Accepted Manuscript

Title: Direct Mass Spectrometry Differentiation of Ectopic and Eutopic Endometrium in Patients with Endometriosis

Author: Leila Adamyan, Natalia Starodubtseva, Anna Borisova, Assia Stepanian, Vitaliy Chagovets, Dinara Salimova, Zhihao Wang, Alexey Kononikhin, Igor Popov, Anna Bugrova, Konstantin Chingin, Andrey Kozachenko, Huanwen Chen, Vladimir Frankevich

PII: S1553-4650(17)31120-2
DOI: http://dx.doi.org/doi: 10.1016/j.jmig.2017.08.658
Reference: JMIG 3266

To appear in: *The Journal of Minimally Invasive Gynecology*

Received date: 17-2-2017
Revised date: 23-8-2017
Accepted date: 28-8-2017

Please cite this article as: Leila Adamyan, Natalia Starodubtseva, Anna Borisova, Assia Stepanian, Vitaliy Chagovets, Dinara Salimova, Zhihao Wang, Alexey Kononikhin, Igor Popov, Anna Bugrova, Konstantin Chingin, Andrey Kozachenko, Huanwen Chen, Vladimir Frankevich, Direct Mass Spectrometry Differentiation of Ectopic and Eutopic Endometrium in Patients with Endometriosis, *The Journal of Minimally Invasive Gynecology* (2017), http://dx.doi.org/doi: 10.1016/j.jmig.2017.08.658.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

1 **Direct Mass Spectrometry Differentiation of Ectopic and Eutopic Endometrium in Patients**
2 **with Endometriosis**

3 Leila Adamyan, MD[1]; Natalia Starodubtseva, PhD[1, 2]; Anna Borisova, MD[1]; *Assia Stepanian,
4 MD[3]; Vitaliy Chagovets, PhD[1]; Dinara Salimova[1]; Zhihao Wang[4]; Alexey Kononikhin, PhD[1, 2];
5 Igor Popov, PhD[1, 2]; Anna Bugrova, PhD[1]; Konstantin Chingin, PhD[4]; Andrey Kozachenko,
6 MD[1]; Huanwen Chen, PhD[4]; Vladimir Frankevich, PhD[1].

7 [1]*Research Center for Obstetrics, Gynecology, Moscow, Russia*

8 [2]*Moscow Institute of Physics and Technology, Moscow, Russia*

9 [3]*Academia of Women's Health and Endoscopic Surgery, Atlanta, USA*

10 [4]*Jiangxi Key Laboratory for Mass Spectrometry and Instrumentation, East China University of*
11 *Technology, Nanchang, China*

12 *Corresponding author: Assia Stepanian, MD, FACOG, Academia of Women's Health &
13 Endoscopic Surgery, 755 Mount Vernon HWY NE, Unit 240, Atlanta, GA 30328, United States;
14 Tel: (404) 549-3224; Fax: (404) 459-0995; Email: astep@migsurgery.com

15

16 **Author Contributions:**

17 Leila Adamyan, MD	Dr. Adamyan has nothing to disclose.
18 Natalia Starodubtseva, PhD	Dr. Starodubtseva has nothing to disclose.
19 Anna Borisova, MD	Dr. Borisov has nothing to disclose.
20 Assia Stepanian, MD	Dr. Stepanian has nothing to disclose.
21 Vitaliy Chagovets, PhD	Dr. Chagovets has nothing to disclose.
22 Dinara Salimova, Medical Student	Dr. Salimova has nothing to disclose.
23 Zhihao Wang, Medical Student	Dr. Wang has nothing to disclose.

Accepted Manuscript

ACCEPTED MANUSCRIPT

24 Alexey Kononikhin, PhD Dr. Kononikhin has nothing to disclose.

25 Igor Popov, PhD Dr. Popov has nothing to disclose.

26 Anna Bugrova, PhD Dr. Bugrova has nothing to disclose.

27 Konstantin Chingin, PhD Dr. Chingin has nothing to disclose.

28 Andrey Kozachenko, MD Dr. Kozachenko has nothing to disclose.

29 Huanwen Chen, PhD Dr. Chen has nothing to disclose.

30 Vladimir Frankevich, PhD Dr. Frankevich has nothing to disclose.

31

32 Précis

33 Direct mass spectrometry identified 15 lipids with potential usefulness as biomarkers for real-

34 time endometriotic tissue determination and differentiation during surgical treatment of

35 endometriosis.

36

Accepted Manuscript

ACCEPTED MANUSCRIPT

37 **Abstract**

38 **Study objective:** To introduce a method for rapid assessment of endometriotic tissues using
39 direct mass spectrometry (MS)-based lipidomics.

40 **Design:** Prospective observational cohort study.

41 **Design classification:** II 2.

42 **Setting:** Department of Operative Gynecology of the Research Centre for Obstetrics,
43 Gynecology and Perinatology.

44 **Patients:** Fifty patients with ovarian cysts and peritoneal endometriosis who underwent
45 laparoscopic surgery between 2014 and 2016.

46 **Intervention:** Differences in mass spectrometric profiles of ectopic endometrium
47 (endometriosis) and eutopic endometrium were analyzed for each patient in combination with
48 morpho-histological evaluation. The lipidomic approach was applied using a direct high-
49 resolution mass spectrometry method.

