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**New class of RNA biomarker for endometriosis diagnosis :  
the potential of salivary piRNA expression**

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## Abstract

**Objectives:** In contrast to miRNA expression, little attention has been given to piwiRNA (piRNA) expression among endometriosis patients. The aim of the present study was to explore the human piRNAome and to investigate a potential piRNA saliva-based diagnostic signature for endometriosis.

**Methods:** Data from the prospective “ENDomiRNA” study (ClinicalTrials.gov Identifier: NCT04728152) were used. Saliva samples from 200 patients were analyzed in order to evaluate human piRNA expression using the piRNA bank. Next Generation Sequencing (NGS), barcoding of unique molecular identifiers and both Artificial Intelligence (AI) and machine learning (ML) were used. For each piRNA, sensitivity, specificity, and ROC AUC values were calculated for the diagnosis of endometriosis.

**Results:** 201 piRNAs were identified, none had an AUC  $\geq 0.70$ , and only three piRNAs (piR-004153, piR001918, piR-020401) had an AUC between  $\geq 0.6$  and  $< 0.70$ . Seven were differentially expressed: piR-004153, piR-001918, piR-020401, piR-012864, piR-017716, piR-020326 and piR-016904. The respective correlation and accuracy to diagnose endometriosis according to the F1-score, sensitivity, specificity, and AUC ranged from 0-0.862 %, 0-0.961%, 0.085-1, and 0.425-0.618. A correlation was observed between the patients' age ( $\geq 35$  years) and piR-004153 ( $p = 0.002$ ) and piR-017716 ( $p = 0.030$ ). Among the 201 piRNAs, four were differentially expressed in patients with and without hormonal treatment: piR-004153 ( $p = 0.015$ ), piR-020401 ( $p = 0.001$ ), piR-012864 ( $p = 0.036$ ) and piR-017716 ( $p = 0.009$ ).

**Conclusion:** Our results support the link between piRNAs and endometriosis physiopathology and establish its utility as a potential diagnostic biomarker using saliva samples. Per se, piRNA expression should be analyzed along with the clinical status of a patient.

**Keywords:** endometriosis; non-coding RNA, piwiRNA; non-invasive biomarker;

## **Introduction**

Endometriosis, defined by the presence of endometrial-like tissue outside the uterus, is thought to affect from 2-10% of women of reproductive age. However, its exact incidence – especially in adolescent and menopausal patients [1,2] and in asymptomatic patients [3] – has not been fully assessed. From a pathogenesis perspective, endometriosis is a multifactorial disease with genetic and epigenetic controls involving multiple pathways such as cell proliferation, cell differentiation, cell adhesion, apoptosis, angiogenesis, steroidogenesis, inflammatory and immune responses, oncogenic suppressors, as well as exposome factors such as persistent organic pollutants [4]. However, despite numerous investigations of these various pathways, the pathophysiology of endometriosis remains incompletely understood.

Recently, numerous studies have focused on the role of non-coding RNAs in the pathophysiology of endometriosis and their potential as diagnostic biomarkers [5–7]. In contrast to miRNAs, that have been extensively investigated in biofluids including blood and saliva [7–9], to our knowledge no study to date has investigated the expression of PIWI-interacting RNAs (piRNA) in patients with endometriosis. Similarly, no data are available about piRNAs as potential diagnostic biomarkers of endometriosis. piRNAs are single-stranded RNAs composed of 23–36 nucleotides [10], and are classified according to their origin and function: repeat-associated piRNAs, piRNAs derived from 3' untranslated mRNAs, and piRNAs originating from intergenic long non-coding RNAs (pachytene piRNAs) [11]. In contrast to other non-coding RNAs processed from a double-stranded RNAs, dicer-dependent, and bound by Argonaute proteins, piRNAs are transcribed from ssRNA transcripts, are dicer-independent, and bound to PIWI (P-element Induced Wimpy testis) proteins [12]. In the human, at least 30,000 piRNAs have been identified as being involved in transposon silencing, transcriptional gene silencing, post-transcriptional gene silencing, transgenerational epigenetic inheritance, exosomal communications, and mRNA decay [13–15]. piRNA

expression has been analyzed in a variety of human tissues and been shown to be involved in different physiological processes (sex determination and differentiation, germ cells) [16], in various benign diseases such as cardiovascular disorders and male infertility [17–20], and in gastric, breast, lung and colorectal cancer [21–26]. However, little data is available relative to piRNA expression in saliva and no data specific to piRNA expression among endometriosis patients is currently available.

