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

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1 **TITLE:** ENDOMETRIOSIS PHENOTYPES ARE ASSOCIATED WITH SPECIFIC SERUM METABOLIC  
2 PROFILES DETERMINED BY PROTON-NUCLEAR MAGNETIC RESONANCE

3

4 **SHORT TITLE:** ENDOMETRIOSIS METABOLOMIC PROFILES

5

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37 **ABSTRACT**

38 *Research question:* To determine if there is a correlation between the serum metabolic  
39 profile and the endometriosis phenotype.

40 *Design:* This was a pilot study nestled in a prospective cohort study at a University hospital,  
41 including forty-six patients with painful endometriosis who had surgery, and 21 controls who  
42 did not have macroscopic endometriotic lesions. Endometriosis was strictly classified into 2  
43 groups of 23 patients each: endometrioma(OMA) and deep infiltrating endometriosis(DIE).  
44 Serum samples were collected before the surgery to perform a metabolomic profiling based  
45 on <sup>1</sup>H-NMR spectroscopy in combination with statistical approaches. Comparative  
46 identification of the metabolites in the serum from endometriosis patients and from controls  
47 was carried out, including an analysis according to the endometriosis phenotype.

48 *Results:* The serum metabolic profiles of the endometriosis patients revealed significantly  
49 lower concentrations of amino acids compared with the controls, while the concentrations  
50 of free fatty acids and ketone bodies were significantly higher. The OMA and the DIE  
51 phenotypes each exhibited a specific metabolic profile, with higher concentrations of two  
52 ketone bodies in the OMA group, and higher concentrations of free fatty acids and lipids in  
53 the DIE group.

54 *Conclusion:* <sup>1</sup>H-NMR-based metabolomics of serum samples was found to have ample  
55 potential for the identification of metabolic changes associated with endometriosis  
56 phenotypes. This information may be useful for obtaining a better understanding of the  
57 pathogenesis of endometriosis.

58

59 **KEYWORDS:**

60 Endometriosis; Metabolomics; <sup>1</sup>H-Nuclear Magnetic Resonance; Endometrioma; Deep  
61 infiltrating endometriosis

62

63 **KEY MESSAGE:**

64 <sup>1</sup>H-NMR-based metabolomics of serum samples has ample potential for the identification of  
65 metabolic changes associated with endometriosis phenotypes. This information may be useful  
66 for obtaining a better understanding of the pathogenesis of endometriosis.

67

## 68 INTRODUCTION

69 Endometriosis, a benign chronic gynecological disorder defined as the presence of  
70 endometrial tissue (e.g., glands and stroma) outside the uterine cavity (Sampson, 1927),  
71 affects 10-15% of reproductive-aged women, causing pain and/or infertility (Chapron et al.,  
72 2019). This disease is heterogeneous in nature, with three main types of lesions: (i) superficial  
73 peritoneal endometriosis (SUP), (ii) ovarian endometrioma (OMA), and (iii) deeply infiltrating  
74 endometriosis (DIE) (Nisolle and Donnez, 1997). Its precise pathophysiology remains to be  
75 fully elucidated, and numerous theories have been put forward including retrograde  
76 menstruation, coelomic metaplasia, stem cells and Müllerian remnants. Immunological,  
77 hormonal, genetic/epigenetic and environmental factors may also be involved (Chapron et al.,  
78 2019; Laganà et al., 2017).

79 A notable characteristic of the disease is a markedly long time to diagnosis and treatment  
80 (Chapron et al., 2011a), resulting in highly adverse consequences on the quality of life of  
81 women with the disease (Chapron et al., 2019). Indeed, the currently available blood tests and  
82 imaging techniques have moderate diagnostic values (Ahn et al., 2017), thus making the  
83 development of new non-invasive diagnostic methods a high-priority objective in the field of  
84 endometriosis research (Thubert et al., 2014).

85 Metabolomics consists of determination of the set of metabolites in biological samples  
86 under normal or pathophysiological conditions, such as diseases (Klassen et al., 2017; Pallet  
87 et al., 2014). This is, therefore, a promising approach to obtain greater insight into the nature  
88 of complex disorders by means of the identification of a specific metabolic signature, and this  
89 could be a way to gain a better understanding of endometriosis pathogenesis as well as new  
90 non-invasive diagnostic biomarkers of this disease. The most commonly used tools for

91 establishing a metabolic profile are mass spectrometry (MS) and nuclear magnetic resonance  
92 (NMR) spectroscopy. In particular, proton-nuclear magnetic resonance ( $^1\text{H-NMR}$ ) has the  
93 advantage of being able to provide a quantitative and non-destructive evaluation of a complex  
94 mixture of metabolites, with minimal handling (Duarte and Gil, 2012). A small number of  
95 studies have recently analyzed the metabolic profile of serum from endometriosis patients,  
96 and they have identified biomarkers involved in inflammation, oxidative stress, cell  
97 proliferation, and angiogenesis (Dutta et al., 2018, 2012; Jana et al., 2013; Vicente-Muñoz et  
98 al., 2016). However, these studies did not correlate the metabolic alterations with the  
99 endometriosis phenotypes.

100 In light of the of paucity of data in the literature in this regard, the aim of the present study  
101 was to use  $^1\text{H-NMR}$  to evaluate the serum metabolic profiles of endometriosis patients versus  
102 control patients, and to determine whether different endometriosis phenotypes have specific  
103 signatures.

104

## 105 **MATERIALS AND METHODS**

### 106 **Study Protocol and Patients**

107 This study was approved by the local institutional review board (approval number 05-2006  
108 provided by the 'Comité de Protection des Personnes et des Biens dans la Recherche  
109 Biomédicale' of Paris Cochin) and all of the participants provided written informed consent.

110 This was a pilot study nestled in a prospective cohort study which details were already  
111 published elsewhere (Chapron et al., 2011c). Forty-six patients with painful endometriosis  
112 who underwent surgery for complete removal of their endometriotic lesions between January  
113 2012 and November 2016 were included. Endometriosis was categorized into two groups

114 according to histological findings: OMA and DIE, as described previously (Chapron et al., 2011).  
115 As these two types of endometriotic lesions are frequently associated, endometriotic patients  
116 were classified in the category of the worst finding. By definition, endometriotic lesions  
117 ranked from least to worst were OMA and DIE (Chapron et al., 2011b). Endometriosis was  
118 scored according to the American Society for Reproductive Medicine (ASRM) (“Revised  
119 American Society for Reproductive Medicine classification of endometriosis,” 1997). Patients  
120 were considered as presenting endometriosis and included only when lesions were  
121 histologically proved.