50 **Measurements and Main Results:** Out of 148 metabolites, 15 showed significant differences
51 between endometriotic tissue and the comparison healthy endometrium of the same patient,
52 considered as a control in this study. Main lipids prevalent in endometriotic tissues were:
53 phosphoethanolamine (PE O-20:0), sphingomyelin (SM 34:1), diglycerides (DG 44:9),
54 phosphatidylcholines (PC 32:1, PC O-36:3, PC 38:7, PC 38:6, PC 40:8, PC 40:7, PC 40:6, PC
55 40:9, PC O-42:1), and triglycerides (TG 41:2, TG 49:4, TG 52:3). Using PLS-DA models, MS
56 demonstrated that the lipidomic profile of endometriotic tissue (peritoneal endometriosis and
57 ovarian endometrioma) was clearly separated from eutopic endometrium, indicating tissue-type
58 differentiation.

59 **Conclusion:** Our results suggest that direct mass spectrometry may play an important role for
60 endometriotic tissue identification. Such approach has potential utility for real-time tissue

Accepted Manuscript

ACCEPTED MANUSCRIPT

61 determination and differentiation during surgical treatment. Lipids of three important classes,
62 sphingolipids, phospholipids, and the fatty acids, di- and tri-glycerides, were identified.
63 Validation is required to determine whether these lipids can be used to discriminate between
64 patients with endometriosis and those with other gynecological diseases.

65 **Keywords:** endometriosis, spectrometry, eutopic endometrium, endometrioma

66 **1. Introduction**

67 Endometriosis is a benign gynecological disorder characterized by the presence of
68 endometrial tissue outside the uterine cavity [1]. It is one of the most common diseases in
69 gynecology, affecting 10-15% of women of reproductive age [2]. Endometriosis, with its
70 features of chronic inflammation, is associated with dysmenorrhea in up to 40–60% of women
71 and infertility in approximately 20–30% [3]. The typical location of endometriosis is in the
72 pelvis. However, endometriosis can be found in extra-pelvic or ectopic sites, including the
73 gastrointestinal tract, anterior abdominal wall, surgical scars, diaphragm, lungs, urinary tract, and
74 the musculoskeletal and nervous systems [4].

75 The diagnosis of endometriosis relies heavily upon direct visualization of suspected
76 lesions during an invasive surgery coupled with histologic confirmation [5]. While the
77 pathogenesis of endometriosis is still unknown, there is on average a 7-11 year delay in
78 diagnosis following the onset of symptoms. Surgical removal of endometriotic foci remains the
79 main method of treatment. Important issues continue to be quality control of the removal of the
80 endometriotic foci as well as prediction and prevention of disease relapse (percentage of
81 recurrence of external genital endometriosis varies greatly in different studies, from 6 to 67%)
82 [6].

83 There are more than 100 proposed markers of endometriosis; however, none of which
84 have demonstrated sufficient diagnostic predictive value [5]. According to the consensus of the
85 World Endometriosis Society [6], the development of a reliable non-invasive test, such as the

Accepted Manuscript

ACCEPTED MANUSCRIPT

86 discovery of a sufficiently sensitive and specific biomarker, is a top research priority. Until now,
87 the search for markers of endometriosis has been mainly limited to targeted compounds, e.g.,
88 metabolites of arachidonic acid and steroids [5, 6]. However, the non-targeted screening of
89 endometriosis has not been reported. One area of particular importance is the role of lipids
90 inasmuch as differences in tissue lipids may be the key to understanding the process that occurs
91 during the invasion and infiltrative growth of endometrial tissue in the ectopic sites.

92 Mass spectrometry (MS) is one of the most widely used and reliable techniques for the
93 analysis of biological samples. MS tests have been introduced in clinical practice for the
94 diagnosis and prognosis of chronic kidney disease (CE/MS) [7] and preeclampsia (SELDI-MS)
95 [8], and for the rapid identification of microorganisms in clinical microbiology laboratories
96 (MALDI-MS) [9]. MS-based proteomics have been also proposed in ovarian, breast, and prostate
97 cancer diagnostics [10] and in newborn and prenatal screening programs, where it has been
98 proposed to detect inherited inborn errors in metabolism [11]. In the past decade, novel
99 improvements in MS have included the introduction of "ambient ionization" (direct MS), which
100 stands out owing to its unique capability of direct analysis of complex samples with no or
101 minimal pretreatment of the samples [12]; and direct-analysis real-time mass spectrometry
102 (DART-MS), an established technique for rapid mass spectral analysis of a large variety of
103 samples [13].

104 In our study, a modified spray-from-tissue ionization method was utilized, which was
105 based on the findings previously reported for brain tissue analysis during neurosurgical
106 procedures that demonstrated real-time lipid profile delineation between brain tumor tissue and
107 surrounding healthy tissue [14]. The analysis of lipid profiles of resected endometriosis lesions
108 (peritoneal endometriosis and ovarian endometrioma) and eutopic endometrium was undertaken
109 in the current study to understand the possible dysregulation in the metabolism and fluxes of
110 specific lipids in women affected by endometriosis. In a pilot experiment using the modified
111 ionization method in 6 patients (data not published), mass spectrometric data demonstrated

Accepted Manuscript

ACCEPTED MANUSCRIPT

112 apparent tissue type differentiation between endometriotic tissue (ectopic endometrium) and
113 uterine endometrium. And as such, the aim of this study was to expand upon these early results.

