Diagnosing endometriosis by measuring plasma micro ribonucleic acids: it may take a miRacle

A pessimist is a well-informed optimist.

Mark Twain

Endometriosis is a common cause of infertility, dyspareunia, dysmenorrhea, and chronic pelvic pain. Apart from detecting an endometrioma by pelvic imaging, current diagnosis of endometriosis is made surgically by the visualization of implants in the pelvis. Surgery is expensive and carries risk; this explains the often decade-long delay in diagnosing endometriosis. Meanwhile, empiric treatment of endometriosis for *all* patients presenting with pelvic pain is a misuse of resources and delays the diagnosis and treatment of alternative causes of pelvic pain. A noninvasive diagnostic biomarker for endometriosis would benefit patients and providers by permitting low-risk, cost-effective, and prompt detection of the disorder. Such a marker might enable early and titrated medical treatment depending on disease severity. A biomarker would benefit the research community by identifying affected patients early in the disease process when study of disease progression, and clinical trials, might be possible.

In this edition of *Fertility and Sterility*, Papari et al. (1) test plasma micro ribonucleic acid (miRNA) profiles as noninvasive markers for diagnosing endometriosis. The miRNAs are processed from long, noncoding, ribonucleic acid (RNA) sequences into functional single-stranded transcripts roughly 22 nucleotides in length. These mature miRNAs are integrated into the RNA-induced silencing complex to post-transcriptionally repress target messenger RNA expression. The estimated 2,200 miRNAs in humans may target more than half of all human genes, regulating a wide array of processes including cell fate, cell cycle, cell metabolism, and cancer progression. Although most RNA species are notoriously vulnerable to degradation, plasma miRNAs are extraordinarily stable due to plasma protein binding and/or localization to exosomes.

In preliminary studies the Papari team (1) performed next-generation sequencing (NGS) on plasma from patients with surgically documented endometriosis (n = 10) or without endometriosis (n = 10), identifying 41 miRNAs that are differentially expressed in endometriosis. Of these, 28 were not previously associated with endometriosis. Candidate miRNAs were confirmed using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) in an additional set of patients with and without endometriosis (n = 30 and n = 15, respectively). From these data, Papari et al. (1) selected 20 plasma miRNAs that differed by more than twofold between study groups and added an additional four miRNAs detected in prior studies (and presumably not identified by NGS) for further study.

The qRT-PCR was then used to test the 24 candidate plasma miRNAs in a retrospective cohort of laparoscopically confirmed endometriosis cases and laparoscopically negative controls. Although Papari et al. (1) started with n = 53 samples from each group, half of the plasma samples were excluded from qRT-PCR due to evidence of hemolysis. Eight miRNAs were confirmed as significantly lower in cases compared with controls. A logistic regression model based on five of the eight miRNAs (miR-17-5p, miR-20a-5p, miR-199a-3p, miR-143-3p, and let-7b-5p) had a sensitivity and specificity for diagnosing endometriosis of 0.96 and 0.79, respectively, with positive and negative predictive values (PPV and NPV) of 0.80 and 0.96, respectively. Although still preliminary, they offer this panel of five miRNAs as noninvasive markers for the diagnosis of endometriosis.

Notably, Vanhie et al. (2) recently reported genome-wide plasma miRNA expression in infertile endometriosis and control patients, most with pain. Their testing of miR-125b-5p, miR-28-5p, and miR-29a-3p in a validation set (n = 30 controls and n = 60 patients with endometriosis) led to poor test specificity.

Strengths of the current report include [1] an unbiased discovery phase using NGS, [2] rigorous phenotyping of cases and controls by laparoscopy, [3] careful selection of circulating reference miRNAs ("housekeeping miRNAs") for which expression is high and remains stable between cases and controls, and [4] elimination of plasma samples with evidence of hemolysis (which is expected to alter the miRNA profile ordinarily present in cell-free plasma). Two experimental methods were used to identify hemolysis (or red blood cell contamination)—spectrophotometry and low expression of the red blood cell-expressed miR-451 relative to the plasma-expressed miR-23a (3).

Limitations of this work include small sample sizes for the purposes of modeling and a low proportion of endometriosis-associated miRNAs identified by NGS that were subsequently confirmed by qRT-PCR. Eight of 24 candidates were confirmed by qRT-PCR. More important, there was no validation of the diagnostic model in a separate patient cohort, leaving open the possibility that the model was overfit to the training dataset. From a practical standpoint, the need to exclude half of the specimens due to hemolysis compromises the clinical utility of this miRNA screening test in its current design. In fact, the high risk of hemolysis in 30%–40% of serum samples represents a potential Achilles heel for many peripheral miRNA biomarker studies (3).

At present more than a dozen teams of investigators have measured peripheral miRNAs in women with and without endometriosis. These studies report disparate findings regarding which plasma miRNAs are altered in patients with endometriosis and inconsistent findings with respect to whether the identified miRNAs are up-regulated or down-regulated in the disorder (4). Interstudy inconsistencies may be attributed to disparities between study populations (including variable disease severity and diverse criteria for selecting the control groups, as well as diverse genetic backgrounds), variable specimens (plasma vs. serum) and specimen handling, different stages of the menstrual cycle,

and variation in plasma miRNA detection platforms (NGS, global qRT-PCR, microarrays). Papari et al. (1) suggest that an additional source of experimental variation may relate to suboptimal choice of reference RNAs when normalizing miRNA expression levels by qRT-PCR (there is evidence that the popular reference RNAs RNU6 and miR-16-5p are not stable in human plasma). Although likely to be true for some studies, this reasoning ignores studies that used alternative reference RNAs and implies failure of multiple research groups to properly validate their methodological approaches.

Despite the disquieting number of inconsistent reports on the subject, an optimist might discern an emerging consensus regarding altered plasma expression of several miRNAs (miR-17-5p?, miR-20a-5p?, miR-125b?) in patients with endometriosis. In aggregate, there remains no consensus on which plasma miRNAs, if any, will predict the presence of endometriosis with sufficient sensitivity and specificity in the clinical setting.

Meanwhile there is emerging evidence that miRNAs play a direct role in the pathogenesis of the endometriosis by regulating essential processes such as inflammation and angiogenesis (5). Whether plasma miRNAs contribute to the pathogenesis of the disorder or are simply markers of existing disease remains unknown. Whether peripheral miRNAs correlate with severity of disease, or the degree of pelvic pain, similarly remains unknown. We must commend Papari et al. (1) for their rigor in phenotyping cases and controls and for excluding samples with evidence of hemolysis. Practical implementation of a peripheral miRNA-based diagnostic test for endometriosis will depend on greater consistency in methods and analysis as well as rigorous replication in validation sets. Given that laparoscopy is required to distinguish cases from controls and that laparoscopy for unexplained infertility or elective sterilization are vanishing practices, it remains possible that the definitive study may never be performed.

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