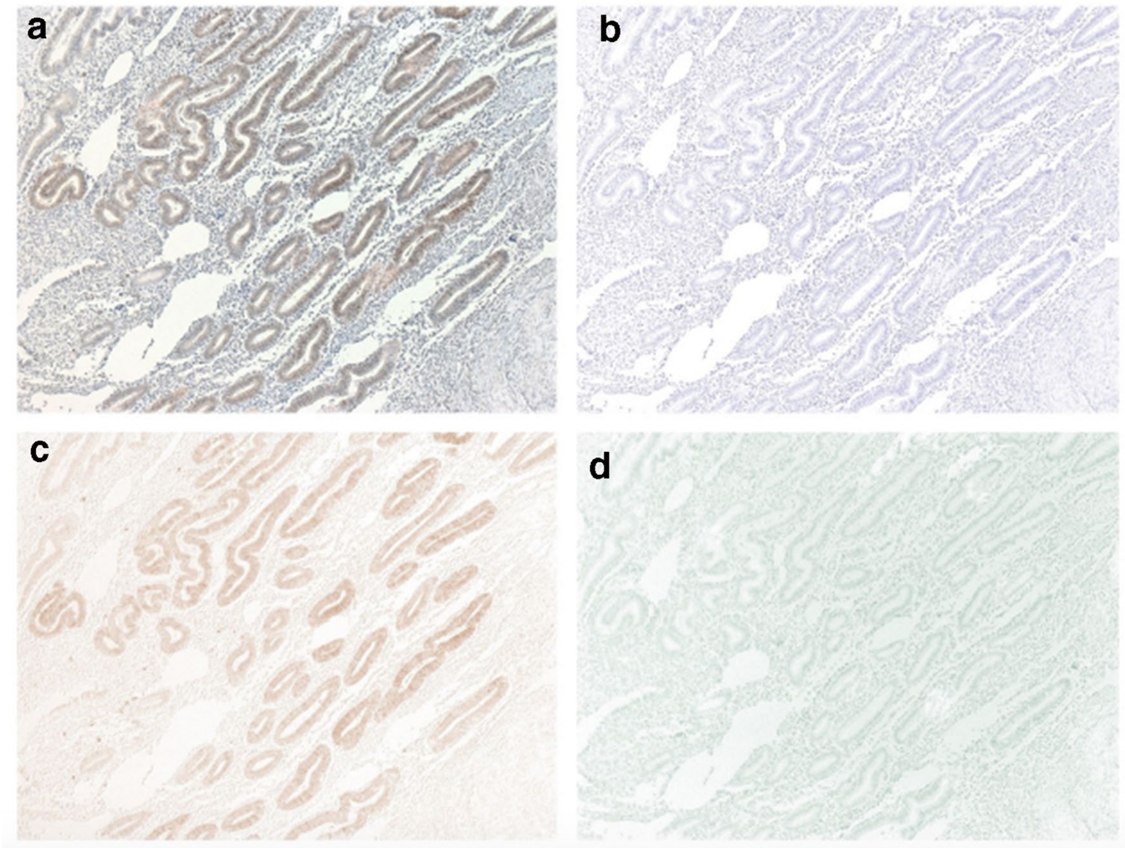

Fig. 2 Protein expression analysis by quantification through ImageJ. Quantification of protein expression performed by ImageJ analysis. **a** Image from one participant of the study without quantification. **b** Image file corresponding to hematoxylin staining of the slide. **c** Image file corresponding to the DAB intensity used to quantify the protein expression. **d** Image file corresponding to the background analysis. The measurement of the (**c**) file provides the mean DAB intensity (i) that is applied to the equation $f=255-i$ to result in the final DAB intensity measurement

corresponding to haematoxylin staining (Fig. 2b), one for diaminobenzidine (DAB) staining (Fig. 2c), and one for background (Fig. 2d), the mean value measured for the DAB file was applied in the Eq. (1) $f=255-i$ to determine the final DAB value, where f = final DAB intensity and i = mean DAB intensity; the value varies from 0 (zero = deep brown, highest expression) to 255 (totally white) [11]. On the slides with heterogeneous expression, up to three images of the region of interest were taken, and the final DAB value was determined by the mean of three images.