114 2. Materials and Methods

115 2.1 Study design

116 Tissue samples were collected from 50 patients with ovarian endometriomas, infiltrative
117 endometriosis, and peritoneal endometriosis who underwent laparoscopic surgery in the
118 Department of a Gynecologic Surgery at the V. I. Kulakov Research Center in Moscow. All
119 patients included in the study provided written informed consent. The Commission of
120 Biomedical Ethics at V.I. Kulakov Research Center for Obstetrics Obstetrics, Gynecology, and
121 Perinatology approved all procedures and study methods.

122 The inclusion criteria for all participants of the study were: reproductive age (15-45
123 years), histologically confirmed diagnosis of stage III or IV endometriosis, late
124 proliferative/early secretory menstrual cycle (days 8 to 21), absence of any chronic pathology
125 (including diabetes mellitus, kidney disease, cardiovascular disease, and inflammatory diseases),
126 and absence of any hormone therapy over 6 months prior to surgery. The disease stage was
127 determined according to the classification system of the American Society for Reproductive
128 Medicine. Endometrioid ovarian cysts and infiltrative endometriosis were identified before
129 surgery in all of cases by transvaginal ultrasound and MRI. Peritoneal endometriotic lesions
130 were revealed during laparoscopic surgery.

131 2.2 Sample collection

132 Patients underwent laparoscopic surgery using the same laparoscope, Karl Storz
133 Endoskope, Tuttlingen, Germany. Hysteroscopy was performed for sampling endometrial tissue
134 for histological and mass spectrometric research. The biopsies of peritoneal endometriotic foci
135 after excision (without thermal exposure), capsules of endometrioid cysts after cystectomy, and
136 endometrium after diagnostic curettage of the uterine cavity were placed in separate sterile tubes

Accepted Manuscript

ACCEPTED MANUSCRIPT

137 (1 ml) and immediately (at the operating room table upon excision) immersed in liquid nitrogen
138 to prevent oxidation of tissue lipids. Samples were then transported to the laboratory, where the
139 MS machine was located, and stored at –80^0 C. The tissue samples were divided into two
140 segments. The first segment of each tissue sample was analyzed by histology and the second
141 segment was studied by mass spectrometry. The diagnosis of endometriosis was confirmed
142 histologically. Normal endometrial tissue from uterine sampling was also histologically assessed.

143 **2.3 Direct MS analysis**

144 The direct-spray-from-tissue method was used for molecular species extraction and
145 simultaneous ionization. Sample wetting with organic solvent (methanol with 0.1% formic acid)
146 was carried out constantly to provide a stable ion current (Fig. 1). All MS spectra were acquired
147 by electrospray ionization at quadruple time of flight mass spectrometer (Maxis Impact, Bruker,
148 Germany) in positive-mode. The scheme was designed with a spray directed to a small fragment
149 of tissue at an angle selected with respect to the mass spectrometer. This design allows for
150 biologic samples to tolerate complex sample matrixes, which makes it feasible to directly
151 analyze biological samples with minimal pretreatment. After ionization, positively charged
152 molecules were sampled through the ion optics system into the mass analyzer for further MS
153 analysis.

154 **2.4. Lipids identification and statistical analysis**

155 Samples were investigated by the MS method to obtain information about their molecular
156 composition. This information was further analyzed with multivariate data analysis (MDA)
157 methods to find out if the MS data was sufficient for the classification of tissues and to find out
158 which chemical compounds were involved in tissue differentiation. Acquired MS spectra were
159 processed with a set of functions developed in R language [15]. Mass spectrometric peaks were
160 filtered with a threshold of 200 arbitrary units to exclude noise signal; afterward, peak
161 information was extracted from MS spectra for each tissue sample under investigation and

Accepted Manuscript

ACCEPTED MANUSCRIPT

162 underwent MDA by the partial least squares discriminant analysis (PLS-DA) method with
163 "ropls" software package [16, 17]. PLS-DA is a supervised modification of the principal
164 components analysis (PCA), which is performed in order to enhance the separation between
165 studied groups of tissue samples. Further, PCA is a statistical procedure that allows for the
166 reduction of data to principal components, describing key aspects of data variance. Pareto scaling
167 was applied to data before MDA [18].

168 As a result of PLS-DA, the statistical models for tissue separation were created. The
169 characteristics of these models (R^2 and Q^2) showed the amount of data (%) that can be described
170 using the latent variables (R^2) and the amount of data (%) that can be predicted by the model
171 according to the cross validation of the values. Thus, R^2 and Q^2 show how accurately the model
172 can be expected to separate new tissues. Models with R^2 and Q^2 of more than 60% are expected
173 to have good predictive ability for tissue differentiation [17].

174 The *m/z* variables that carried information about different tissue separation were
175 determined based on the results of the PLS-DA study. Chemical compounds corresponding to
176 the obtained *m/z* were identified using accurately measured masses, with 5 – 10 ppm accuracy,
177 and information from tandem mass spectra concerning characteristic fragmentation was
178 obtained. Statistical analysis of the identified lipids was conducted using t-test with Bonferroni
179 correction for multiple comparisons.