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

134 The study analysis used a prospectively managed database. For each patient, their personal  
135 history data were obtained during face-to-face interviews, which were conducted by the  
136 surgeon in the month prior to the surgery. A highly structured previously published  
137 questionnaire was used for all of the patients (Chapron et al., 2010b, 2010a). The following



138 items were recorded: age, parity, gravidity, height, weight, body mass index (BMI), the  
139 existence and duration of infertility, lifestyle habits, the history of hormonal and/or surgical  
140 treatments for symptomatic endometriosis, and the existence of gynecological pain  
141 symptoms (dysmenorrhea, deep dyspareunia, non-cyclic chronic pelvic pain (NCCPP)), and  
142 gastrointestinal and lower urinary tract symptoms. A 10-cm visual analog scale was used  
143 (Huskisson, 1974) in order to evaluate the pain intensity preoperatively. The patients who had  
144 no hormonal treatment at the time of the surgery had a minimum wash-out period of three  
145 months. The indications of using hormonal treatments in both study groups were: invalidating  
146 chronic pelvic pain symptoms and/or a need for contraception. Different hormonal  
147 treatments could be used according to the efficacy, tolerability and cost, including combined  
148 oral contraceptives (COC), progestins (P) or gonadotropin-releasing hormone agonists  
149 (GnRHa).

#### 150 **Sample collection and NMR sample preparation**

151 For each subject, a serum sample was collected after overnight fasting and before the  
152 surgery. The blood samples were centrifuged at 800g for 12 min at 4°C, and the serum  
153 supernatants were collected. Aliquots of the supernatants were stored at -70°C until they  
154 were analyzed.

155 NMR samples were prepared by taking 300 µL from each serum sample supernatant after  
156 thawing on ice, followed by the addition of 300 µL of a solution containing a phosphate buffer  
157 with D<sub>2</sub>O to obtain a final buffer concentration of 38 mM with a pH value of 7.4, that contained  
158 2.3 mM NaN<sub>3</sub> as well as 2.3 mM sodium trimethylsilylpropionate salt (TSP) as the NMR  
159 chemical shift reference. D<sub>2</sub>O (12% (v/v)) was used for the field-frequency lock and shimming  
160 on the NMR spectrometer. A total of 600 µL was then transferred into 5-mm NMR tubes for

161 the metabolomic analysis.

## 162 **NMR spectroscopy and spectral processing**

163 The NMR data were recorded at 300 K on a 500 MHz Bruker Avance II spectrometer  
164 equipped with a 5-mm  $^{13}\text{C}/^1\text{H}$  cryoprobe, using a sampleXpress<sup>TM</sup> automation sample changer.  
165  $^1\text{H}$  spectra were obtained for each sample using a Carr-Purcell-Meiboom-Gill (CPMG) pulse  
166 sequence (Meiboom and Gill, 1958) to remove broad peaks originating from macromolecules.  
167 For each experiment, a total of 512 scans with 32768 points were recorded using a recycle  
168 delay of 2 s, and a chemical shift spectral window of 20.65 ppm, thereby resulting in a total  
169 experimental time of 33 min. Other parameters were as follow: a CPMG spin-echo train of 64  
170 ms with a spin-echo delay of 400  $\mu\text{s}$ , and an acquisition time of 1.6 s. Processing of the NMR  
171 spectra was carried out using NMRProcflow software (Jacob et al., 2017). The spectra were  
172 zero-filled to 65536 points and processed with an exponential apodization function using a  
173 line broadening of 0.3 Hz prior to Fourier transformation. They were then phased, baseline  
174 corrected and finally, referenced to the alanine methyl group signal at 1.47 ppm. Individual  
175 metabolites were assigned using the Chenomx NMR suite 7.1 software (Chenomx Inc.  
176 Edmonton, Canada), the human metabolome database (HMDB) (Wishart et al., 2007) as well  
177 as previously published data (Dutta et al., 2012; Vicente-Muñoz et al., 2016).

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

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

## 188 **Statistical Analysis**

189 The continuous data are presented as mean and standard deviations; the categorical data,  
190 as numbers and percentages. For comparison of the patients' general characteristics, we used  
191 a Pearson's  $X^2$  test or Fisher's exact probability test for the qualitative variables and the  
192 Student's *t*-test for the quantitative variables.

193 In the present study, an untargeted approach whereby no assumption was made about the  
194 metabolites or the metabolic pathways affected by the disease was used in order to obtain an  
195 overall profile for endometriosis patients. The calculation of a sample size did not appear to  
196 be necessary. Both univariate and multivariate statistical approaches were used. Univariate  
197 analysis was carried out using the Student's *t*-test and ANOVA as implemented in  
198 MetaboAnalyst (Chong et al., 2018; Xia et al., 2009). Multivariate analysis was performed using  
199 SIMCA software (Sartorius Stedim Biotech). A supervised orthogonal projections to latent  
200 structures discriminant analysis (OPLS-DA) (Trygg and Wold, 2002) was carried out, taking into  
201 account the class of the different samples to maximize class discrimination. The quality of the  
202 models thus obtained was assessed with the quality factors provided by the SIMCA software,  
203 *i.e.* the cumulative explained variance of the observed data  $X$ ,  $R^2X$ , the cumulative explained  
204 variance of assignment  $Y$ ,  $R^2Y$ , and the estimate of predictive ability,  $Q^2$ . The performance of  
205 the models was further tested using CV-ANOVA (Eriksson et al., 2008) with a *p*-value < 0.05  
206 being considered statistically significant. The main variables responsible for the class

207 discrimination, were identified with the Variable Importance on Projection (VIP) values of the  
208 OPLS-DA models. Variables were considered relevant only when their VIP score was greater  
209 than 1.

## 210 **RESULTS**

### 211 **Study Population**

212 Forty-six women with endometriosis and 21 disease-free women were included in the  
213 study. The patients' characteristics are presented in Table 1. No significant differences were  
214 found in terms of age, BMI, gravidity, parity, and infertility between the study and the control  
215 groups. Neither the percentage of patients undergoing hormonal treatment at the time of the  
216 surgery nor the menstrual cycle phases for the patients without hormonal treatment differed  
217 significantly between the two study groups. The distribution of the hormonal treatments in  
218 the study population was as follows: COC, 5 patients (23.8%), P, 12 patients (57.1%) and  
219 GnRHa, 4 patients (19.1%) in the endometriosis group, versus COC, 1 patient (12.5%), P, 5  
220 patients (62.5%) and GnRHa, 2 patients (25%) in the control group ( $p = 0.999$ ). In the  
221 endometriosis group, the phenotypes were distributed as follows: OMA, 23 patients (50%)  
222 and DIE, 23 patients (50%), including 9 patients (39.1%) with associated OMA. The mean ASRM  
223 stage was  $2.96 \pm 1.08$ .

