Nuclear magnetic resonance metabolomic profiling of urine provides a noninvasive alternative to the identification of biomarkers associated with endometriosis

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Objective: To investigate whether urine metabolomic profile can be used to identify biomarkers associated to endometriosis.
Design: Prospective study. For each subject, a urine sample was collected after overnight fasting and before surgery.
Setting: University medical center.
Patient(s): The clinical cohort included 45 endometriosis patients, diagnosed at early (n = 6) and advanced (n = 39) stages of the disease, and 36 healthy women. All women underwent diagnostic laparoscopy to visually confirm the presence or absence of endometriotic lesions.
Intervention(s): Metabolomic profiling of urine samples based on ^{1}H-nuclear magnetic resonance (NMR) spectroscopy in combination with statistical approaches.
Main Outcome Measure(s): Comparative identification of metabolites present in urine from endometriosis patients and healthy women.
Result(s): The urine metabolomic profile of endometriosis patients exhibited higher concentrations of N^{1}-methyl-4-pyridone-5-carboxamide, guanidinosuccinate, creatinine, taurine, valine, and 2-hydroxyisovalerate and decreased concentrations of lysine compared with healthy women. Most of these metabolites are involved in inflammation and oxidative stress processes. These pathophysiologic events had been previously described to be present in ectopic endometrial proliferation foci.
Conclusion(s): Overall, the results demonstrate the potential of ^{1}H-NMR–based metabolomics, a rapid and noninvasive approach, to identify metabolic changes associated to endometriosis in urine samples. This information could be useful to get a better understanding of the pathogenesis of endometriosis, thus providing support to the noninvasive diagnosis of this pathology. (Fertil Steril® 2015;104:1202–9. ©2015 by American Society for Reproductive Medicine.)
Key Words: Endometriosis, metabolomics, ^{1}H-NMR spectroscopy, urine, biomarkers

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Endometriosis is a chronic gynecologic disease, estrogen dependent, and associated with pelvic pain and infertility. Endometriosis is defined by the presence of endometrial tissue outside the uterine cavity (1) and represents one of the most common benign gynecologic disorders. The exact prevalence of endometriosis is unknown, but estimates range from 10% in women of reproductive age to up to 50% in women with pelvic pain and/or infertility (2, 3). Endometriosis symptoms are highly variable, often nonspecific, and common in the general population. The wide range of clinical manifestations complicates the diagnosis of endometriosis based

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on symptoms or clinical examination. Furthermore, available imaging techniques and blood tests have limited value as diagnostic tools (4). The compounding effects of all these factors contribute to diagnostic delays of an average of 8 years (5).

Currently, the definitive method to diagnose and classify endometriosis is direct visualization at surgery, usually via laparoscopy (2). But laparoscopy is an invasive procedure and inappropriate for periodic restaging of the disease after treatment. In this context, a biomarker, or a combination of them, that could be measured in an easily accessible biofluid would provide a starting point for the noninvasive diagnosis of endometriosis. However, despite numerous efforts carried out during the past years focused on the discovery of endometriosis biomarkers, nowadays, there is no simple test that can be used to diagnose endometriosis with enough statistical power to be applied in clinical practice. Thus, the discovery of biomarkers in biologic fluids remains an unresolved challenge.

Metabolomics focuses on the characterization of patterns of metabolites present in body fluids or tissues and is leading to advanced diagnostics and therapeutics (6). Metabolites are low-molecular-weight compounds, end-products of metabolism (7), that can be objectively measured and evaluated as indicators of normal physiologic or pathologic processes, pharmacologic responses to treatment, etc. (8). Disease status is known to alter the metabolic composition of biofluids, in qualitative and quantitative terms, by generating metabolic signatures that correlate with a given pathologic condition (9). The most commonly used analytic techniques for metabolic profiling are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). In particular, metabolomics with the use of ^{1}H-NMR provides a robust and reliable platform for the simultaneous measurement and quantification of a wide range of metabolites in a given sample (10) with minimal sample handling (11).

The aim of the present study was the characterization of the urine metabolomic profile of endometriosis patients and its comparison with that of healthy women. Our results show that metabolites involved in inflammation and oxidative stress processes play a very important role in this disease. This information could be useful for getting a better understanding of the molecular mechanisms involved in the pathogenesis of endometriosis and contribute to the noninvasive diagnosis of this disease.

MATERIALS AND METHODS

Study Design and Subjects

Patient recruitment was carried out at the Hospital Universitario La Fe (Valencia, Spain), and measurement and analysis of the urine metabolomic profiles were carried out at the Centro de Investigación Príncipe Felipe (Valencia, Spain). Samples were collected from 81 volunteers recruited from November 2009 to December 2012. Clinical diagnosis and classification of subjects was performed with the use of laparoscopy to visually confirm the presence or absence of endometriosis. The group of women diagnosed with endometriosis consisted of 45 patients aged 18–43 years, presenting endometriosis-associated symptoms, who were scheduled for laparoscopy for diagnosis and surgical treatment. Women diagnosed with endometriosis were staged from I to IV according to the revised American Society for Reproductive Medicine score (12). The control group was composed of 36 healthy women aged 26–45 years, who underwent routine diagnostic laparoscopy for tubal sterilization and, after discarding the presence of endometriosis, were included in the healthy individuals cohort. Menstrual cycle phases (MCPs) were classified as follicular phase (cycle days 1–14) or luteal phase (cycle days 15–28) after adjustment to a 28-day cycle. Women with previous history of endometriosis or other gynecologic diseases, including fibroids, other ovarian cysts, or pelvic inflammatory disease were excluded from the study. None of the participating women had received hormonal therapy for ≥ 1 month before surgery. In addition, women diagnosed with any other disease or under pharmacologic treatment were not included in the study. Clinical information associated with each sample group is summarized in Table 1.

Patient recruitment and sampling procedures were performed in accordance with the Declaration of Helsinki and applicable local regulatory requirements and laws and after approval from the Ethics Committee of the Hospital Universitario La Fe. Written informed consent was obtained from each participant before being included in this study.

Sample Collection and NMR Sample Preparation

Urine samples were collected from each participant after overnight fasting and before surgery into labeled tubes containing 0.05% sodium azide (NaN_3) to avoid bacterial growth (13). After collection, samples were rapidly frozen in liquid nitrogen and stored at −80°C until NMR analysis. For NMR analysis, urine samples were thawed at room temperature and gently mixed before sample preparation. 700 μL urine was centrifuged at 6000*g* for 10 minutes, 540 μL of the supernate was added to 60 μL 1.5 mol/L potassium phosphate buffer (pH 7.4) containing 0.1% trimethylsilylpropionic acid–d4 sodium salt (TSP) as internal standard compound, and 0.05% NaN_3 in D_2O. Then samples were transferred to 5-mm NMR tubes and stored at 4°C until their analysis.

TABLE 1

Clinical characteristics of the samples included in the urine metabolomic study.

Characteristic	Healthy women (n = 36)	Endometriosis patients (n = 45)
Age (y), mean ± SD	35.47 ± 5.15	32.29 ± 6.59
Menstrual cycle phase		
Follicular	30	30
Luteal	6	15
Stage of the disease		
Minimal/mild (I–II)	–	6
Moderate/severe (III–IV)	–	39

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