Accepted Manuscript

180 **3. Results**

181 **3.1 Demographics data**

182 Select demographic and clinical characteristics of patients (N=50) are presented in Table
183 1. On admission more than half of the women (60%) had complaints of chronic pelvic pain, 29%
184 suffered from dysmenorrhea, and 9% had dyspareunia. Almost half of the women (48%) suffered
185 from sterility, while infertility/miscarriages were reported in seven women (14%); irregular
186 menstrual pattern and metrorrhagia were observed in 16% of patients. The majority of patients

ACCEPTED MANUSCRIPT

187 (83%) had a normal BMI of 18.5 to 24.9; two patients were overweight, one was obese, and two
188 were underweight. Prior to study enrollment, the duration of the clinical manifestations of
189 disease was of 3.5 ± 0.6 years, ranging from 5 months to 10 years.

190 Transvaginal ultrasound and subsequent laparoscopy revealed that 32 women (64%) had
191 no concomitant gynecological pathology, ten women (20%) were diagnosed with adenomyosis,
192 and eight women (16%) with uterine fibroids; 12 women (24%) had undergone previous surgery
193 for endometriosis (ovarian resection and excision and coagulation of the peritoneal
194 endometriosis foci). After the first surgical treatment, 7 out of 12 women (58%) received
195 postoperative hormonal therapy for 3-6 months (GnRH agonists, synthetic progestins).

196 **3.2. Molecular composition**

197 Samples of peritoneal endometriosis and ovarian endometriomas were analyzed in all 50
198 cases. We identified that 26 women had healthy endometrium, with no signs of inflammation or
199 other endometrial pathology. From these women with healthy endometrium, we collected tissue
200 from the capsule of the cyst (n=28) and peritoneal foci (n= 27). Using PLS-DA models, we
201 observed that endometriotic tissue samples (peritoneal endometriosis and ovarian
202 endometriomas) were clearly separated from eutopic endometrium. In the score plots shown in
203 Figure 2, endometriosis is represented by red dots and eutopic endometrium is represented by
204 grey dots. The graphs show a comparative analysis of eutopic endometrium with ovarian
205 endometrioma (A) and peritoneal foci (B), respectively. Each dot on the graph corresponds to
206 one sample (mass spectrum).

207 The PLS-DA models for tissue differentiation were developed. The PLS-DA statistical
208 model (R^2 = 80% and Q^2 = 66%) showed good predictive ability for tissue classification in cases
209 of separation of the ovarian endometrioma *vs.* eutopic endometrium. The PLS-DA statistical
210 model for the separation of the peritoneal endometriosis *vs.* eutopic endometrium (R^2 = 94% and
211 Q^2 = 83%) had even better accuracy in tissue differentiation. Sensitivity and specificity of such

Accepted Manuscript

ACCEPTED MANUSCRIPT

212 classification method for pelvic endometriotic tissue versus eutopic (normal) endometrium are
213 0.93 and 0.97, respectively; and for the ovarian cysts compared to the eutopic endometrium are
214 0.90 and 0.92. The variable importance of the projection (VIP) values were obtained from PLS-
215 DA models and determined that 15 compounds proved beneficial for tissue differentiation.
216 Identification of these compounds was performed in accordance with accurate mass and
217 characteristic MS/MS spectra. Most of the determined chemical compounds in the direct MS
218 spectra from tissues were found to be lipids, and their distribution provided sufficient
219 information to distinguish between endometriosis and eutopic endometrium.

220 **3.3. Lipids**

221 Figure 3 shows the typical masses of lipids that were obtained from the three types of
222 tissues: ovarian endometrioma, peritoneal endometriosis, and eutopic endometrium. Among the
223 most important lipids were: phosphoethanolamine (PE O-20:0), sphingomyelin (SM 34:1),
224 diglycerides (DG 44:9), phosphatidylcholines (PC 32:1, PC O-36:3, PC 38:7, PC 38:6, PC 40:8,
225 PC 40:7, PC 40:6, PC 40:9, PC O-42:1), and triglycerides (TG 41:2, TG 49:4, TG 52:3). It
226 should be noted that the amount of some lipids were particularly elevated in endometriotic
227 tissues: sphingomyelin SM 34:1, phosphoethanolamine PE O-20:0, triglyceride TG 41:2 and
228 phosphatidylcholines - PC 38:7, PC 40:8, PC 40:7.

229 The level of some types of phosphatidylcholine (PC 38:6, PC 40:6, PC 40:9, PC 32:1)
230 was higher in eutopic endometrium than in endometriotic tissue. More than 70% of the identified
231 lipids included some polyunsaturated fatty acids. The amount of phospholipid (PC O-42:1) and
232 triglyceride (TG 52:3) in peritoneal endometriosis foci was 10 times higher in the capsules of
233 endometrioma and normal endometrium. We considered that such a high level of triglycerides
234 (TG 52:3) might have been caused by inclusion of some fragment of adipose tissue into the
235 sample during the excision of peritoneal lesions (fat cells are known to consist of up to 85%
236 triglycerides). Statistical analysis of the identified lipids was conducted using a t-test with

Accepted Manuscript

ACCEPTED MANUSCRIPT

237 Bonferroni correction (Table 2). This analysis showed that despite significant difference (p<.05)
238 between endometriosis and eutopic endometrium as it relates to individual lipids present in the
239 tissue, there were no such significant differences between the tissues of endometrioid cyst of the
240 ovaries and peritoneal endometriosis. These results suggest that the observed lipids are specific
241 for the endometriotic process and not for peritoneal tissue or the ovary separately.