Therefore, using data from the prospective “ENDO-miRNA” study [27], the aims of the present work were: (i) to describe the piRNAs differentially expressed in the saliva of patients with and without endometriosis; (ii) to estimate their value in terms of diagnostic accuracy; and (iii) to gain insights into the pathophysiology of endometriosis based on piRNA expression.

## **Methods**

### **Study population**

We used data from the prospective “ENDO-miRNA” study (ClinicalTrials.gov Identifier: NCT04728152). Data collection and analysis were carried out under the Research Protocol n° ID RCB: 2020-A03297-32. All patients gave written consent to participate to the study. The ENDO-miRNA study [27] included 200 saliva samples obtained from patients with chronic pelvic pain suggestive of endometriosis. In brief, as previously published, all patients underwent a laparoscopic procedure (either therapeutic or diagnostic laparoscopic) and/or MRI imaging detecting endometriosis by the presence of endometrioma and/or deep endometriosis with colorectal involvement. All the saliva samples were collected between January 2021 and June 2021. Analysis was performed blinded to the surgical and imaging findings. In case of endometriosis, the patients were stratified according to the revised American Society of Reproductive Medicine (rASRM) classification [28]. The main characteristics of the patients included in the ENDO-miRNA study were previously published [9] and are displayed in Supplementary Table 1 .

### **Saliva sample analysis**

Saliva collection and processing has been previously described by our team [29]

RNA-sequencing libraries were prepared using the QIAseq miRNA Library Kit (Qiagen) according to the manufacturer’s instructions. Samples were indexed in batches of 96, with a targeted sequencing depth of 17 million reads per sample. Reads were first processed by trimming off the 3’ adapter and low-quality bases using cutadapt (cutadapt.readthedocs.io/en/stable/guide.html). Reads with no adapter sequence were tallied (no\_adapter\_reads). Following trimming, the insert sequences and UMI sequences were

identified. Reads with less than 16 bp insert sequences (`too_short_reads`) or less than 10 bp UMI sequences (`UMI_defective_reads`) were discarded. At each step, only unmapped sequences passed to the next step. Read counts for each RNA category (miRBase mature, miRBase hairpin, piRNA, tRNA, rRNA, mRNA and otherRNA) were calculated from the mapping results (`miRNA_Reads`, `hairpin_Reads`, `piRNA_Reads`, etc.). piRNABank was used for piRNA [30]. For each sample, all reads assigned to a particular miRNA or piRNA ID were counted, and associated UMIs were clustered to count unique molecules [31].

## **Bioinformatics**

Sequencing reads were processed using the data processing pipeline. FastQ files were trimmed to remove adapter sequences using Cutadapt version v.1.18 and were aligned using Bowtie version 1.1.1 to the following transcriptome databases: the human reference genome available from NCBI (<https://www.ncbi.nlm.nih.gov/genome/guide/human/>), and piRNABank (piRNAs) using the piRDeep2 v0.1.0 package. The raw sequencing data quality was assessed using FastQC software v0.11.7 [32–36].

Differential expression analysis of the piRNAs was quantified by piRDeep2 [37]. Differential expression tests were then conducted in DESeq2 for piRNAs with read counts in  $\geq 1$  of the samples. DESeq2 integrates methodological advances with several novel features to facilitate a more quantitative analysis of comparative RNA-seq data using shrinkage estimators for dispersion and fold change [38] [39]. piRNAs were considered as differentially expressed if the absolute value of log<sub>2</sub>-fold change was  $>1.5$  (upregulated) and  $<0.5$  (downregulated). The P value adjusted for multiple testing was  $<.05$  [38].

To evaluate the accuracy of each piRNA, sensitivity, specificity, and ROC analysis have been performed, and the ROC AUC was calculated [40,41]



Additional statistical analysis was based on the Chi<sup>2</sup> test as appropriate for categorical variables as well as Wilcoxon-test. Values of P <.05 were considered to denote significant differences. Data were managed with an Excel database (Microsoft, Redmond, WA) and analyzed using R 2.15 software, available online (<http://cran.r-project.org/>).

## **Results**

### **Global overview of piRNA transcriptome**

Small RNA-seq of 200 saliva samples yielded ~4 642 M raw sequencing reads (from ~13.7 M to ~39.3 M reads/sample). Pre-filtering and filtering steps retained 70% (~3 205 M) of initial raw reads. Most of the filtered reads were of short read length. Quantification of the filtered reads and identification of known piRNAs yielded ~2,9 M sequences that were mapped to 2495 piRNAs from piRNABank. The number of expressed piRNAs ranged from 7 (outlier) to 247 per sample (169 = mean). The distribution of expressed piRNAs in the 200 saliva samples and the overall composition of processed reads is shown in Figure 1 – 3.