Results

The data regarding the characterization of the women that provided the samples for this study are summarized in Table 1. According to the statistical analysis of these data, there was no significant difference between the control and adenomyosis groups in any of the variables analysed. The participants showed a mean age of 44.28 ± 5.76 and 45.81 ± 4.86 years old in the control and adenomyosis groups, respectively ($p=0.23$; Student's t test). In the control group, the mean body mass index (BMI) was 28.07 ± 4.98 kg/m^2, and in the adenomyosis group, the mean BMI was 28.31 ± 3.94 kg/m^2 ($p=0.84$; Student's t test). Both groups showed no significant difference regarding mean age at menarche, which was 12.75 ± 1.87 years old for the control group and 12.86 ± 1.98 years old for the adenomyosis group ($p=0.84$; Student's t test). There was also no significant difference between the groups regarding the type of hysterectomy. In the control group, 72.9% ($n=26$) of participants had undergone a hysterectomy by videolaparoscopy, and 27.8% ($n=10$) participants had undergone a hysterectomy via open abdominal surgery. In the adenomyosis group, 80.6% ($n=29$) of participants had undergone a hysterectomy by videolaparoscopy, and 19.4% ($n=7$) of participants undergone a hysterectomy via abdominal surgery ($p=0.58$; Chi-square test). Considering the type of birth, 32 (45.1%) participants had only had vaginal births, 18 (25.4%) of them had only

Table 1 Characteristics of the sample in study considering the absence and presence of adenomyosis

Variables	Groups		p value
	Control (n = 36)	Adenomyosis (n = 36)	
Type of hysterectomy			0.58[a]
Videolaparoscopy	26 (72.9)	29 (80.6)	
Abdominal	10 (27.8)	7 (19.4)	
Age (years)	44.28 ± 5.76	45.81 ± 4.86	0.23[b]
BMI (kg/m^2)	28.07 ± 4.98	28.31 ± 3.94	0.84[b]
Menarche age (years)	12.75 ± 1.87	12.86 ± 1.98	0.84[b]
Number of gestations	2.50 (2.00–4.00)	3.00 (2.00–3.75)	0.74[c]
Number of live births	2.00 (2.00–3.00)	2.00 (2.00–3.00)	0.42[c]
Abortion			0.95[a]
No	28 (77.8)	26 (74.3)	
Yes	8 (22.2)	9 (25.7)	
Smoke habits			0.35[a]
No	17 (51.5)	24 (66.7)	
Yes	10 (30.3)	6 (16.7)	
Former smoker	6 (18.2)	6 (16.7)	

Characteristics of the sample that underwent the study according to the groups; BMI stands for body mass index; C section stands for caesarean-section surgery; variables are presented. According to their distribution, the ones that are symmetrical are presented as mean ± standard deviation (SD), the ones that are asymmetrical are presented as median (percentile 25–75), and for variables that are categorical, they are presented as absolute frequency and relative frequency, n (n%), respectively; p values have different letters according to the statistical analysis performed for each variable

[a]Chi-square

[b]Student's t test

[c]Mann–Whitney

caesarean-section births, and the same absolute and relative frequencies, n (n%), previously described, applies to the participants that had both types of birth as well. Only one (1.4%) out of 72 participants did not have this datum registered in their medical record. There was no significant difference between groups when analysing the type of birth distribution ($p = 0.07$; Chi-square test).

Taking into account the participants' medical records for hormonal use, we found a prevalence of 87.5% of use ($n = 63$), while nine (12.5%) had no information about this in their file. When segregated into both groups and by the type of hormone ($p = 0.64$; Chi-square test), in the control group, 41.9% ($n = 13$) used only progesterone, 12.9% ($n = 4$) used a combined hormone formula with progesterone and oestradiol, and 45.2% ($n = 14$) declared no use of hormones. In the adenomyosis group, only 31.3% ($n = 10$) used progesterone alone, 18.8% ($n = 6$) used both hormones, and 50% ($n = 16$) used no hormones as a treatment for adenomyosis symptoms.

When analysing the presence of symptoms, only one participant (1.4%) declared not having any symptoms in their medical records, while the other 71 participants (98.6%) reported having at least one physical symptom. 47.2% ($n = 34$) of the women reported two symptoms, with dysmenorrhea and metrorrhagia being the most frequent, at 51.4% ($n = 37$) and 41.7% ($n = 30$), respectively.