242 **4. Discussion**

243 Direct mass-spectrometry is actively used for the analysis of cancer-involved tissues with
244 high specificity and sensitivity [19, 20]. However, this method has not been studied in women
245 with_endometriosis to establish the extent of excision or for the intraoperative identification of
246 lesions questionable for presence of endometriosis. Lipids play an integral role in the
247 development of fundamental reactions underlying almost any pathological process, such as
248 inflammation, oxidative stress, proliferation, and angiogenesis; all of which are involved in the
249 pathogenesis of endometriosis [21, 22]. In the framework of this study, we proposed a new
250 principle of utilizing tissue analysis and a new ionization source based on electrospray for
251 patients with endometriosis.

252 We first set out to establish that differences in lipids between healthy endometrium and
253 endometriosis exists; and then, to identify the lipids that demonstrate such difference. Direct
254 mass spectrometry is a unique technique for analyzing a sample without any sample preparation.
255 The time required for the analysis of one sample takes 3-6 minutes. Currently available method
256 of tissue freezing, sectioning, and staining, followed by microscopic examination is time-
257 consuming, limiting the number of samples that can be processed in a timely manner. It is,
258 therefore, not feasible for rapid guidance of excision of endometriosis margins. In view of this,
259 the utilization of a method for express analysis of tissues involved with endometriosis, by using
260 such a highly sensitive method as mass spectrometry, is extremely appealing. Because we did
261 not have access to a portable mass spectrometry machine (our machine is large and located
262 within our laboratory department), all tissues excised for analysis were frozen in the operating

Accepted Manuscript

ACCEPTED MANUSCRIPT

263 room (OR) in liquid nitrogen to prevent oxidation of lipids, which can occur within 30 minutes if
264 not frozen, and then transported to the laboratory. Despite the fact that delivery of the specimen
265 from the OR to the laboratory uniformly took place within 15 minutes and hence the specimen
266 quality would probably not have been affected without freezing, we wanted to provide standard
267 conditions for all specimens. Therefore, each specimen was frozen at the OR table site
268 immediately upon excision. This step would not be necessary for existing centers in the world
269 where mass spectrometers are installed directly in operating rooms.

270 The purpose of our study was to identify the lipid composition of endometriotic tissues.
271 We found that the mass spectrometry method allowed identification, with high sensitivity, of
272 endometriotic tissue from that of eutopic endometrium in an individual patient with
273 endometriosis. We identified 148 lipids, but significance was shown only for a panel consisting
274 of 15 lipids. As demonstrated by PLS-DA (Fig. 2), there was a significant difference in lipid
275 content between endometriosis and eutopic endometrium, but not in the composition between
276 pelvic endometriosis and ovarian endometrioma. This observation suggests that these lipids may
277 be specific for the endometriotic process per se, and not for differentiation between peritoneal
278 and ovarian endometriosis. Three classes of lipids showed marked differences between
279 endometriotic and endometrial tissue in studied patients – sphingolipids, phospholipids, and the
280 fatty acids, di- and tri-glycerides.

281 As is the case of all of the lipids identified in this study, the role of sphingolipids in
282 endometriosis has not been fully investigated; however, they are a distinctive and highly
283 important class of lipids functioning in different biological processes, including signal
284 transduction and cell fate determination [23]. As such, sphingolipids are increasingly known to
285 be important bioactive signaling molecules [24-29]. Since endometriosis enhances cellular
286 growth, it can be postulated that an imbalance in the sphingolipid metabolic pathway may result
287 in intrinsic accelerated proliferation of endometrial cells. This may cause implantation and
288 growth of lesions at ectopic sites. Additionally, the bioactive sphingolipid metabolite,

Accepted Manuscript

ACCEPTED MANUSCRIPT

289 sphingosine-1-phosphate (S1P), enhances the proliferative potential of the cells, provides their
290 resistance to apoptosis, and stimulates angiogenesis and cell migration. S1P stimulates
291 expression of the local immune cells TNFα and IL8, which activate COX2 and prostaglandin
292 synthesis. Prostaglandins are known to participate in the development of pain and infertility in
293 endometriosis [30]. Additionally, Vouk et al demonstrated that continuing processes of
294 denervation followed by re-innervation in ectopic endometrium are connected with elevated
295 levels of sphingomyelins [29], strongly suggesting that elevated levels of sphingomyelins are the
296 primary source of pain in patients with endometriosis. These observations suggest that the
297 presence of elevated levels of sphingolipids and phosphatidylcholines, and imbalances in their
298 metabolic pathways in the endometriotic tissue, may be directly involved in the implantation as
299 well as the heightened proliferative and decreased apoptotic properties of endometriotic tissue,
300 with a resultant growth of lesions and associated pain.

