Endometriosis phenotypes are associated with specific serum metabolic profiles determined by proton-nuclear magnetic resonance

**KEY MESSAGE**
Proton-nuclear magnetic resonance-based metabolomics of serum samples has ample potential for the identification of metabolic changes associated with endometriosis phenotypes. This information may be useful for obtaining a better understanding of the pathogenesis of endometriosis.

**ABSTRACT**

**Research question:** What is the correlation between serum metabolic profile and endometriosis phenotype?

**Design:** A pilot study nestled in a prospective cohort study at a university hospital, including 46 patients with painful endometriosis who underwent surgery and 21 controls who did not have macroscopic endometriotic lesions. Endometriosis was strictly classified into two groups of 23 patients each: endometrioma (OMA) and deep infiltrating endometriosis (DIE). Serum samples were collected before surgery for metabolomic profiling based on proton-nuclear magnetic resonance spectroscopy in combination with statistical approaches. Comparative identification of the metabolites in the serum from endometriosis patients and from controls was carried out, including an analysis according to endometriosis phenotype.

**Results:** The serum metabolic profiles of the endometriosis patients revealed significantly lower concentrations of several amino acids compared with the controls, whereas the concentrations of free fatty acids and ketone bodies were significantly higher. The OMA and the DIE phenotypes each had a specific metabolic profile, with higher concentrations of two ketone bodies in the OMA group, and higher concentrations of free fatty acids and lipids in the DIE group.

**Conclusion:** Proton-nuclear magnetic resonance-based metabolomics of serum samples were found to have ample potential for identifying metabolic changes associated with endometriosis phenotypes. This information may improve our understanding of the pathogenesis of endometriosis.

**KEYWORDS**

- H-nuclear magnetic resonance
- Deep infiltrating endometriosis
- Endometriosis
- Metabolomics
INTRODUCTION

Endometriosis is a benign chronic gynaecological disorder defined as the presence of endometrial tissue, e.g. glands and stroma, outside the uterine cavity (Sampson, 1927). It affects 10–15% of reproductive-aged women, causing pain, infertility, or both (Chapron et al., 2019). This disease is heterogeneous in nature, with three main types of lesions: superficial peritoneal endometriosis; ovarian endometrioma (OMA); and deeply infiltrating endometriosis (DIE) (Nicolle and Donnez, 1997). Its precise pathophysiology remains to be fully elucidated, and numerous theories have been forwarded, including retrograde menstruation, coelomic metaplasia, stem cells and Mullerian remnants. Immunological, hormonal, genetic, epigenetic and environmental factors may also be involved (Lagonà et al., 2017; Chapron et al., 2019).

A notable characteristic of the disease is a markedly long time to diagnosis and treatment (Chapron et al., 2010a), resulting in highly adverse consequences on the quality of life of women with the disease (Chapron et al., 2019). Indeed, the currently available blood tests and imaging techniques have moderate diagnostic values (Ahn et al., 2017), thus making the development of new non-invasive diagnostic methods a high-priority objective in the field of endometriosis research (Thubert et al., 2014).

Metabolomics consists of determining the set of metabolites in biological samples under normal or pathophysiological conditions, such as diseases (Pillet et al., 2014; Klassen et al., 2017). This is, therefore, a promising approach for obtaining greater insight into the nature of complex disorders by means of identifying a specific metabolic signature, and this could be a way to gain a better understanding of endometriosis pathogenesis as well as new non-invasive diagnostic biomarkers of this disease. The most commonly used tools for establishing a metabolic profile are mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy. In particular, proton-nuclear magnetic resonance (1H-NMR) has the advantage of being able to provide a quantitative and non-destructive evaluation of a complex mixture of metabolites, with minimal handling (Duarte and Gil, 2012).

A small number of studies have recently analysed the metabolic profile of serum from endometriosis patients, and they have identified biomarkers involved in inflammation, oxidative stress, cell proliferation and angiogenesis (Duarte et al., 2012; 2018; Jong et al., 2013; Vicente-Muñoz et al., 2016). These studies, however, did not correlate the metabolic alterations with the endometriosis phenotypes.