### 224 **Serum metabolic profiles of the endometriosis patients compared with the controls**

225 Figure 1 depicts a representative serum  $^1\text{H-NMR}$  spectrum from an endometriosis patient.  
226 The assignment of the most significant metabolites is also indicated. Following univariate  
227 analysis, a total of 22 variables with a  $p$ -value  $< 0.1$  (of which 18 variables had  $p$ -values  $< 0.05$ )  
228 were identified as relevant regions in the discrimination. These were used to identify the  
229 metabolites that had altered levels in the endometriosis patients, as shown in Supplemental

230 Table 1 and Figure 2. The concentrations of several amino acids, such as glutamine (GLN),  
231 valine (VAL), threonine (THR), histidine (HIS), leucine (LEU), isoleucine (ILE), and glutamic acid  
232 (GLU) were significantly lower in the endometriosis patients compared with the control  
233 patients. Conversely, the concentrations of free fatty acids ( $\alpha$ -CH<sub>2</sub> FFA) such as 2-octanoate,  
234 and ketone bodies (KB) such as acetone (Ac) and 3-hydroxybutyrate (3HB), were significantly  
235 higher in the endometriosis group.

236 The supervised OPLS-DA score plot, presented in Figure 3 showed a clear separation between  
237 the endometriosis and the control samples, with relatively good model quality factors (R2X =  
238 0.13, R2Y = 0.71, and Q2 = 0.33). The corresponding CV-ANOVA analysis (Eriksson et al., 2008)  
239 led to a p-value of  $5.03 \times 10^{-5}$ , further confirming the good performance of the model. The  
240 main variables contributing to the differences between the two groups of samples were  
241 identified and plotted as a VIP score plot (Figure 4). Assignment of these variables revealed  
242 the same metabolites as those obtained with the univariate analysis, thereby underscoring  
243 their relevance to the pathology.

244 To be noted, no validated model was obtained according to the cycle phase or the type of  
245 hormonal treatment using a supervised method (p-value = 1, and Q2 values = - 0.18 and - 0.28,  
246 respectively, typical of unrepredictive models (full data not shown)).

#### 247 **Serum metabolic profiles according to the endometriosis phenotypes**

248 The results of the univariate analysis comparing the serum metabolic profiles of the control  
249 women versus the OMA patients and the DIE patients are displayed in Figure 5. In the OMA  
250 group, a significantly higher concentration of two KB (Ac, 3HB) was found compared with the  
251 control group and the DIE group (p < 0.05). In the DIE group, the concentrations of free fatty  
252 acids and lipids were significantly higher compared with the other two groups (p < 0.05). The

253 multivariate analysis comparing the three groups confirmed these results, with the OPLS-DA  
254 score plot underlining a significant discrimination between the three types of samples ( $p =$   
255  $3 \times 10^{-3}$ ) (Figure 6).

256

## 257 **DISCUSSION**

### 258 **Principal findings**

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

### 265 **Results**

266 The search for biomarkers involved in endometriosis is challenging, not only because  
267 it could become a non-invasive diagnostic tool, but also because it could help with obtaining  
268 a better understanding of the pathophysiological processes underlying the disease. A small  
269 number of untargeted NMR-based metabolomics studies have recently analyzed blood  
270 samples of endometriosis patients relative to controls (Dutta et al., 2018, 2012; Jana et al.,  
271 2013; Vicente-Muñoz et al., 2016), and they have highlighted the relevance of amino acids  
272 and metabolites involved in oxidative stress and glycolytic pathway.

### 273 **Clinical/research implications and Biological rational of the findings**

274 In the present study, several amino acids appeared to be statistically discriminant  
275 between the patient and the control samples, with lower levels observed in endometriosis

276 patients. It is well known that endometriosis is a chronic inflammatory and immune condition  
277 (Ahn et al., 2015; Coelho Riccio et al., 2018; Lousse et al., 2012), characterized by increased  
278 levels of various cytokines (Lambert et al., 2014; Luckow Invitti et al., 2018; Santulli et al.,  
279 2013, 2012). The production of a number of these cytokines by inflammatory cells has been  
280 shown to stimulate the acute-phase response (APR) in the liver, leading to the secretion of  
281 acute-phase proteins (APP) (Gabay and Kushner, 1999). The reduction in the amino acids  
282 levels could hence be due to their use in the synthesis of APP, as observed in other chronic  
283 inflammatory conditions such as chronic kidney disease (Suliman et al., 2005).

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

289 Among the affected amino acids, GLN, GLU, VAL, ILE, THR and HIS are glucogenic amino acids.  
290 Their reduced levels in serum samples of endometriosis patients may also reflect their  
291 increased use in gluconeogenesis, to meet the higher energy requirements of endometriotic  
292 cells, which have been shown to have a proliferative capacity similar to that of cancer cells  
293 (Wingfield et al., 1995). Indeed, enhanced gluconeogenesis has been observed in various  
294 types of cancers (Chen et al., 2015), suggesting that the level of circulating glucose is not  
295 sufficient for optimal cell proliferation. As endometriotic cells have features in common with  
296 cancer cells, such as their ability to proliferate, migrate, and invade (Maignien et al., 2019),  
297 gluconeogenesis may also be increased in endometriosis, thereby accounting for the reduced  
298 levels of gluconeogenic amino acids. These results are consistent with the study of Jana and  
299 coworkers, who also highlighted this metabolic alteration (Jana et al., 2013).