301 In this study, we also observed elevated levels of various phospholipid (PL) subclasses in
302 patients with endometriosis. Biologically active PLs implement a variety of cellular functions
303 such as enzymatic regulation, transcription, signal transduction of second messengers, as well as
304 transport substances [31]. Phosphatidylcholine (ChoGpl) is the most important lipid in the
305 human body. It plays an important role in the formation of the membrane structure and cell
306 signaling. The increase of ChoGpl is the basis for the synthesis of phospholipase A2 (PA2), an
307 enzyme that is overexpressed in endometriotic lesions. PA2 is, in turn, responsible for the
308 production of lysophosphatidic acid, a lipid involved in cell proliferation related to cancer and
309 endometriosis [32]. ChoGpl is considered a marker of high proliferation of malignant tissue**;** and
310 as such, it can be postulated as a potential biomarker for endometriosis.

311 Additionally, the role of diacylglycerol (DG) in the metabolism of glycerolipids is of
312 primary importance; it is also a precursor of numerous lipid molecules, including
313 phosphatidylcholine, phosphoethanolamine, triacylglycerol, and phosphatidic acid [33].
314 Moreover, DG is important in the signaling of glycerolipids since it activates protein kinase C,

ACCEPTED MANUSCRIPT

315 which plays an important role in various biological processes, including cell differentiation and
316 proliferation [34]. Interestingly, DG counteracts the effect ceramides, bioactive sphingolipids,
317 have against apoptosis, but not against the cell cycle, which suggests the protective role of
318 diglyceride [35]. Changes in the levels of ceramide and diglyceride can cause prolonged and
319 presumably permanent reprogramming of cell function through the regulatory mechanism of
320 apoptosis and the cell cycle [36]. Most often and most noticeably, DG mediates agonist-induced
321 stimulation of cell growth and proliferation, while ceramides contribute transduction agonists
322 activation in antiproliferative pathways [37]. Consequently, having the concentration of ceramide
323 and diacylglycerol determined can be important in regulating cell growth and viability in that
324 disturbance of the ratio of DG to ceramides may lead to abnormal cellular responses and cause
325 malignant disorders, aging processes, and, presumably, endometriosis [38].

326 In this study, we compared direct mass spectrometry-based lipidomics of the healthy
327 endometrium of women with endometriosis with that of excised endometriotic tissue (peritoneal
328 endometriosis and ovarian endometrioma). We considered that the best way to isolate a specific
329 substance in targeted tissue (endometriosis) is to compare it with the tissue with least variability
330 from the tissue studied (endometrium). Furthermore, while the endometrium of a healthy woman
331 without endometriosis could be a better control, the endometrium of the same woman allows for
332 the least lipidomic differences as compared with her own endometrial tissue of different position
333 and quality (endometriosis). The differences in biochemical composition of the endometriosis
334 and endometrium are minimized in a sample originating from the same host. We therefore
335 consider eutopic endometrium of a woman with endometriosis to be the best control/comparator
336 for her endometriosis lesions. Studies involving the inclusion of tissues from healthy patients
337 may be of additional benefit.

338 We do not conclude, based on our findings, that surgeons can replace conventional
339 histological methods with the mass spectrometric method outlined herein. We continue to
340 consider histopathology to be the gold-standard in evaluation of excised tissue. Based on our

Accepted Manuscript

ACCEPTED MANUSCRIPT

341 review of the literature, a lipid analysis using mass spectrometry to compare endometriotic tissue
342 with eutopic endometrium has been carried out for the first time in our study. These findings lay
343 the groundwork for possible intraoperative clinical applications. Future studies focusing on
344 comparisons between healthy tissue and the same tissue involved with endometriosis are needed
345 to further confirm and/or refine the lipidomic criteria for establishing the presence or absence of
346 the disease and to corroborate our results and conclusions. Continued exploration may bring us
347 closer to understanding the pathobiology of the disease and to elaborating new ways of early
348 diagnostics and heightened surgical capacity for tissue discernment as it relates to surgery for
349 endometriosis.

350 **5. Conclusions**

351 This study identified, using modified spray-from-tissue ionization mass spectrometry, a
352 consistent lipid profile present in histopathologically-confirmed endometriotic tissue that was
353 different than that of the comparison healthy endometrium of the same woman. In this study,
354 endometriotic tissue was shown to be associated with elevated levels of lipids of three important
355 classes: sphingolipids, phospholipids, and the fatty acids, di- and tri-glycerides. These lipids
356 have been previously described as directly involved in the implantation and the heightened
357 proliferative and decreased apoptotic properties of endometriotic tissue, as well as contributing
358 to pain syndromes of affected patients. Validation of these lipids as biomarkers in future studies
359 is warranted.

360

361 **Acknowledgements**

362 This work was supported by Ministry of Education and Science of the Russian Federation grant
363 (agreement No. 14.613.21.0059, RFMEFI61316X0059).
364 Konstantin Chingin and Huanwen Chen acknowledged the National Natural Science Fund of
365 China (no. 21305012), the Program for Changjiang Scholars and Innovative Research Team

Accepted Manuscript

ACCEPTED MANUSCRIPT

366 (PCSIRT) (no. IRT13054), the Science and Technology Planning Project at the Ministry of
367 Science and Technology of Jiangxi Province, China (no. 20152ACB21013).

368

369 **References**

370 1. Signorile PG, Baldi A. New evidence in endometriosis. *Int J Biochem Cell Biol.* 2015;
371 60:19–22.