The results regarding protein expression are presented in Figs. 3, 4, 5. Each human PIWI protein tested was compared to the control group and to its paired normal endometrial tissue.

The analysis of PIWIL1 expression showed a median of 19.51 [13.03–25.75] and 13.26 [4.31–20.87] of final DAB intensity in the control and adenomyosis group, respectively ($p = 0.003$; Mann–Whitney) (Fig. 3a). We also compared its expression in the eutopic and ectopic endometrium from the participants that were positive for an adenomyosis diagnosis. A median of 13.26 [4.31–20.87] of final DAB intensity in the adenomyoma itself and a median of 13.74 [7.21–21.30] of final DAB intensity in the eutopic endometrium ($p = 0.0001$; Wilcoxon) (Fig. 3b) were revealed. When analysing the PIWIL1 pattern of expression in the endometrium tissue, we observed a mean of final DAB intensity of 20.40 ± 9.46 in the control group and a mean of final DAB intensity of15.36 ± 10.39 in the eutopic endometrium from the adenomyosis group ($p = 0.03$; Student's t test) (Fig. 3c).

The median of final DAB intensity of PIWIL2 was 18.09 [9.25–29.84] and 33.04 [23.70–52.60] when comparing the control and adenomyosis groups, respectively ($p = 0.0001$; Mann–Whitney) (Fig. 4a). The eutopic and ectopic endometrium of the adenomyosis group showed a median of 35.42 [25.80–48.20] and 33.04 [23.70–52.60] ($p = 0.0001$; Wilcoxon) (Fig. 4b). We also analysed PIWIL2 expression by comparing the control group and the eutopic endometrium of the adenomyosis group; the analysis revealed a median of 18.09 [9.25–29.84] and 35.42 [25.80–48.20], respectively ($p = 0.0001$; Mann–Whitney) (Fig. 4c).

When analysing the PIWIL4 expression pattern in the control and the adenomyosis groups, the median final DAB intensities were 45.55 [33.15–51.83] and 34.58 [16.46–48.00], respectively ($p = 0.05$; Mann–Whitney) (Fig. 5a). We also performed a comparison between the eutopic and ectopic endometrium in the adenomyosis group. The expression of PIWIL4 analysis revealed mean final DAB intensities of 34.31 ± 18.60 and 33.97 ± 18.47, respectively ($p = 0.94$; paired t test) (Fig. 5b). The final analysis was protein expression in the control group and in the eutopic endometrium of the adenomyosis group; PIWIL4 showed a final DAB intensity median of 45.44 [33.15–51.83] and 33.81 [16.36–49.09], respectively ($p = 0.07$; Mann–Whitney) (Fig. 5c).