300 Secondly, we found higher KB concentrations in the serum of endometriosis patients. KB are  
301 formed as a result of lipolysis through the action of lipases, when the circulating level of  
302 glucose is not sufficient, and they can be an alternative source of energy. The production  
303 pathways of KB could, therefore, be activated to meet the high energy requirements of highly  
304 proliferative endometriotic cells. In support of this hypothesis, the levels of glycerol and free  
305 fatty acids such as 2-octenoate, which are produced during the first steps of lipolysis, were  
306 also higher in the patient samples compared to the controls. In addition to their role as energy-  
307 rich compounds, they can be involved in a range of functions including signal transduction  
308 (Puchalska and Crawford, 2017). In particular, many studies have pointed out the pro-  
309 inflammatory role of KB and their link with increased cytokines levels (Chriett et al., 2019;  
310 Kurepa et al., 2012; Shi et al., 2014), as well as their role in promoting oxidative stress (Cheng  
311 et al., 2019; Kanikarla-Marie and Jain, 2015; Shi et al., 2016). KB levels can hence be expected  
312 to increase in endometriosis, consistent with the inflammatory and oxidative character of this  
313 disease. To illustrate these hypotheses, an overview of the putative altered metabolic  
314 pathways in endometriosis patients is presented in Figure 7. In summary, these metabolic  
315 alterations associated with energy pathways are very similar to those observed in prolonged  
316 fasting. This may be explained by an impaired glucose metabolism or reduced mitochondrial  
317 respiration (Atkins et al., 2019), leading to enhanced gluconeogenesis and KB formation to  
318 meet the high energy requirements of endometriotic cells. Of note, enhanced lipolysis as well  
319 as the effects of KB on reduced perception of hunger and food intake (Paoli et al., 2015) could  
320 favor leanness in patients, which is consistent with the documented relationship between  
321 endometriotic patients and a low BMI (Pillet et al., 2012). Moreover, 3HB has recently been  
322 shown to influence pathways commonly believed to be part of the pathophysiology of  
323 migraine (Gross et al., 2019), which could account for the significant association between



324 migraine and endometriosis (Maitrot-Mantelet et al., 2019). In the same way, 3HB has been  
325 linked to abdominal pain (van Rijt et al., 2020), and was found in increased levels in the serum  
326 of celiac disease (CD) patients, as in the serum of endometriosis patients. In a few publications,  
327 the gluten-free diet contributed to restore normal levels of 3HB and to improve CD patients'  
328 quality of life (Bertini et al., 2009; Calabrò et al., 2014). Our findings could therefore contribute  
329 to explain the potential benefits of gluten-free diet in endometriosis patients (Marziali et al.,  
330 2012; Marziali and Capozzolo, 2015).

331 In finishing, for the first time in the literature we have shown that endometriosis phenotypes  
332 are characterized by specific metabolic profiles. Indeed, the OMA samples exhibited  
333 significantly higher concentrations of two KB (Ac and 3HB), while the DIE patients had higher  
334 concentrations of lipids. One explanation could be that the estradiol concentration appears to  
335 be higher in OMA compared with DIE (Huhtinen et al., 2012), which could increase lipases'  
336 activity, as previously shown by Cox-York et al. and Palin et al. (Cox-York et al., 2017; Palin et  
337 al., 2003). Lipolysis would, therefore, be enhanced in OMA compared to DIE, thereby resulting  
338 in higher KB levels in OMA samples, as opposed to higher lipid levels in DIE samples, as  
339 observed in our study. These observations of phenotypic differences between the two  
340 endometriosis phenotypes are in agreement with previous reports underlining different  
341 molecular features in OMA and DIE patients (Nisolle and Donnez, 1997; Sanchez et al., 2014;  
342 Tosti et al., 2015), and this calls for each subtype as being considered to be a separate disease  
343 entity.

#### 344 **Strengths and limitations**

345 The main strengths of this study lie in the novelty of the subject and the  
346 methodological design: (i) as far as we are aware, this is the first study to correlate the serum

347 metabolic profiles with well-defined endometriosis phenotypes (OMA or DIE) based on  
348 stringent pre-operative imaging criteria and surgical exploration; (ii) only patients who had  
349 undergone a thorough surgical evaluation of the peritoneal cavity and for whom histological  
350 proof confirmed the disease were included, and thus the endometriosis state was assessed  
351 with a high degree of precision according to a previously described classification (Chapron et  
352 al., 2006); (iii) all of the control patients also underwent a surgical examination, which  
353 definitively ruled out the presence of endometriosis; (iv) we employed a statistical analysis  
354 using an untargeted approach, measuring and comparing many metabolite signals  
355 simultaneously, to identify those involved in the disease with no prior knowledge.

356 However, our findings may nonetheless be subject to several biases: (i) the study included  
357 women who were undergoing hormonal treatments, or who were at different stages of the  
358 menstrual cycle. As the serum metabolic profile can be altered by these parameters (Draper  
359 et al., 2018; Ruoppolo et al., 2014; Wallace et al., 2010), our results could have been affected.  
360 Nevertheless, no significant difference was found regarding the percentage of patients under  
361 hormonal treatments or the distribution of the menstrual cycle phases between the two  
362 groups, which makes this scenario less likely. Moreover, no cluster according to the menstrual  
363 cycle phase or the presence of hormonal treatment was found in our analysis; (ii) the study  
364 was undertaken in a referral center for the disease and, therefore, included patients with  
365 particularly severe forms of endometriosis, which may have exacerbated the differences in  
366 the serum metabolic profile between the endometriosis patients and the controls; (iii) our  
367 control group included patients operated for benign gynecological conditions (e.g., tubal  
368 infertility, uterine fibroids, or ovarian cysts) which could conceivably also result in altered  
369 metabolic patterns (Yang et al., 2017), and may therefore not be representative of the  
370 metabolic profile of disease-free patients; (iv) we did not include patients with isolated

371 superficial peritoneal endometriotic lesions. As it was a preliminary study, we chose to focus  
372 on the most severe endometriosis phenotypes (Chapron et al., 2019), to see if significant  
373 differences were found regarding the metabolic serum signatures. Further analysis, including  
374 superficial lesions, are currently being handled. Moreover, some of the endometriosis patients  
375 had both types of endometriotic lesions (i.e. DIE with associated OMA: 9 patients, 19.6%),  
376 which could have biased the evaluation of the metabolic profiles according to the two  
377 endometriosis phenotypes (i.e. OMA and DIE). We thus performed a supplemental  
378 multivariate analysis, comparing the three subgroups of endometriosis patients (i.e. OMA  
379 only, DIE only, and DIE with associated OMA) and failed to find any significant differences  
380 between the serum metabolic profiles, possibly due to the small sample size of each subgroup  
381 (data not shown). Further analysis with a larger number of patients would be interesting to  
382 perform to find out if the metabolomic analysis could discriminate the three subgroups of  
383 phenotypes.

#### 384 **Conclusion**

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

392

393 **AUTHORS' ROLES**

394 PS, CC and GB conceived of and designed the study. CM, CC, FK, LM, MB, KP and PS contributed  
395 to the data collection. All of the authors analyzed and interpreted the data. FK, GB and PS  
396 supervised and reviewed the statistical analysis. CM, FK, PS, GB, and CC authored the  
397 manuscript. All of the authors read and approved the final version of the manuscript.