372 2. Mehedintu C, Plotogea MN, Ionescu S, Antonovici M. Endometriosis still a challenge. *J*
373 *Med Life*. 2014; 7(3):349–57.

374 3. Farquhar C. Endometriosis. *BMJ.* 2007; 334:249–53.

375 4. Emre A, Akbulut S, Yilmaz M, Bozdag Z. Laparoscopic Trocar Port Site Endometriosis:
376 A Case Report and Brief Literature Review. *Int Surg*. 2012; 97(2):135–9.

377 5. Fassbender A, Burney RO, O DF, D'Hooghe T, Giudice L. Update on Biomarkers for the
378 Detection of Endometriosis. *Biomed Res Int*. 2015; 2015:130854.

379 6. Johnson NP, Hummelshoj L, World Endometriosis Society Montpellier Consortium.
380 Consensus on the current management of endometriosis. *Hum Reprod*. 2013; 28:1552–68.

381 7. Mischak H. Pro: Urine proteomics as a liquid kidney biopsy: no more kidney punctures!
382 *Nephrol Dial Transplant.* 2015; 30:532–7.

383 8. Buhimschi LA, Nayeri UA, Zhao G, et al. Protein misfolding, congophilia,
384 oligomerization, and defective amyloid processing in preeclampsia. *Sci Transl Med.* 2014;
385 6(245):245ra92.

386 9. Meng QH. Mass Spectrometry Applications in Clinical Diagnostics. *J Clinic Experiment*
387 *Pathol*. 2013; S6.

388 10. Pietrowska M, Marczak L, Polanska J, Behrendt K, Nowicka E, Walaszczyk A, Chmura
389 A, Deja R, Stobiecki M, Polanski A, Tarnawski R, Widlak P. Mass spectrometry-based serum
390 proteome pattern analysis in molecular diagnostics of early stage breast cancer. *J Transl Med.*
391 2009; 7:60.

Accepted Manuscript

ACCEPTED MANUSCRIPT

392 11. Pourfarzam M, Zadhoush F. Newborn Screening for inherited metabolic disorders; news
393 and views. *J Res Med Sci.* 2013; 18(9):801–8.
394 12. Takáts, Z, Wiseman JM, Gologan B, Cooks RG. Mass spectrometry sampling under
395 ambient conditions with desorption electrospray ionization. *Science.* 2004; 306(5695):471–3.
396 13. Gross JH. Direct analysis in real time--a critical review on DART-MS. *Anal Bioanal*
397 *Chem.* 2014; 406(1):63–80.
398 14. Kononikhin A, Zhvansky E, Shurkhay V, Popov I, Bormotov D, Kostyukevich Y,
399 Karchugina S, Indeykina M, Bugrova A, Starodubtseva N, Potapov A, Nikolaev E. A novel
400 direct spray-from-tissue ionization method for mass spectrometric analysis of human brain
401 tumors. *Anal Bioanal Chem.* 2015; 407:7797–805.
402 15. R Development Core Team. R: A language and environment for statistical computing. R
403 Foundation for Statistical Computing, Vienna, Austria, 2008. ISBN 3-900051-07-0, URL
404 http://www.R-project.org.
405 16. Thevenot EA, Roux A, Xu Y, Ezan E and Junot C. Analysis of the human adult urinary
406 metabolome variations with age, body mass index and gender by implementing a comprehensive
407 workflow for univariate and OPLS statistical analyses. *J Proteome Res.* 2015; 14(8):3322–35.
408 17. Wold S, Sjöström M, Eriksson L. PLS-regression: a basic tool of chemometrics. *Chemom*
409 *Intell Lab Syst*. 2001; 58(2):109–30.
410 18. Eriksson L, Johansson E, Kettaneh-Wold N, Wold S. Introduction to multi- and
411 megavariate data analysis using projection methods (PCA & PLS) Umetrics; 1999. Scaling; pp.
412 213–225.
413 19. Whitney B. Pope. Intraoperative mass spectrometry of tumor metabolites. *Proc Natl Acad*
414 *Sci USA*. 2014; 111(30):10906–7.
415 20. Santagata S, et al. Intraoperative mass spectrometry mapping of an onco-metabolite to
416 guide brain tumor surgery. *Proc Natl Acad Sci USA*. 2014; 111:11121–6.

Accepted Manuscript

ACCEPTED MANUSCRIPT

417 21. Polak G, Barczyński B, Kwaśniewski W, Bednarek W, Wertel I, Derewianka-Polak M,
418 Kotarski J. Low-Density Lipoproteins Oxidation and Endometriosis. Mediators of Inflammation.
419 2013; 1–4. http://dx.doi.org/10.1155/2013/624540.

420 22. Vouk K, Ribič-Pucelj M, Adamski J, Rižner TL. Altered levels of acylcarnitines,
421 phosphatidylcholines, and sphingomyelins in peritoneal fluid from ovarian endometriosis
422 patients. J Steroid Biochem Mol Biol. 2016; 159:60–9.

423 23. Lee J, Yeganeh B, Ermini L, Post M. Sphingolipids as cell fate regulators in lung
424 development and disease. *Apoptosis.* 2015; 20(5):740–57.