In light of the paucity of published research in this area, the aim of the present study was to use 1H-NMR to evaluate the serum metabolic profiles of endometriosis patients compared with control patients, and to determine whether different endometriosis phenotypes have specific signatures.

MATERIALS AND METHODS

Study protocol and participants

This study was approved by the local Institutional Review Board (approval number 05-2006 provided by the ‘Comité de Protection des Personnes et des Biens dans la Recherche Biomédicale’ of Paris Cochin on 24 February 2006), and all participants provided written informed consent.

This was a pilot study nested in a prospective cohort study of which details have been published elsewhere (Chapron et al., 2011c). Forty-six patients with painful endometriosis who underwent surgery for complete removal of their endometriotic lesions between January 2012 and November 2016 were included. Endometriosis was categorized into two groups according to histological findings (OMA and DIE) as described previously (Chapron et al., 2006). As these two types of endometriotic lesions are frequently associated, patients with endometriosis were classified to the category of the worst finding. By definition, endometriotic lesions ranked from least to worst were OMA and DIE (Chapron et al., 2011b). Endometriosis was scored according to the American Society for Reproductive Medicine (Revised American Society for Reproductive Medicine classification of endometriosis, 1997). Patients were considered as presenting endometriosis and included only when lesions were histologically proved.

The control patients included 21 non-pregnant women without any macroscopic endometriotic lesions based on the results of a preoperative magnetic resonance imaging (MRI) and a thorough examination of the abdominopelvic cavity during surgery. The indications for surgery in these control patients were as follows: tubal infertility, non-endometriotic ovarian cysts or uterine myoma. All patients underwent a preoperative imaging work-up by senior radiologists, including a MRI, to stage the endometriosis accurately according to previously published criteria (Kinkel et al., 2006; Medeiros et al., 2015; Millischer et al., 2015) in the endometriosis group and to rule out the presence of DIE lesions in the control group, as small nodules may be difficult to identify during surgical examination (Kaninckx et al., 2012). Women aged over 42 years who had cancer or chronic viral infections, e.g. hepatitis and HIV, who did not undergo a preoperative MRI, serum sampling the morning of the surgery, or both, or who did not provide consent, were excluded from the study.

The study analysis used a prospectively managed database. For each patient, their personal history data were obtained during face-to-face interviews, which were conducted by the surgeon in the month before surgery. A highly structured previously published questionnaire was used for all of the patients (Chapron et al., 2010a; 2010b). The following items were recorded: age, parity, gravidity, height, weight, body mass index (BMI), the existence and duration of infertility, lifestyle habits, history of hormonal, surgical treatments for symptomatic endometriosis, or both, and the existence of gynaecological pain symptoms, i.e. dysmenorrhoea, deep dyspareunia, non-cyclic chronic pelvic pain, and gastrointestinal and lower urinary tract symptoms. A 10-cm visual analogue scale was used (Huskisson, 1974) to evaluate the pain intensity preoperatively. The patients who had no hormonal treatment at the time of surgery had a minimum wash-out period of 3 months. The indications of using hormonal treatments in both study groups were as follows: invalidating chronic pelvic pain symptoms, a need for contraception, or both. Different hormonal treatments could be used according to the efficacy, tolerability and cost, including combined oral contraceptives (COC), progestins or gonadotrophin-releasing hormone agonists (GnRHAs).
Sample collection and nuclear magnetic resonance sample preparation

For each patient, a serum sample was collected after overnight fasting and before surgery. The blood samples were centrifuged at 800 g for 12 min at 4°C, and the serum supernatants were collected. Aliquots of the supernatants were stored at −70°C until they were analysed.

After thawing on ice, NMR samples were prepared by taking 300 μl from each serum sample supernatant, followed by the addition of 300 μl of a solution containing a phosphate buffer with D2O to obtain a final buffer concentration of 38 mM with a pH value of 7.4, which contained 2.3 mM NaN3 as well as 2.3 mM sodium trimethylsilylpropionate salt as the NMR chemical shift reference. D2O (12% [v/v]) was used for the field-frequency lock and shimming on the NMR spectrometer. A total of 600 μl was then transferred into 5-mm NMR tubes for the metabolic analysis.