398

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404

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672

## 673 TABLES

674 TABLE 1. Baseline characteristics of the patients.

Patient characteristics	Endometriosis (n = 46)	Controls (n = 21)	P-value <sup>d</sup>
Age (years) <sup>a</sup>	33.9 ± 5.8	33.1 ± 5.8	0.515
BMI (kg/m <sup>2</sup> ) <sup>a</sup>	22.1 ± 3.8	23.3 ± 3.8	0.165
Parity <sup>a</sup>	0.3 ± 0.7	0.5 ± 0.7	0.207
Gravidity <sup>a</sup>	0.5 ± 1	0.9 ± 1	0.130
Infertility			0.386
No	32 (70%)	13 (60%)	
Yes	14 (30%)	8 (40%)	
Length of infertility (months)	34.4 ± 26.7	36.6 ± 24.6	0.844
Painful symptoms (VAS score)			
Dysmenorrhea	7.7 ± 2.6	6.1 ± 2.5	0.028
Deep dyspareunia	4.2 ± 3.5	3.3 ± 3.5	0.235
Non-cyclic chronic pelvic pain	3.9 ± 2.9	1.6 ± 3.2	0.004
Gastrointestinal symptoms	4.7 ± 3.4	1.8 ± 3.4	< 0.001
Lower urinary tract symptoms	1.6 ± 2.7	0.2 ± 2.7	0.007
Hormonal treatment before surgery (n, %)			0.071
Yes	21 (45.7%)	8 (38%)	
Cycle phase if no hormonal treatment			
Proliferative phase	8 (32%)	9 (69.2%)	
Secretory phase	14 (56%)	2 (15.4%)	



Missing data	3 (12%)	2 (15.4%)
Surgical classification of endometriosis (n, %) <sup>b</sup>		
OMA	23 (50%)	NA
DIE	23 (50%)	NA
Intestinal DIE	9 (39.1%)	
Associated OMA	9 (39.1%)	
ASRM total score <sup>a, c</sup>	31.2 ± 34.1	NA
ASRM implants score <sup>a, c</sup>	18.4 ± 15	NA
ASRM adhesions score <sup>a, c</sup>	16.7 ± 19.6	NA

675

676 *Note: BMI: body mass index; VAS: visual analogue scale; OMA; ovarian endometrioma; DIE:*  
677 *deeply infiltrating endometriosis; NA: not applicable; ASRM: American Society for Reproductive*  
678 *Medicine*

679 <sup>a</sup> *Data are presented as mean ± standard deviation*

680 <sup>b</sup> *According to a previously published surgical classification for deeply infiltrating endometriosis*  
681 *by Chapron et al. (Chapron, Chopin et al. 2006)*

682 <sup>c</sup> *Score according to the American Society for Reproductive Medicine classification (“Revised*  
683 *American Society for Reproductive Medicine classification of endometriosis,” 1997)*

684 <sup>d</sup> *Statistical analysis was performed using Fisher’s exact test or Pearson’s Chi-square for*  
685 *qualitative variables and Student’s t test for quantitative variables*

686 *Statistical significance: p < 0.05*

687



689 **FIGURE LEGENDS**

690 **FIGURE 1.** Representative 500 MHz  $^1\text{H}$ -Nuclear Magnetic Resonance spectrum of a serum  
691 sample from one of the endometriosis patients.

692  *$^1\text{H}$ -NMR spectrum of an endometriosis patient serum sample obtained at 300 K on a  
693 spectrometer operating at a proton frequency of 500 MHz.*

694 *Assignment of statistically relevant metabolites is indicated above their corresponding NMR  
695 signal. (1a) Full spectrum; (1b) Enlargement of the left part of the spectrum, corresponding  
696 mainly to the aromatic signals; (1c) Enlargement of the right part of the spectrum  
697 corresponding mainly to the aliphatic signals.*

698

699 **FIGURE 2.** Boxplots showing the significant differences in the concentrations of metabolites  
700 between the control samples (n=21) and the endometriosis samples (n=46).

701 *The results are presented as boxplots showing the difference in the concentrations of  
702 metabolites using the Student's t-test, with statistically significant differences between the  
703 two groups indicated by an asterisk (\* p-value < 0.1 and \*\* p-value < 0.05).*

704 *The data are presented as medians, interquartile ranges, and extremes.*

705 **FIGURE 3.** Score plot of the supervised multivariate analysis obtained using orthogonal partial  
706 least square discriminant analysis (OPLS-DA) scores for multivariate analysis of serum samples  
707 collected from the control patients (n=21) and the endometriosis patients (n=46).

708 *The corresponding quality factors were as follows:  $R^2X = 0.13$ ,  $R^2Y = 0.71$ , and  $Q^2 = 0.33$ .*

709 *The OPLS-DA model was validated with its corresponding p-value of  $5.03 \times 10^{-5}$  obtained using*  
710 *CV-ANOVA*

711

712 **FIGURE 4.** Variable importance in projection (VIP) analysis for the multivariate analysis of  
713 serum samples collected from the control patients (n=21) and the endometriosis patients  
714 (n=46).

715 *VIP showing the variables contributing the most to the group discrimination in the OPLS-DA*  
716 *model. The variables are indicated on the X-axis as the center of the chemical shift of the*  
717 *corresponding bucket, whereas the Y-axis corresponds to the VIP score, i.e., the weight of each*  
718 *variable in the group separation. The variables are sorted according to their VIP score, with a*  
719 *threshold of 1 in order for the variables to be considered statistically relevant. The metabolites*  
720 *that had increased levels in the endometriosis patients are indicated in black; those that had*  
721 *reduced levels are indicated in white.*

722

723 **FIGURE 5.** Boxplots showing the significant differences in the concentrations of metabolites  
724 between the control samples (n=21) and the endometriosis samples according to the  
725 phenotype (OMA, n=23; DIE, n=23).

726 *The results are presented as boxplots showing the difference in the concentrations of*  
727 *metabolites using one-way ANOVA. Statistically significant differences between pairs of*  
728 *groups are indicated by an asterisk (\* p-value < 0.1, and \*\* p-value < 0.05).*

729 *The data are presented as medians, interquartile ranges, and extremes.*

730

731 **FIGURE 6.** Score plot of the supervised multivariate analysis obtained using orthogonal partial  
732 least square discriminant analysis (OPLS-DA) scores for multivariate analysis of serum samples  
733 collected from the control patients (n=21), the OMA patients (n=23) and the DIE patients  
734 (n=23).