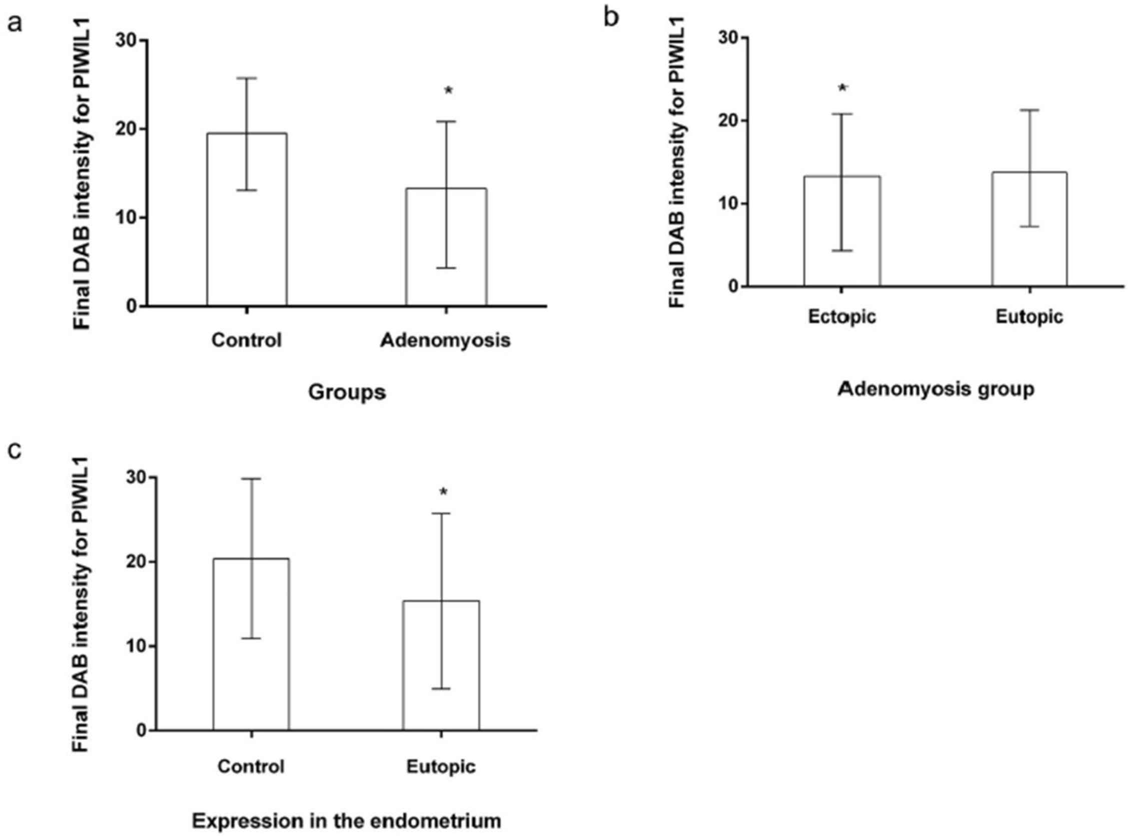

Fig. 3 Expression pattern of PIWIL1 in the sample studied. Analysis of PIWIL1 expression pattern in the sample that was studied. **a** Comparison of the expression of PIWIL1 in the control and adenomyosis group ($p < 0.05$; Mann–Whitney). **b** Comparison of the PIWIL1 expression pattern in the adenomyosis group by analysing the DAB intensity in the ectopic (adenomyoma itself) and the eutopicendometrium ($p < 0.05$; Wilcoxon). **c** Comparison of the expression pattern of PIWIL1 in the endometrium of the control group and in the eutopic endometrium of the adenomyosis group ($p < 0.05$; Student's *t* test)

To verify and confirm the PIWI protein quantification, we performed a blind qualitative quantification, in which the pathologist did not have any knowledge about the previous protein quantification results obtained using ImageJ software. For PIWIL1, the qualitative quantification resulted in the identification of the protein in 61.1% ($n = 22$) of the control group, in 27.8% ($n = 10$) of the ectopic endometrium of the adenomyosis group and in 63.9% ($n = 23$) of the eutopic endometrium of the adenomyosis group. When analysing the data for PIWIL2 expression, its presence was confirmed in 83.3 ($n = 30$), 69.4% ($n = 25$) and 82.9% ($n = 29$) of the control and adenomyosis ectopic and eutopic endometrium, respectively. We also analysed the qualitative quantification of PIWIL4, and it was expressed in all 36 participants in the control group (100%), in 34 participants in the ectopic endometrium of the adenomyosis group (94.4%), and in all participants from the adenomyosis group when analysing the eutopic endometrium.

Discussion

This study is a pioneer in the identification and characterization of human PIWI proteins in women with adenomyosis. For the first time in the scientific community, we were able to establish that PIWI proteins are present in adenomyosis and that they may be involved in its physiopathology.

The objective of this study was to investigate the presence of PIWI proteins in tissue samples from women diagnosed with adenomyosis that underwent a hysterectomy. Our findings regarding protein expression showed that PIWIL1, PIWIL2 and PIWIL4 were present in the samples studied. According to our data, PIWIL1 was downregulated in the adenomyosis group in comparison to the control group. The same pattern occurs when we compared the endometrium of the adenomyosis group, segregating it into the eutopic and ectopic endometrium, in which the