Nuclear magnetic resonance spectroscopy and spectral processing

The NMR data were recorded at 300 K on a 500 MHz Bruker Avance II spectrometer equipped with a 5-mm 1H cryoprobe, using a sampleXpressTM automation sample changer. 1H spectra were obtained for each sample using a Carr–Purcell–Meiboom–Gill pulse sequence (Meiboom and Gill, 1958) to remove broad peaks originating from macromolecules. For each experiment, a total of 512 scans with 32,768 points were recorded using a recycle delay of 2 s, and a chemical shift spectral window of 20.65 ppm, thereby resulting in a total experimental time of 33 min. Other parameters were as follows: a Carr–Purcell–Meiboom–Gill spin-echo train of 64 ms with a spin-echo delay of 400 μs, and an acquisition time of 1.6 s. Processing of the NMR spectra was carried out using NMRProcflow software (Jacob et al., 2017). The spectra were zero-filled to 65,536 points and processed with an exponential apodization function using a line broadening of 0.3 Hz before Fourier transformation. They were then phased, baseline corrected and, finally, referenced to the alanine methyl group signal at 1.47 ppm. Individual metabolites were assigned using the Chenomx NMR suite 7.1 software (Chenomx Inc. Edmonton, Canada), the Human Metabolome Database (Wishart et al., 2007) as well as previously published data (Dutta et al., 2012; Vicente-Muñoz et al., 2016).

The spectral data were further reduced by dividing each spectrum into unevenly spaced buckets using the variable size bucketing method as implemented in NMRProcflow (Jacob et al., 2017) to avoid peaks lying in different consecutive integration regions. The spectral regions corresponding to sodium trimethylsilylpropionate salt (ppm), water (ppm), as well as to the baseline noise (ppm), were excluded from the analysis. A total number of 121 buckets were obtained and further integrated to build the matrix of variables used in the statistical analysis.

Before statistical analysis, the matrix of buckets integral values was normalized using a Probabilistic Quotient Normalization (Dieterle et al., 2006) to account for the difference in samples concentrations, and then finally scaled to unit variance to afford the same weight to each variable, using MetaboAnalyst (Xia et al., 2009; Chong et al., 2018).

Statistical analysis

The continuous data are presented as mean and SD, and the categorical data as numbers and percentages. For comparison of the patients’ general characteristics, a Pearson’s chi-squared test or Fisher’s exact probability test was used for the qualitative variables and the Student’s t-test for the quantitative variables.

In the present study, an untargeted approach whereby no assumption was made about the metabolites or the metabolic pathways affected by the disease was used to obtain an overall profile for endometriosis patients. The calculation of a sample size did not seem to be necessary. Both univariate and multivariate statistical approaches were used. Univariate analysis was carried out using the Student’s t-test and analysis of variance as implemented in MetaboAnalyst (Ko et al., 2009; Chong et al., 2018). Multivariate analysis was carried out using SIMCA software (Sartorius Stedim Biotech). A supervised orthogonal projections to latent structures discriminant analysis (OPLS-DA) (Trygg and Wald, 2002) was carried out, taking into account the class of the different samples to maximize class discrimination. The quality of the models thus obtained was assessed with the quality factors provided by the SIMCA software, i.e. the cumulative explained variance of the observed data X, R2Y, the cumulative explained variance of assignment Y, R2Y, and the estimate of predictive ability, Q2. The performance of the models was further tested using cross-validated analysis of variance (Eriksson et al., 2008) with P < 0.05 being considered statistically significant. The main variables responsible for the class discrimination were identified with the Variable Importance on Projection (VIP) values of the OPLS-DA models. Variables were considered relevant only when their VIP score was greater than 1.