### **Comparison of piRNAs expressed in patients with and without endometriosis**

The distribution of the piRNAs according to the AUC values is given in Supplementary Table 2. None had an AUC  $\geq 0.70$ , and only three piRNAs (piR-004153, piR-001918, piR-020401) had an AUC between  $\geq 0.6$  and  $< 0.70$ .

Among the 201 piRNAs detected, none were up-regulated and 33 were down-regulated (Table 1). Four piRNAs were differentially expressed in patients with and without endometriosis: piR-004153, piR-012864, piR-017716, and piR-016904 (Figure 4). Only one piRNA had both an AUC  $> 0.60$  and p-value using Wilcoxon-test  $< 0.05$ . The Boxplots for the four piRNAs differentially expressed are given in Figure 5.

The respective relation and accuracy to diagnose endometriosis according to the F1-score, sensitivity, specificity, and AUC ranged from 0-0.862 %, 0-0.961%, 0.085-1, and 0.425-0.618.

### **Relation between epidemiological characteristics of the population and piRNA expression.**

Relations between piRNAs expression and age, alcohol, smoking, BMI and infertility were evaluated using Mann-Whitney test. No relation was found between piRNAs and age < 30 years. Using a cut-off of 35 years, a relation was observed between piR-004153 (p = 0.002) and piR-017716 (p = 0.030), and the diagnostic of endometriosis. Among patients with endometriosis, no relation was found between piRNAs expression and infertility. No relation was observed between piRNAs expression and BMI (< or >= 30 kg/m<sup>2</sup>), alcohol consumption nor smoking.

### **Relation between piRNAs expression and dysmenorrhea, disease stage or hormonal treatment**

No relation was observed between dysmenorrhea intensity using Visual Analogic Scale (VAS) or disease stage according to ASRM classification and piRNAs expression. Among patients with endometriosis, four piRNAs were differentially expressed in patients with and without hormonal treatment: piR-004153 (p = 0.015), piR-020401 (p = 0.001), piR-012864 (p = 0.036) and piR-017716 (p = 0.009). In both endometriosis and control groups, three other piRNAs were differentially expressed in patients using hormonal treatment: piR-011370 (p=0.011), piR-022296 (p=0.005) and piR-006701 (p=0.029),

### **Relation between pathophysiology of endometriosis and piRNA expression.**

Among piRNAs of interest, only one piRNA, analyzed in blood (piR-004153), was previously reported in colorectal cancer [42]. No pathophysiological signaling pathway associated with piR-004153 expression was clearly identified. No saliva expression was previously reported for this piRNA. Among the three remaining piRNAs differentially

expressed, none was reported either in benign or malignant disorders. None of these piRNAs was previously reported in endometriosis.

## Discussion

In the current study, we demonstrated that saliva piRNAs were differentially expressed in women with and without endometriosis. This finding is particularly relevant for identifying piRNAs as potential diagnostic biomarkers. Moreover, we demonstrated the relation between saliva piRNA expression and hormonal treatment and age. Finally, our analysis underlines the overall lack of data to designate the specific contribution of piRNAs in terms of pathophysiology.

In the present study, saliva piRNA expression was evaluated using NGS with Unique Molecular Identifier (UMI) barcoding that is the recommended approach to avoid amplification bias [31]. In the current study, using Poisson regression and the Wilcoxon test, we observed that none of the piRNAs detected had an AUC  $\geq 0.70$ , and that only three piRNAs (piR-004153, piR001918, piR-020401) had an AUC between  $\geq 0.6$  and  $< 0.70$  for the diagnosis of endometriosis. Four saliva piRNAs appear particularly relevant for the diagnosis of endometriosis (piR-004153, piR-012864, piR-017716 and piR-016904). None of them were previously reported in endometriosis. Thus, further studies are required to confirm the implication of piRNAs in endometriosis and their potential contribution as a diagnostic biomarker.