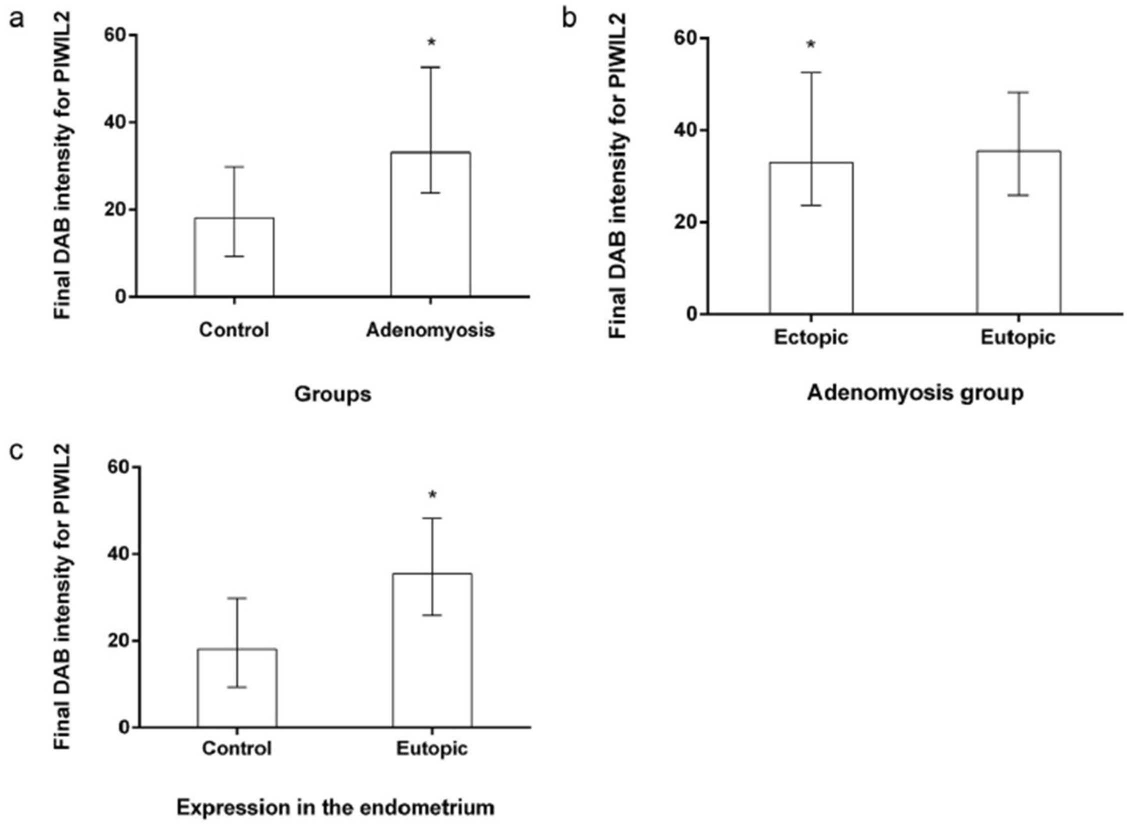

Fig. 4 Expression pattern of PIWIL2 in the sample studied. Analysis of PIWIL2 expression pattern in the sample that was studied. **a** Comparison of the expression of PIWIL2 in the control and adenomyosis group ($p < 0.05$; Mann–Whitney). **b** Comparison of the PIWIL2 expression pattern in the adenomyosis group by analysing the DAB intensity in the ectopic (adenomyoma itself) and the eutopicendometrium ($p < 0.05$; Wilcoxon). **c** Comparison of the expression pattern of PIWIL2 in the endometrium of the control group and in the eutopic endometrium of the adenomyosis group ($p < 0.05$; Mann–Whitney)

ectopic endometrium shows lower levels of PIWIL1. After analysing the eutopic endometrium from the control and adenomyosis groups, PIWIL1 was also downregulated in the eutopic endometrium of the adenomyosis group. Our data are in agreement with previous results reported in the literature, in which a lower PIWIL1 expression was found in both seminoma and non-seminoma tumours [12].