RESULTS

Study population

Forty-six women with endometriosis and 21 controls without endometriosis were included in the study. The patients’ characteristics are presented in Table 1. No significant differences were found in age (P = 0.515), BMI (P = 0.165), gravidity (P = 0.130), parity (P = 0.207) and infertility (P = 0.386) between the study and the control groups. Neither the percentage of patients undergoing hormonal treatment at the time of surgery nor the menstrual cycle phases for the patients without hormonal treatment differed significantly between the two study groups (P = 0.071). The distribution of the hormonal treatments in the study population was as follows: COC (n = 5 [23.8%]), progesterins (n = 12 [57.1%]) and GnRHa (n = 4 [19.1%]) in the endometriosis group, versus COC (n = 1 [12.5%]), progesterins (n = 5 [62.5%]) and GnRHa (n = 2 [25%]) in the control group (P = 0.999). In the endometriosis group, the phenotypes were distributed as follows: OMA (n = 23 [50%]) and DIE (n = 23 [50%]), including nine patients (39%) with associated OMA. The mean American Society for Reproductive Medicine stage was 2.96 ± 1.08. As expected in the endometriosis group (Berkley et al., 2005), almost all visual acuity scores for chronic painful symptoms were significantly higher compared with the controls, including dysmenorrhoea (P = 0.028), non-cyclic chronic pelvic pain (P = 0.004), gastrointestinal symptoms (P < 0.001) and low urinary tract symptoms (P = 0.007).

Serum metabolic profiles of endometriosis patients compared with controls

A representative serum 1H-NMR spectrum from an endometriosis patient
is presented in FIGURE 1. The assignment of the most significant metabolites is also indicated. After univariate analysis, a total of 22 variables with $P < 0.1$ (of which 18 variables had $P < 0.05$) were identified as relevant regions in the discrimination. These were used to identify the metabolites that had altered levels in the endometriosis patients (Supplementary Table 1 and FIGURE 2). The concentrations of several amino acids, such as glutamine (GLN) ($P = 0.001$), valine (VAL) ($P = 0.012$), threonine (THR) ($P = 0.011$), histidine (HIS) ($P = 0.004$), tyrosine (TYR) ($P = 0.063$), leucine (LEU) ($P = 0.039$), isoleucine (ILE) ($P = 0.084$), and glutamic acid (GLU) ($P = 0.027$) were significantly lower in the endometriosis patients compared with the control patients. Conversely, the concentrations of free fatty acids ($\alpha$-CH$_2$FFA) such as 2-octenoate ($P = 0.062$), and ketone bodies such as acetone ($P = 0.021$) and 3-hydroxybutyrate (3HB) ($P = 0.029$), were significantly higher in the endometriosis group.

The supervised OPLS-DA score plot (FIGURE 3) showed a clear separation between the endometriosis and the control samples, with relatively good model quality factors ($R^2X = 0.13$, $R^2Y = 0.71$ and $Q^2 = 0.33$). The corresponding cross-validated analysis of variance (Eriksson et al., 2008) led to a $P$-value of $5.03 \times 10^{-5}$, further confirming the good performance of the model. The main variables contributing to the differences between the two groups of samples were identified and plotted as a VIP score plot (FIGURE 4). Assignment of these variables revealed the same metabolites as those obtained with the univariate analysis, thereby underscoring their relevance to the pathology.