The most relevant data of the present study is the relation between saliva piRNA expression and hormonal treatment – including the contraceptive pill, progestins, and GnRH analog – which is the first-line treatment for endometriosis [43,44]. In the present study, among the piRNAs of interest, four were differentially expressed in patients with and without hormonal treatment (piR-004153,  $p=0.015$ ; piR-020401,  $p=0.001$ ; piR-012864,  $p=0.036$ ; and piR-017716,  $p=0.009$ ), and three additional piRNAs were differentially expressed in patients using hormonal treatment (piR-011370,  $p=0.011$ ; piR-022296,  $p=0.005$ ; and piR-006701,  $p=0.029$ ) regardless the endometriosis status. So far, none of these piRNAs have been

reported to be associated with hormonal treatment whatever the disease opening new avenues to elucidate the role of hormonal therapies and their mechanisms of action in endometriosis. Only piR-004153 has previously been evaluated, in the specific context of colorectal cancer, but without evaluating the associated signaling pathways. Despite the lack of data about the mechanistic role in the estrogenic piRNA response [45], previous reports have highlighted that piRNA expression is differentially regulated by estrogens [46]. Moreover, Zhang et al (2010) indicated that piRNA pathways are regulated by sex steroids [47]. Previous studies [48] have shown that the piR-31470/PIWIL4 complex could regulate the methylation level of glutathione S-transferase pi 1 (GSTP1) by recruiting multiple DNA methylation enzymes. This is relevant as a recent meta-analysis [49] demonstrated that only five of 28 polymorphisms were associated with endometriosis including GSTP1 rs1695. In transcriptional gene silencing (TGS), which mainly occurs with PIWI proteins with low catalytic activity, Cheng et al reported that the piRNA/PIWI complex mediates chromatin silencing by collaborating with histone-modifying enzymes or DNA methyltransferases hence suppressing mRNA transcription [50]. Finally, evidence of chromatin remodeling, de novo methylation, and direct transcriptional regulation shows the multifaceted roles of piRNAs [51–75] and the special role of piRNAs in epigenetic modification [76,77].

PiRNAs could contribute to a better understanding of the physiopathology of endometriosis, improvement in these patients management when infertile, as well as the development of new therapies [78]. Indeed, Expression of PIWI proteins in somatic stem cells [79] suggests that piRNA may also be involved in stem cell function regulation. However, the mechanism, major functions, and pathways regulated by the Piwi-piRNA complex remain poorly understood. Regarding fertility, the small RNA population of both the oocyte and cumulus cells during in vitro maturation (IVM) have been sequenced and the portfolio of endo-siRNA, miRNA, and piRNA demonstrated in pigs [80]. Moreover, since piRNAs

regulate protein-coding genes involved in the development of various diseases, they can be considered molecules useful as innovative epidrugs [81].

Another interesting finding is the variation in piRNA expression according to the epidemiological characteristics of the population. In the current study, piRNAs were differentially expressed according to the age of the patients. Although no difference was observed for patients under 30 years, differential expression was noted for piR-004153 ( $p=0.002$ ) and piR-017716 ( $p=0.030$ ) for patients over 35. So far, no data exist about the relation between piRNA expression and age in patients with endometriosis. However, Erwin and Blumenstiel have previously reported that piRNA biosynthesis increases with aging [82]. Moreover, in drosophila, Feltzin et al indicated a role of the exonuclease Nibbler on age-associated processes to modulate the length of small non-coding RNAs including piRNAs [83]. Wang et al described that normal ageing was associated with 3' shortening of piRNAs coupled with a decline in piRNA abundance [84]. Finally, Rounge et al found that ageing was the strongest factor associated with small-non-coding RNA expression, regardless of the class. However, none of their top five piRNAs that were differentially expressed in patients according to age were common with those found in our study [85]. This could be explained by differences in age between the studies: our population was composed of women between 18 and 43 years with few women over 40 years, while Rounge et al found a difference in piRNA expression for patients of 40-60 years. In contrast to age, we found no difference in piRNA expression according to BMI and smoking unlike the findings of a previous study [85] highlighting the need of further studies to assess not only the pathology involved but also the epidemiological characteristics of the population.

Some limits of the present study deserve to be underlined. First, in contrast to miRNA analysis in endometriosis [5–9], the crucial issue is the difference in the naming and numbering rules of piRNAs from one database to another [86] giving rise to different names

in various studies [86,87]. For example, piR-30025 and piRNA\_30025 are listed in the piRNAdb and piRNAQuest databases, respectively, but have different chromosomal positions [88]. Moreover, it has been pointed out that most of the small RNAs in somatic tissues that are annotated as piRNAs and map on the same genomic location as other ncRNAs, are not functional piRNAs but only fragments of other ncRNAs [87,89]. In the current study, we decided to evaluate piRNA expression using the piRNABank database only, with the potential risk of restricting the number of piRNAs evaluated. Second, although the present prospective study is the first to evaluate saliva piRNA expression in women with and without endometriosis, it is important to note that the study focused on a specific population in terms of age with a potential bias linked to the exclusion of adolescents and patients over 43 years old as well as menopausal women. Third, the absence of a relation between piRNA expression and some characteristics such as fertility, obesity and smoking could be explained by the relatively small sample size.