PIWIL1 is involved in methylation of targeted DNA for its posterior silencing to guarantee correct cellular division and maintenance of DNA integrity [13]. This activity may be compromised when considering that after their migration to the myometrium, endometrial cells may not need to continue to undergo replication and cellular proliferation to generate the proliferative endometrium; this could explain why PIWIL1 was found at lower levels in adenomyosis since there is no need to maintain DNA integrity. Another hypothesis for its lower levels could be because of the hostile environment of the myometrium and its immunological response to cellular invasion [14]. Indeed, Tremellen et al. (2012) identified a higher density of macrophages and natural killer cells in diffuse adenomyosis when compared to mild adenomyosis or no disease. Our findings open a new perspective in terms of immunomodulation and/or monoclonal target treatment in these patients with adenomyosis in the future.

The analysis of the data regarding protein expression showed that PIWIL2 was highly expressed in the adenomyosis group in comparison to the control group. The same characteristic was found when we investigated its expression in the adenomyosis group by analysing its pattern in the eutopic and ectopic endometrium. The same expression pattern was also observed when we compared the endometrium from the control group and the eutopic endometrium of the participants with adenomyosis. These data are in agreement with data found in the literature [15, 16]. A systematic review performed by Vannuccini et al. (2017) proposed that one explanation for endometrial invasion could be due to basal endometrium characteristics, which are non-cyclic and have an anti-apoptotic activity that is associated with oestrogen receptors (ERs) and *Bcl-2* gene expression [17, 18]. As a matter of fact, Goumenou et al. (2001) showed that *Bcl-2* expression is higher in the ectopic endometrium