### TABLE 1: BASELINE CHARACTERISTICS OF THE PATIENTS

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Endometriosis ($n = 46$)</th>
<th>Controls ($n = 21$)</th>
<th>$P$-value$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years$^a$</td>
<td>33.9 ± 5.8</td>
<td>33.1 ± 5.8</td>
<td>0.515</td>
</tr>
<tr>
<td>BMI, kg/m$^2$</td>
<td>22.1 ± 3.8</td>
<td>23.3 ± 3.8</td>
<td>0.165</td>
</tr>
<tr>
<td>Parity$^a$</td>
<td>0.3 ± 0.7</td>
<td>0.5 ± 0.7</td>
<td>0.207</td>
</tr>
<tr>
<td>Gravidity$^a$</td>
<td>0.5 ± 1</td>
<td>0.9 ± 1</td>
<td>0.130</td>
</tr>
<tr>
<td>Infertility, n (%)</td>
<td></td>
<td></td>
<td>0.386</td>
</tr>
<tr>
<td>No</td>
<td>32 (69.6)</td>
<td>13 (61.9)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>14 (30.4)</td>
<td>8 (38.1)</td>
<td></td>
</tr>
<tr>
<td>Length of infertility, months</td>
<td>34.4 ± 26.7</td>
<td>36.6 ± 24.6</td>
<td>0.844</td>
</tr>
<tr>
<td>Painful symptoms (VAS score)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dysmenorrhoea</td>
<td>77 ± 2.6</td>
<td>61 ± 2.5</td>
<td>0.028</td>
</tr>
<tr>
<td>Deep dyspareunia</td>
<td>42 ± 3.5</td>
<td>33 ± 3.5</td>
<td>0.235</td>
</tr>
<tr>
<td>Non-cyclical chronic pelvic pain</td>
<td>39 ± 2.9</td>
<td>16 ± 3.2</td>
<td>0.004</td>
</tr>
<tr>
<td>Gastrointestinal symptoms</td>
<td>47 ± 3.4</td>
<td>18 ± 3.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lower urinary tract symptoms</td>
<td>1.6 ± 2.7</td>
<td>0.2 ± 2.7</td>
<td>0.007</td>
</tr>
<tr>
<td>Hormonal treatment before surgery, n (%)</td>
<td></td>
<td></td>
<td>0.071</td>
</tr>
<tr>
<td>Yes</td>
<td>21 (45.7)</td>
<td>8 (38.1)</td>
<td></td>
</tr>
<tr>
<td>Cycle phase if no hormonal treatment, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferative phase</td>
<td>8 (32)</td>
<td>9 (69.2)</td>
<td></td>
</tr>
<tr>
<td>Secretory phase</td>
<td>14 (56)</td>
<td>2 (15.4)</td>
<td></td>
</tr>
<tr>
<td>Missing data</td>
<td>3 (12)</td>
<td>2 (15.4)</td>
<td></td>
</tr>
<tr>
<td>Surgical classification of endometriosis, n (%)$^b$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OMA</td>
<td>23 (50)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>DIE</td>
<td>23 (50)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Intestinal DIE</td>
<td>9 (39.1)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Associated OMA</td>
<td>9 (39.1)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>ASRM total score$^c$</td>
<td>31.2 ± 34.1</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>ASRM implants score$^c$</td>
<td>18.4 ± 15</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>ASRM adhesions score$^c$</td>
<td>16.7 ± 19.6</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Data are presented as mean ± SD.

$^b$ According to a previously published surgical classification for deeply infiltrating endometriosis by Chapron et al. (2006).

$^c$ Score according to the American Society for Reproductive Medicine classification (Revised American Society for Reproductive Medicine classification of endometriosis, 1997).

$^d$ Statistical analysis was performed using Fisher’s exact test or Pearson’s Chi-square for qualitative variables and Student’s t test for quantitative variables; statistical significance: $P < 0.05$.

ASRM, American Society for Reproductive Medicine; BMI, body mass index; DIE, deeply infiltrating endometriosis; NA, not applicable; OMA, ovarian endometrioma; VAS, visual analogue scale.
To be noted, no validated model was obtained according to the cycle phase or the type of hormonal treatment using a supervised method ($P = 1$ and $Q^2$ values = –0.18 and –0.28, respectively, typical of unpredictive models [full data not shown]).

Serum metabolic profiles according to the endometriosis phenotypes
The results of the univariate analysis comparing the serum metabolic profiles of the control women versus the OMA patients and the DIE patients are presented in figures. In the OMA group, a significantly higher concentration of two ketone bodies (acetone, 3HB) ($P = 0.005$ and $P = 0.016$, respectively) and of an unknown metabolite (U2) ($P = 0.039$) was found compared with the control group and the DIE group. In the DIE group, the concentrations of free fatty acids ($\beta$-CH$_2$ FFA, $P = 0.010$) and lipids were significantly higher compared with the other two groups ($=\text{CH-CH}_2\text{-CH}_2$ LIPIDS, $P = 0.025$ and $=\text{CH-CH}_2$ LIPIDS, $P = 0.018$). The multivariate analysis comparing the three groups confirmed these results, with the OPLS-DA score plot underlining a significant discrimination between the three types of samples ($P = 3 \times 10^{-3}$) (figure 6).