In conclusion, despite some limits, our results from a large cohort of saliva samples are the first to suggest that piRNAs play a role in endometriosis. A diagnostic tool developed around saliva piRNAs would have the advantage of being non-invasive and simple to administer. Further studies are necessary to confirm our findings since no data are available on piRNA expression in women with endometriosis using NGS and UMI in saliva, blood, or serum.





## **Declarations**

**Ethics approval and consent to participate:** The authors state that the data used are from the prospective ENDO-miRNA study (ClinicalTrials.gov Identifier: NCT04728152). Data collection and analysis were carried out under Research Protocol n° ID RCB: 2020-A03297-32. Informed consent was obtained from all subjects involved in the study.

**Consent for publication:** not applicable

**Availability of data and materials:** All relevant data are presented in the manuscript or in the supplementary files. Authors are available to provide any complementary information if asked.

**Competing interests:** S. Suisse is a former employee of Ziwig, Inc. The remaining authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript.

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### **Author's contributions**

Methodology and Design: SB, ED, PD, FG

Data collection: SB, YD, AP, CT, LD, MP

Biologic data collection: YD, LJ, SF, DB, YM

Analysis: SS, SB, ED, FG, PD, YM, SF

Data Interpretation: MP, SB, SS, LJ, YD, AP, CT et ED.

Manuscript writing: SS, YD, MP, SB, ED, LD.

All authors reviewed the manuscript for critical intellectual content.

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## **Figures Caption**

Figure 1: Overall composition of piRNAs in the processed reads

Figure 2: Distribution of expressed piRNAs in the 200 samples.

Figure 3: Distribution of expressed piRNAs in the samples per diagnosis (0 = controls, 1 = cases with endometriosis).

Figure 4: ROC curves analysis of the four piRNAs differentially expressed in patients with and without endometriosis.

Figure 5: Boxplot of the four piRNAs of interest for the diagnostic of endometriosis.

Down - regulated	Up-regulated
hsa_piR_004153/gb/DQ575660/Homo	-
hsa_piR_012864/gb/DQ587426/Homo	-
hsa_piR_021740/gb/DQ599789/Homo	-
hsa_piR_020582/gb/DQ598312/Homo	-
hsa_piR_006701/gb/DQ579162/Homo	-
hsa_piR_002980/gb/DQ574006/Homo	-
hsa_piR_004800/gb/DQ576604/Homo	-
hsa_piR_015149/gb/DQ590703/Homo	-
hsa_piR_019675/gb/DQ596992/Homo	-
hsa_piR_016659/gb/DQ592932/Homo	-
hsa_piR_000848/gb/DQ571067/Homo	-
hsa_piR_020455/gb/DQ598110/Homo	-
hsa_piR_004152/gb/DQ575658/Homo	-
hsa_piR_019912/gb/DQ597341/Homo	-
hsa_piR_014539/gb/DQ589909/Homo	-
hsa_piR_001104/gb/DQ571422/Homo	-
hsa_piR_015150/gb/DQ590704/Homo	-
hsa_piR_010024/gb/DQ583491/Homo	-
hsa_piR_011374/gb/DQ585363/Homo	-
hsa_piR_014879/gb/DQ590348/Homo	-
hsa_piR_016658/gb/DQ592931/Homo	-
hsa_piR_017295/gb/DQ593909/Homo	-
hsa_piR_017833/gb/DQ594619/Homo	-
hsa_piR_017936/gb/DQ594740/Homo	-
hsa_piR_014635/gb/DQ590029/Homo	-
hsa_piR_003672/gb/DQ574991/Homo	-
hsa_piR_018749/gb/DQ595765/Homo	-
hsa_piR_007540/gb/DQ580277/Homo	-
hsa_piR_020809/gb/DQ598639/Homo	-
hsa_piR_011186/gb/DQ585093/Homo	-
hsa_piR_004019/gb/DQ575471/Homo	-
hsa_piR_020668/gb/DQ598445/Homo	-
hsa_piR_015511/gb/DQ591196/Homo	-

Table 1 : piRNAs up and down-regulated in the population