735 *The corresponding quality factors were as follows:  $R^2X = 0.15$ ,  $R^2Y = 0.42$ , and  $Q^2 = 0.10$ .*

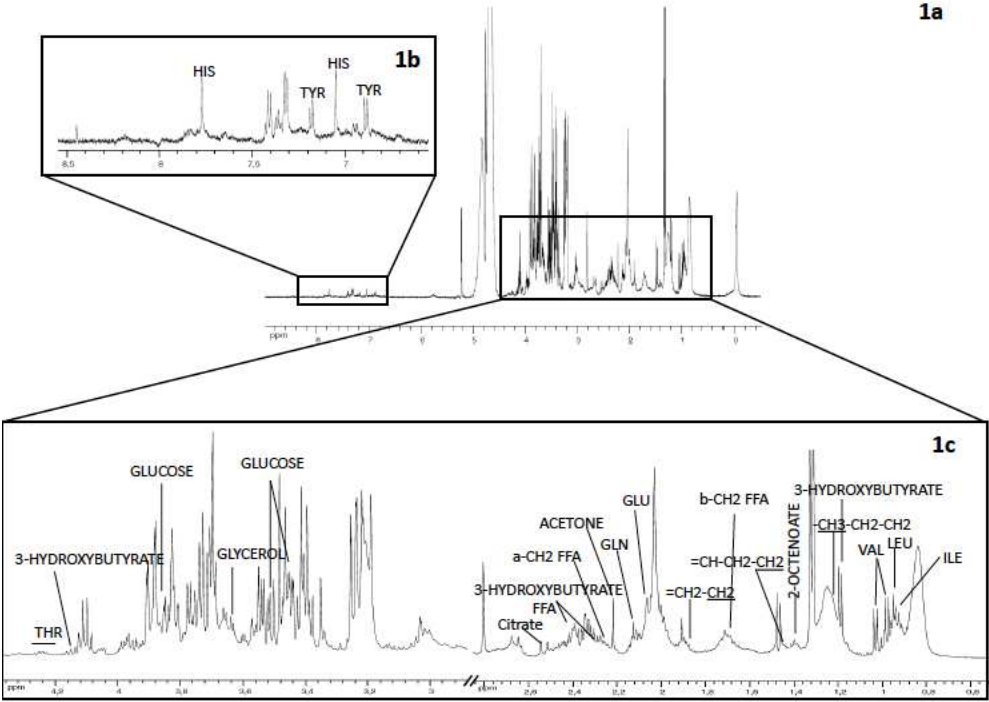
736 *The OPLS-DA model was validated with its corresponding p-value of  $3 \times 10^{-3}$  obtained using CV-*  
737 *ANOVA.*

738 **FIGURE 7.** Schematic representation of the putative metabolic pathways altered in  
739 endometriosis.

740 *(Top) Oxidative stress resulting in amino acid oxidation, or the activation of the acute phase*  
741 *proteins synthesis as a response to inflammation, could result in enhanced amino acids*  
742 *catabolism.*

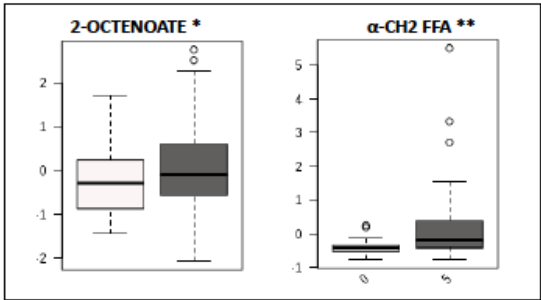
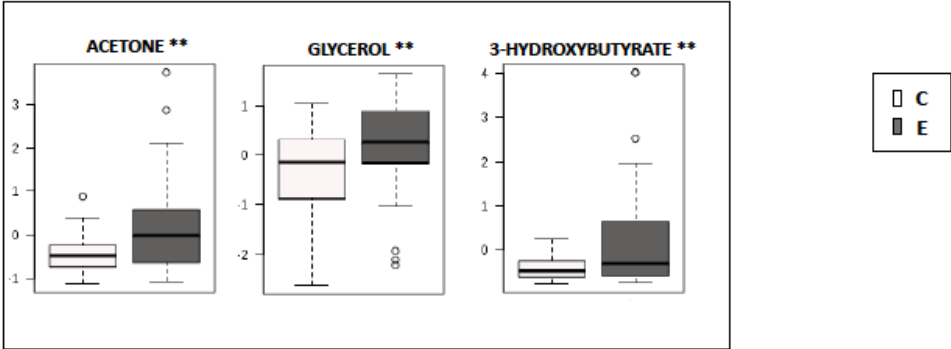
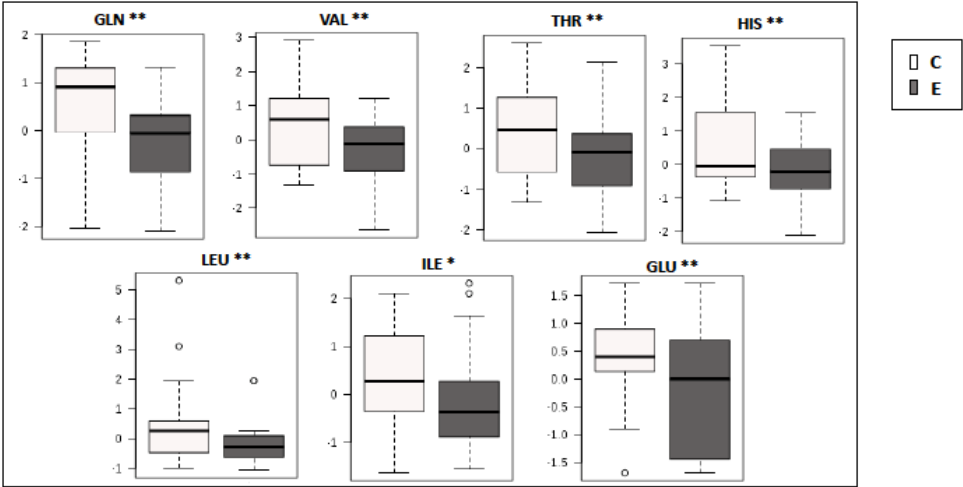
743 *(Bottom) Activation of lipases results in the  $\beta$ -oxidation of lipids, leading to pronounced release*  
744 *of glycerol and acetyl-CoA, which is then converted into ketone bodies. On the other hand,*  
745 *whereas ketogenic amino acids such as leucine could also enter the ketone body formation*  
746 *pathways, glucogenic amino acids could be recruited for the formation of the TCA cycle*  
747 *intermediates that are substrates of gluconeogenesis, thereby giving rise to glucose. All of*  
748 *these reactions take place primarily in the liver.*

**FIGURE 1.**



Note: HIS: histidine; TYR: tyrosine; GLN: glutamine; VAL: valine; THR: threonine; LEU: leucine; ILE: isoleucine; GLU: glutamic acid; TYR: tyrosine; FFA: free fatty acid