DISCUSSION
Principal findings
This study highlights the potential role of using $^1$H-NMR-metabolomics of serum samples to identify metabolic changes associated with endometriosis and endometriosis phenotypes. We showed that the concentrations of several amino acids were lower in the serum of endometriosis patients. Concomitantly, we found that the concentrations of ketone bodies and free fatty acids concentrations were higher. Moreover, women with OMA and DIE exhibited specific metabolic profiles.

Results
The search for biomarkers involved in endometriosis is challenging, not only because it could become a non-invasive diagnostic tool, but also because it could help with obtaining a better understanding of the pathophysiological processes underlying the disease. A small number of untargeted NMR-based metabolomics studies have recently analysed blood samples of endometriosis patients relative to controls (Dutta et al., 2012; 2018; Jana et al., 2013; Vicente-Muñoz et al., 2016), and they have highlighted the relevance of amino acids and metabolites involved in oxidative stress and glycolytic pathway.

Clinical and research implications and biological rationale of the findings
In the present study, several amino acids seemed to be statistically discriminant between the patient and the control samples, with lower levels observed in endometriosis patients. It is well
known that endometriosis is a chronic inflammatory and immune condition (Lousse et al., 2012; Ahn et al., 2015; Coelho Riccio et al., 2018), characterized by increased levels of various cytokines (Santulli et al., 2012; 2013; Lambert et al., 2014; Luckow Invitti et al., 2018). The production of a number of these cytokines by inflammatory cells has been shown to stimulate the acute-phase response in the liver, leading to the secretion of acute-phase proteins.
The corresponding quality factors were as follows: $R^2_X = 0.13$, $R^2_Y = 0.71$ and $Q^2 = 0.33$.

The OPLS-DA model was validated with its corresponding $P$-value of $5.03 \times 10^{-5}$ obtained using cross-validated analysis of variance. C, control samples; E, endometriosis samples.

Oxidative stress could also be one of the causes of variation in serum amino acid levels. Indeed, endometriosis is associated with enhanced oxidative stress (Ngô et al., 2009; Jana et al., 2013; Santulli et al., 2015; Donnez et al., 2016; Scutiero et al., 2017) and increased reactive oxygen species levels in the patients’ samples could promote amino acids oxidation, thus explaining their reduced levels.

Among the affected amino acids, GLN, GLU, VAL, ILE, THR and HIS

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**FIGURE 3** Score plot of the supervised multivariate analysis obtained using orthogonal partial least square discriminant analysis (OPLS-DA) scores for multivariate analysis of serum samples collected from the control patients ($n = 21$) and the endometriosis patients ($n = 46$). The OPLS-DA model was validated with its corresponding $P$-value of $5.03 \times 10^{-5}$ obtained using cross-validated analysis of variance. C, control samples; E, endometriosis samples.

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**FIGURE 4** Variable importance in projection (VIP) analysis for the multivariate analysis of serum samples collected from the control patients ($n = 21$) and the endometriosis patients ($n = 46$). The VIP showing the variables contributing the most to the group discrimination in the OPLS-DA model. The variables are indicated on the X-axis as the centre of the chemical shift of the corresponding bucket, whereas the Y-axis corresponds to the VIP score, i.e. the weight of each variable in the group separation. The variables are sorted according to their VIP score, with a threshold of 1 for the variables to be considered statistically relevant. The metabolites that had increased levels in the endometriosis patients are indicated in black; those that had reduced levels are indicated in white. FFA, free fatty acid; GLN, glutamine; GLU, glutamic acid; HIS, histidine; ILE, isoleucine; LEU, leucine; THR, threonine; TYR, tyrosine; U1, unknown metabolite 1; VAL, valine. *Results from the univariate analysis using the Student’s t-test ($P < 0.1$); **results from the univariate analysis using the Student’s t-test ($P < 0.05$).
are glucogenic amino acids. Their reduced levels in serum samples of endometriosis patients may also reflect their increased use in gluconeogenesis, to meet the higher energy requirements of endometriotic cells, which have been shown to have a proliferative capacity similar to that of cancer cells (Wingfield et al., 1995). Indeed, enhanced gluconeogenesis has been observed in various types of cancers (Maignien et al., 2019), suggesting that the level of circulating glucose is not sufficient for optimal cell proliferation.