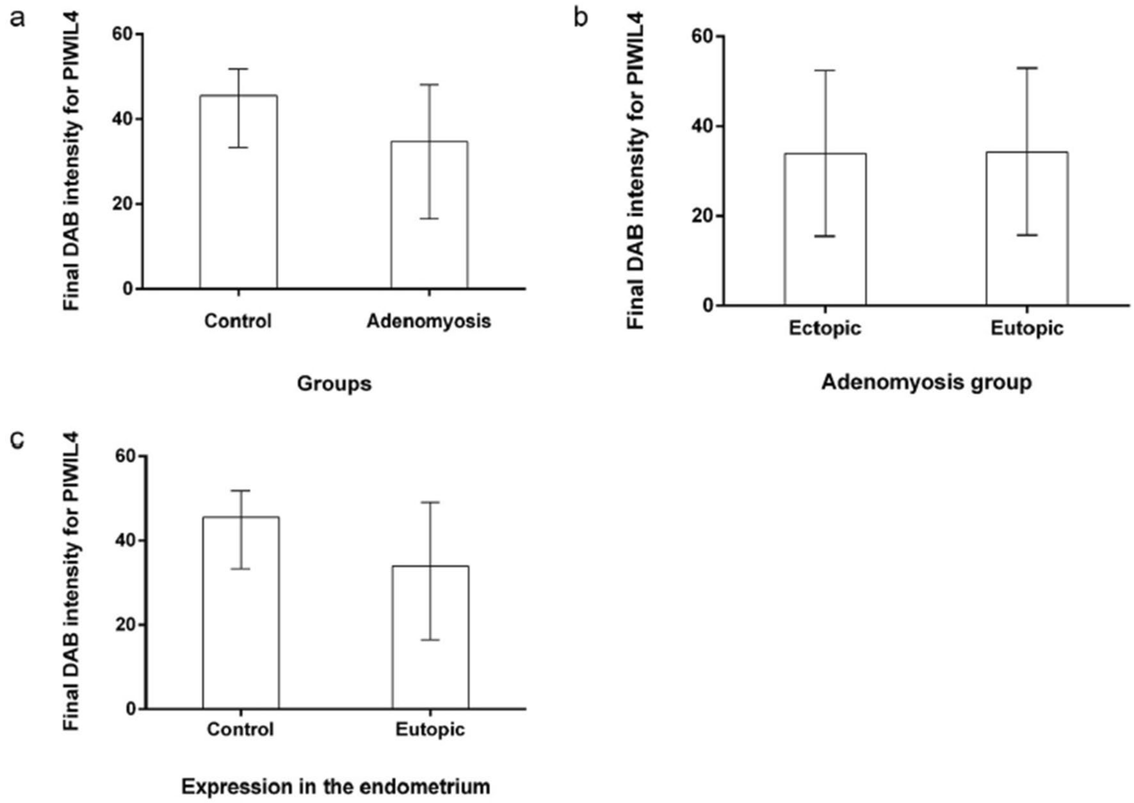

Fig. 5 Expression pattern of PIWIL4 in the sample studied. Analysis of PIWIL4 expression pattern in the sample that was studied. **a** Comparison of the expression of PIWIL4 in the control and adenomyosis group ($p \geq 0.05$; Mann–Whitney). **b** Comparison of the PIWIL4 expression pattern in the adenomyosis group by analysing the DAB intensity in the ectopic (adenomyoma itself) and the eutopic endometrium ($p \geq 0.05$; Paired *t* test). **c** Comparison of the expression pattern of PIWIL4 in the endometrium of the control group and in the eutopic endometrium of the adenomyosis group ($p \geq 0.05$; Mann–Whitney)

in comparison to the eutopic endometrium of women with adenomyosis during the proliferative phase and significantly lower in the secretory phase [19].

In addition, Lee et al. showed that PIWIL2 acts as an oncogene in the promotion of cellular proliferation and the inhibition of apoptosis [20]. This PIWI protein may also be involved in endometrium cell proliferation and therefore may be part of the pathogenesis of adenomyosis. Wang et al. identified that when highly expressed, PIWIL2 can lead to cisplatin resistance at the cellular level in some types of cancer. They also reported that when silencing PIWIL2, there was an activation of ovarian cancer cell apoptosis because of the lack of activity of PIWIL2 in the *Stat/Bcl-*X_L pathway [16].

In this study, we also investigated the presence of PIWIL4 in the tissue samples of hysterectomized patients. Indeed, the same pattern of expression was observed when comparing the eutopic and ectopic endometrium as well as when analysing the endometrium of the control group and the eutopic endometrium from the adenomyosis group. This pattern of expression has also been reported in epithelial ovarian cancer [21]. Although PIWIL4 is known to be responsible for cellular proliferation, its levels in adenomyosis could be due to the loss of function of the endometrial tissue after it invades the myometrium. This may also explain why its levels are higher in the control group because in its natural environment, the cells need to form proliferative endometrium.

We also conducted qualitative quantification of protein expression as well as quantitative quantification using ImageJ software. Since they are based on different methodological aspects, they cannot be directly compared, although we can argue that the qualitative analysis is variable due to observer experience and subjective analysis of the intensity of colour and comparisons between slides. On the other hand, the quantitative analysis performed is based on an image from a slide and gives us a number that represents the final intensity of the diaminobenzidine (DAB) substrate. Moreover, it is the result of a mathematical equation, but it is not fool proof since it can register residue on the slide as DAB intensity and it also depends on the individual's experience with the software.

Our study had a few limitations. We could not investigate the expression pattern of human PIWI proteins according to menstrual cycle phase due to lack of this information in the participants medical files. Additionally, our sample was composed only by diffuse adenomyosis, we cannot confirm if this pattern of expression exists in profound adenomyosis. We were not able to establish the beginning of adenomyosis and we do not know if PIWI proteins are expressed in women that did not underwent a hysterectomy. In spite of these limitations, our study was able to identify that PIWIL1, PIWIL2, and PIWIL4 are expressed in adenomyosis.

In conclusion, we were able to study the expression patterns of PIWI proteins in adenomyosis for the first time. Moreover, these proteins might be related to adenomyosis aetiology because each one of the PIWI proteins may present global complementary activities that could explain the occurrence of this disease. Clearly, more studies involving these proteins in, different adenomyosis phenotypes, are needed to corroborate our data and to help clarify adenomyosis physiopathology.

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Author contributions MMCM and JSLCF were responsible for the planning and execution of the project, data collection/analysis, statistical analysis, and draft of the manuscript. VKG and CABS were responsible for data collection, execution of the experiments and draft of the manuscript. PRO was responsible for ethical committee approval, planning of the project and draft of the manuscript. ACPF was responsible for participants' selection, analysis of the immunohistochemistry results and draft of the manuscript.

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Data availability The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interests The authors declare that they have no competing interests.

Consent to participate All participants signed an informed consent form to be part in the study.

Ethics approval This study was approved by the Hospital de Clínicas de Porto Alegre's (HCPA) ethics committee, and it is registered under number 16-0639 and CAAE: 63591516.0.0000.5327 at Plataforma Brasil.

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