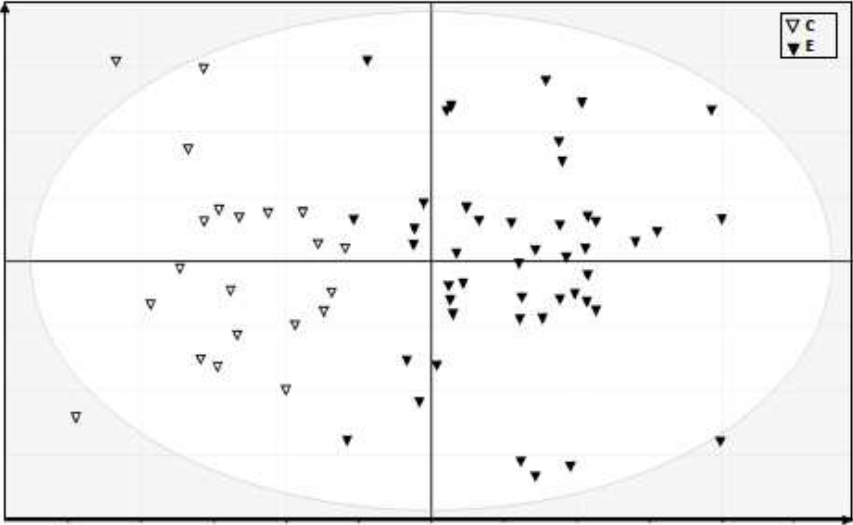
**FIGURE 2.**



*Note: C: control samples; E: endometriosis samples; GLN: glutamine; VAL: valine; THR: threonine; HIS: histidine; LEU: leucine; ILE: isoleucine; GLU: glutamic acid ; FFA : free fatty acid*



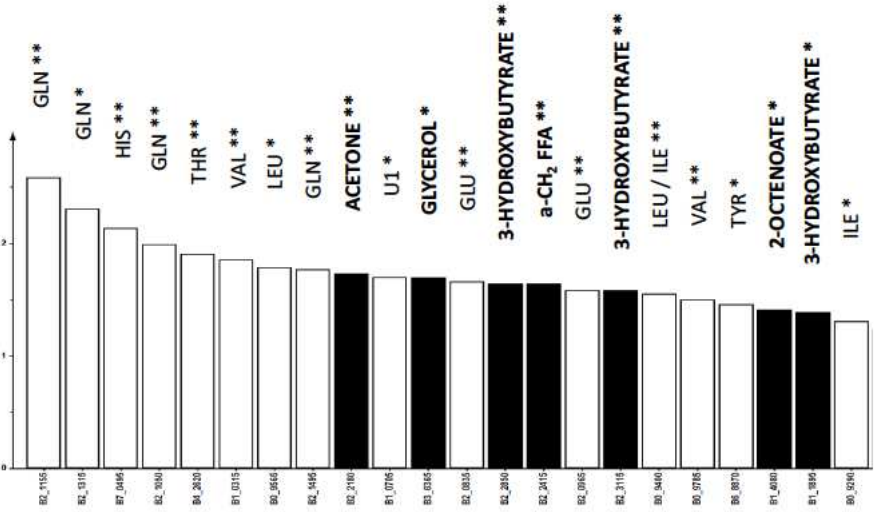
FIGURE 3.



$R^2X(\text{cum}) = 0.13$ ,  $R^2Y(\text{cum}) = 0.71$ ,  $Q2 = 0.33$ ,  $p\text{-value} = 5.03 \cdot 10^{-3}$

Note: C: control samples; E: endometriosis samples

**FIGURE 4.**



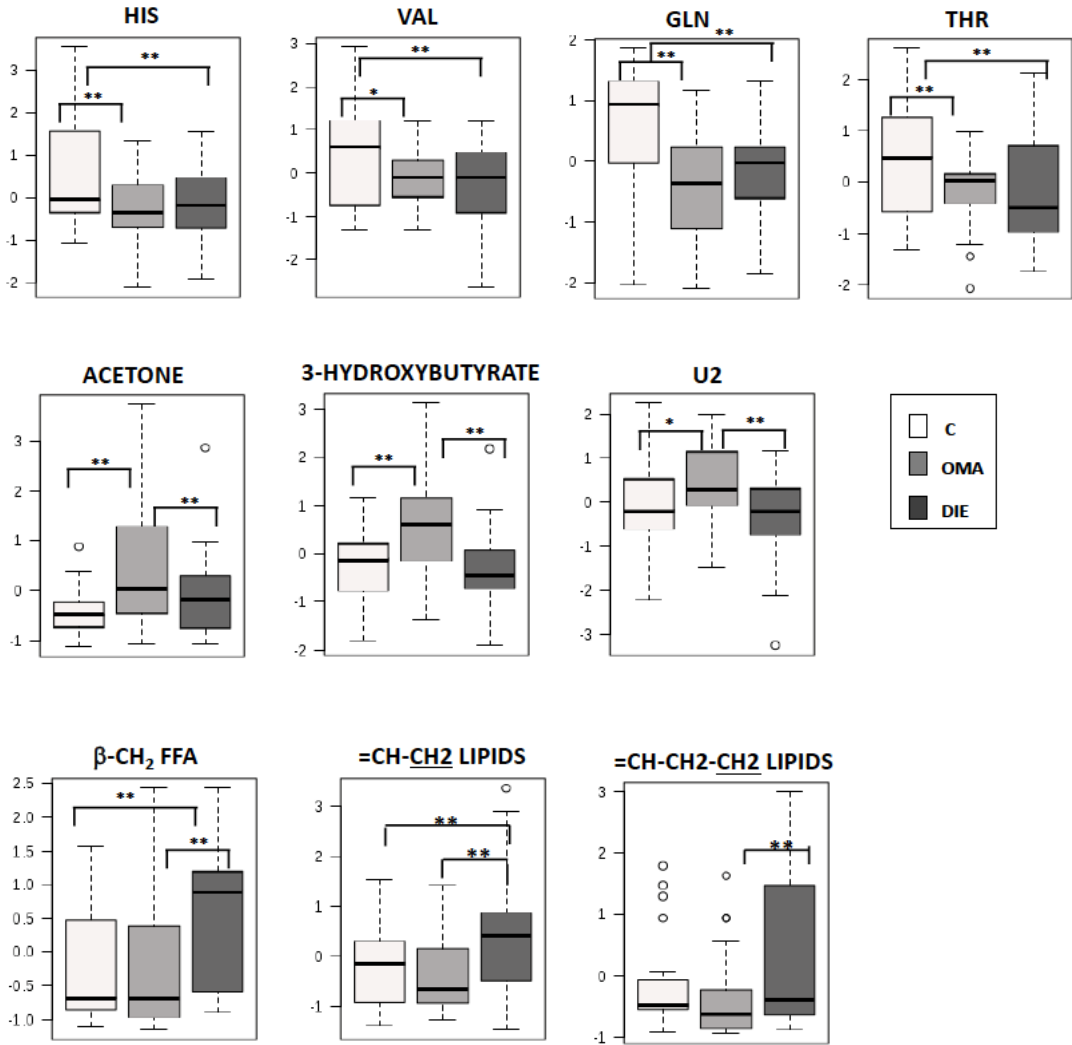
Note: GLN: glutamine; VAL: valine; THR: threonine; HIS: histidine; LEU: leucine; ILE: isoleucine;

GLU: glutamic acid; FFA: free fatty acid; TYR: tyrosine; U1: unknown metabolite 1.