As endometriotic cells have features in common with cancer cells, such as their ability to proliferate, migrate and invade (Maignien et al., 2019), gluconeogenesis may also be increased in endometriosis, thereby accounting for the reduced levels of gluconeogenic amino acids. These results are consistent with the study of Jana et al. (2013), who also highlighted this metabolic alteration.

Second, we found higher ketone body concentrations in the serum of endometriosis patients. Ketone bodies are formed as a result of lipolysis through the action of lipases, when the circulating level of glucose is not sufficient, and they can be an alternative source of energy. The production pathways of ketone bodies could, therefore, be activated to meet the high energy requirements of highly proliferative endometriotic cells. In support of this hypothesis, the levels of glycerol and free fatty acids such as 2-octenoate, which are produced during the first steps of lipolysis, were also higher in the patient samples compared with the controls.
In addition to their role as energy-rich compounds, they can be involved in a range of functions, including signal transduction (Puchalska and Crawford, 2017). In particular, many studies have pointed out the pro-inflammatory role of ketone bodies and their link with increased cytokines levels (Kurepa et al., 2012; Shi et al., 2014; Chriett et al., 2019), as well as their role in promoting oxidative stress (Kanikarla-Marie and Jain, 2015; Shi et al., 2016; Cheng et al., 2019). Ketone body levels can hence be expected to increase in endometriosis, consistent with the inflammatory and oxidative character of this disease. To illustrate these hypotheses, an overview of the putative altered metabolic pathways in endometriosis patients is presented in Figure 7. In summary, these metabolic alterations associated with energy pathways are similar to those observed in prolonged fasting. This may be explained by an impaired glucose metabolism or reduced mitochondrial respiration (Atkins et al., 2019), leading to enhanced gluconeogenesis and ketone body formation to meet the high-energy requirements of endometriotic cells. Of note, enhanced lipolysis as well as the effects of ketone bodies on reduced perception of hunger and food intake (Paoli et al., 2015) could favour leanness in patients, which is consistent with the documented relationship between endometriotic patients and a low BMI (Pilet et al., 2012). Moreover, 3HB has recently been shown to influence pathways commonly believed to be part of the pathophysiology of migraine (Gross et al., 2019), which could account for the significant association between migraine and endometriosis (Maitrot-Mantelet et al., 2019). In the same way, 3HB has been linked to abdominal pain (van Rijt et al., 2020), and was found in increased levels in the serum of patients with coeliac disease, as in the serum of endometriosis patients. In a few publications, the gluten-free diet contributed to restore normal levels of 3HB and to improve the quality of life of patients with coeliac disease (Bertini et al., 2009; Colabrò et al., 2014). Our findings could, therefore, contribute to an explanation of the potential benefits of gluten-free diet in endometriosis patients (Marziali et al., 2012; Marziali and Capozzolo, 2015).

To the best of our knowledge we have shown, for the first time, that endometriosis phenotypes are characterized by specific metabolic profiles. Indeed, the OMA samples had significantly higher concentrations of two ketone bodies (acetone and 3HB), whereas the DIE patients had higher concentrations of lipids. One explanation could be that the oestradiol concentration seems to be higher in OMA compared with DIE (Huhtinen et al., 2012), which could increase lipase activity, as previously shown by Polin et al. (2003) and Cox-York et al. (2017). Lipolysis would, therefore, be enhanced in OMA compared with DIE, thereby resulting in higher ketone body levels in OMA samples, compared with higher lipid levels in DIE samples, as observed in our study. These observations of phenotypic differences between the two endometriosis phenotypes are in agreement with previous reports underlining different molecular features in OMA and DIE patients (Nisolle and Donnez, 1997; Sanchez et al., 2014; Tosti et al., 2015), and this calls for each subtype as being considered to be a separate disease entity.

Strengths and limitations
The main strengths of this study lie in the novelty of the subject and the methodological design: as far as we are aware, this is the first study to correlate the serum metabolic profiles with well-defined endometriosis phenotypes (OMA or DIE) based on...
stringent preoperative imaging criteria and surgical exploration; only patients who had undergone a thorough surgical evaluation of the peritoneal cavity and for whom histological proof confirmed the disease were included and, therefore, the endometriosis state was assessed with a high degree of precision according to a previously described classification (Chapron et al., 2006); all of the control patients also underwent a surgical examination, which definitively ruled out the presence of endometriosis; we used a statistical analysis using an untargeted approach, measuring and comparing many metabolite signals simultaneously, to identify those involved in the disease with no prior knowledge.

Our findings, however, may nonetheless be subject to several biases: the study included women who were undergoing hormonal treatments, or who were at different stages of the menstrual cycle. As the serum metabolic profile can be altered by these parameters (Wallace et al., 2010; Ruoppolo et al., 2014; Draper et al., 2018), our results could

FIGURE 7 The putative metabolic pathways altered in endometriosis. (Top) Oxidative stress resulting in amino acid oxidation, or the activation of the acute phase proteins synthesis as a response to inflammation, could result in enhanced amino acids catabolism; (bottom) Activation of lipases results in the β-oxidation of lipids, leading to pronounced release of glycerol and acetyl-CoA, which is then converted into ketone bodies. On the other hand, whereas ketogenic amino acids such as leucine could also enter the ketone body formation pathways, glucogenic amino acids could be recruited for the formation of the tricarboxylic acid cycle (TCA) cycle intermediates that are substrates of gluconeogenesis, thereby giving rise to glucose. All of these reactions take place primarily in the liver. OS, oxidative stress; TCA, tricarboxylic acid cycle. Large grey arrows indicate the variation in the metabolite concentrations in the endometriosis serum samples.
have been affected. Nevertheless, no significant difference was found in the percentage of patients under hormonal treatments or the distribution of the menstrual cycle phases between the two groups, which makes this scenario less likely. Moreover, no cluster according to the menstrual cycle phase or the presence of hormonal treatment was found in our analysis; the study was undertaken in a referral centre for the disease and, therefore, included patients with particularly severe forms of endometriosis, which may have exacerbated the differences in the serum metabolic profile between the endometriosis patients and the controls; our control group included patients operated for benign gynaecological conditions, e.g. tubal infertility, uterine fibroids or ovarian cysts, which could conceivably also result in altered metabolic patterns (Yang et al., 2017), and may therefore not be representative of the metabolic profile of disease-free patients; and we did not include patients with isolated superficial peritoneal endometriotic lesions. As it was a preliminary study, we chose to focus on the most severe endometriosis phenotypes (Chapron et al., 2019), to see if significant differences were found in the metabolic serum signatures. Further analysis, including superficial lesions, are currently being handled. Moreover, some of the endometriosis patients had both types of endometriotic lesions, i.e. DIE with associated OMA (n = 9 [19.6%]), which could have biased the evaluation of the metabolic profiles according to the two endometriosis phenotypes, i.e. OMA and DIE. We therefore conducted a supplemental multivariate analysis, comparing the three subgroups of endometriosis patients, i.e. OMA only, DIE only, and DIE with associated OMA, and failed to find any significant differences between the serum metabolic profiles, possibly owing to the small sample size of each subgroup (data not shown). Further analysis with a larger number of patients would be interesting to perform to establish whether metabolomic analysis could discriminate the three subgroups of phenotypes.

In conclusion, 1H-NMR-based metabolomics of serum samples, which is a rapid and non-invasive approach, was shown to have ample potential for identifying the characteristic metabolic changes associated with endometriosis and endometriosis phenotypes. This information may be useful for obtaining greater insight into the pathogenesis of endometriosis and it could help with non-invasive diagnosis of the disease. Although further research with larger cohorts of patients is needed to confirm these results, 1H-NMR metabolomics seems to be a promising tool for increasing what is known regarding this complex disorder.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2020.06.019.

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