\* Results from the univariate analysis using the Student’s t-test (p-value < 0.1)

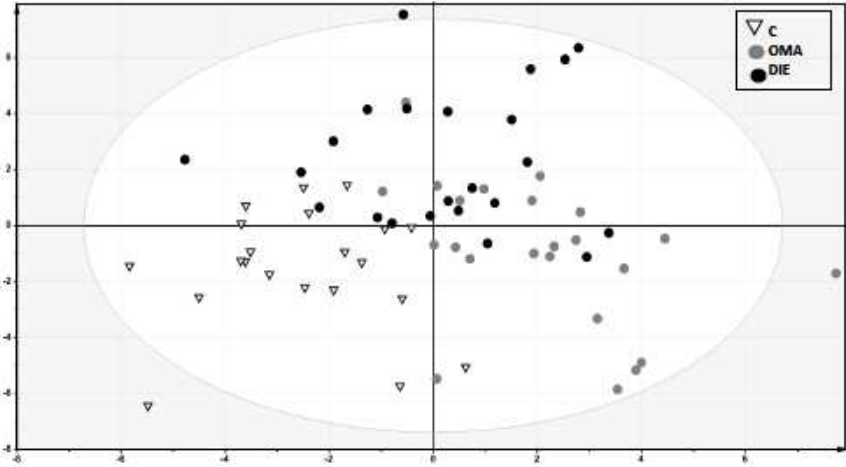
\*\* Results from the univariate analysis using the Student’s t-test (p-value < 0.05)

**FIGURE 5.**



Note: C: control samples; OMA: endometrioma samples; DIE: deep infiltrating endometriosis samples; HIS: histidine; VAL: valine; GLN: glutamine; THR: threonine; U2: unknown metabolite 2; FFA: free fatty acid

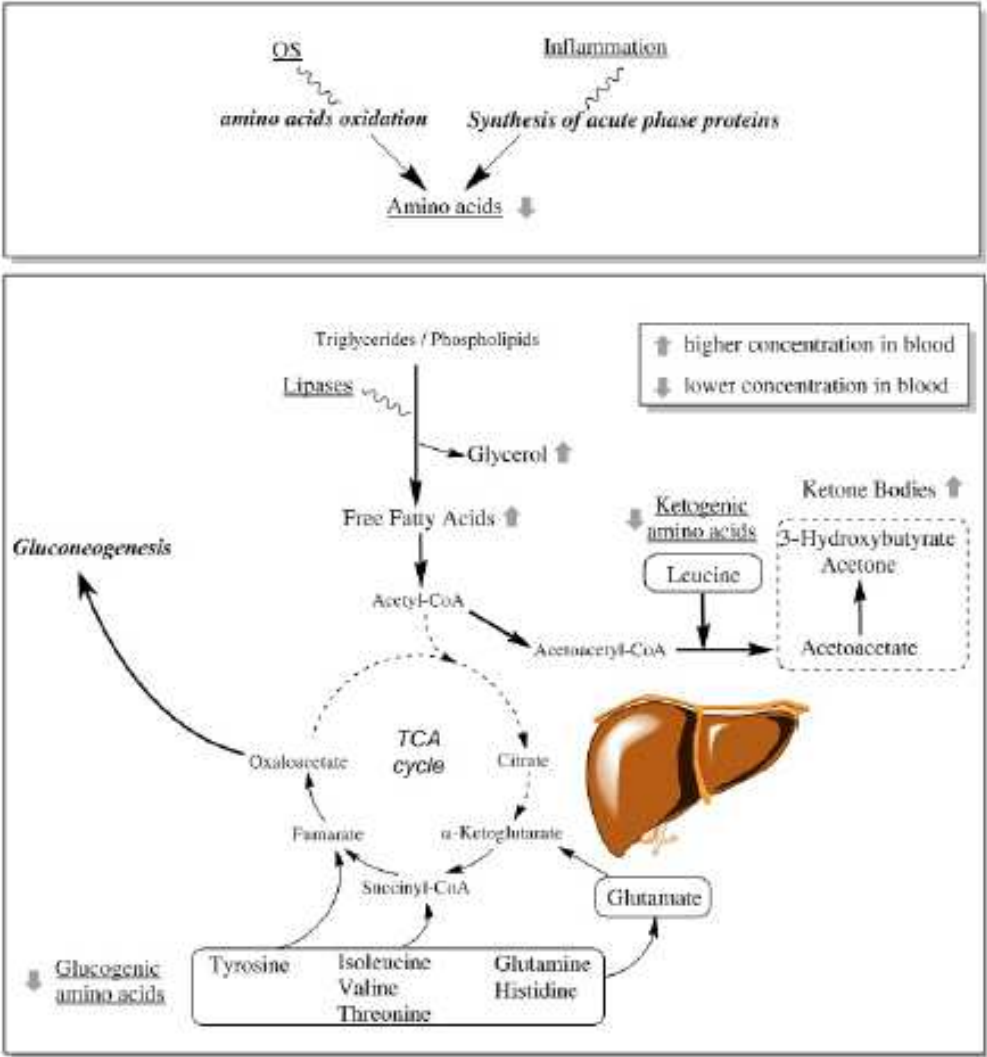
FIGURE 6.



$R^2X(\text{cum}) = 0.15$ ,  $R^2Y(\text{cum}) = 0.42$ ,  $Q^2 = 0.1$  and  $p\text{-value} = 3.10^{-3}$

*Note: C: control samples; OMA: endometrioma samples; DIE: deep infiltrating endometriosis samples*

**FIGURE 7.**



*Note: OS: oxidative stress; TCA: tricarboxylic acid cycle*

*Large grey arrows indicate the variation in the metabolite concentrations in the endometriosis serum samples.*