425 24. Chrobak A, Sieradzka U, Sozański R, Chełmońska-Soyta A, Gabryś M, Jerzak M.
426 Ectopic and eutopic stromal endometriotic cells have a damaged ceramide signaling pathway to
427 apoptosis. *Fertil Steril.* 2009; 92(6):1834–43.

428 25. Gault CR, Obeid LM, Hannun YA. An overview of sphingolipid metabolism: from
429 synthesis to breakdown. *Adv Exp Med Biol.* 2010; 688:1–23.

430 26. Lee YH, Tan CW, Venkatratnam A, Tan CS, Cui L, Loh SF, Griffith L, Tannenbaum SR,
431 Chan JK. Dysregulated Sphingolipid Metabolism in Endometriosis. *J Clin Endocrinol Metab.*
432 2014; 99(10):E1913-21.

433 27. Martinez TN, Chen X, Bandyopadhyay S, Merrill AH, Tansey MG. Ceramide
434 sphingolipid signaling mediates Tumor Necrosis Factor (TNF)-dependent toxicity via caspase
435 signaling in dopaminergic neurons. *Mol Neurodegener.* 2012; 7:45.

436 28. Merrill AH Jr. Sphingolipid and glycosphingolipid metabolic pathways in the era of
437 sphingolipidomics. *Chem Rev.* 2011; 111(10):6387-422.

438 29. Vouk K, Hevir N, Ribic-Pucelj M, Haarpaintner G, Scherb H, Osredkar J, et al.
439 Discovery of phosphatidylcholines and sphingomyelins as biomarkers for ovarian endometriosis.
440 *Human Reprod.* 2012; 27:2955–65.

441 30. Funk CD, Song WC, FitzGerald GA. Prostaglandins and Other Lipid Mediators in
442 Reproductive Medicine. *Yen & Jaffe's Reproductive Endocrinology.* Elsevier. 2009; 6:121–37.

ACCEPTED MANUSCRIPT

443 31. Davies SS, Guo L. Lipid Peroxidation Generates Biologically Active Phospholipids
444 Including Oxidatively N-Modified Phospholipids. *Chem Phys Lipids*. 2014; 0: 1–33.
445 32. Ye X, Chun J. Lysophosphatidic acid (LPA) signaling in vertebrate reproduction. *Trends*
446 *Endocrinol Metab.* 2010; 21(1):17–24.
447 33. Tijburg LBM, Geelen MJH, Van Golde LMG. Regulation of the biosynthesis of
448 triacylglycerol, phosphatidylcholine and phosphatidylethanolamine in the liver. *Biochim Biophys*
449 *Acta*. 1989; 1004(1):1–19.
450 34. Nishizuka Y. Protein kinase C and lipid signaling for sustained cellular responses.
451 *FASEB J.* 1995; 9(7):484–96.
452 35. Merrill AH, Jones DD. An update of the enzymology and regulation of sphingomyelin
453 metabolism. *Biochim Biophys Acta.* 1990; 1044:1–12.
454 36. Jayadev S, Liu B, Bielawska AE, Lee JY, Nazaire F, Pushkareva MY, Obeid LM,
455 Hannun YA. Role for ceramide in cell cycle arrest. *J Biol Chem.* 1995; 270(5):2047–52.
456 37. Bladergroen BA, Bussière M, Klein W, Geelen MJ, Van Golde LM, Houweling M.
457 Inhibition of phosphatidylcholine and phosphatidylethanolamine biosynthesis in rat-2 fibroblasts
458 by cell-permeable ceramides. *Eur J Biochem.* 1999; 264(1):152–60.
459 38. Al-Zoughbi W, Huang J, Paramasivan GS, Till H, Pichler M, Guertl-Lackner B, Hoefler
460 G. Tumor macroenvironment and metabolism. *Semin Oncol.* 2014; 41(2):281–95.

461

Accepted Manuscript

ACCEPTED MANUSCRIPT

462 Fig. 1 Scheme of the ion source for Direct Tissue Analysis.

463 Fig. 2 PLS-DA score plots for MS data on tissue samples of ectopic and eutopic endometrium:
464 A) ovarian endometrioma (red dots) and eutopic endometrium (grey dots); B) pelvic
465 endometriosis (red dots) and eutopic endometrium (grey dots).

466 Fig. 3 Comparison of the substances of three types of tissue (red – pelvic endometriosis; yellow
467 – endometrioid ovarian cysts (endometriomas); green – eutopic endometrium).

468

469

470

Accepted Manuscript

ACCEPTED MANUSCRIPT

471 **Table 1** Clinical and demographic data of the patients

	Patients (N=50)	Frequency (%)
Age category		
<25 years	10	20
26–29.9 years	12	24
30–35.9 years	17	34
36–40.9 years	10	20
>41 years	1	2
Menstrual phase		
Proliferative	2	4
Late proliferative/early secretory	46	92
Secretory	2	4
Concomitant gynecologic pathology		
Adenomyosis	10	20
Myoma uteri	8	16
None	32	64
Ethnicity		
Caucasian	46	92
Others	4	8
Reproductive function		
Sterility	24	48
Infertility/previous miscarriage	7	14
No willingness to conceive	12	24
Recurrence of endometriosis		
Previously operated for endometriosis	12	24

472

Accepted Manuscript

ACCEPTED MANUSCRIPT

473 Table 2. The results of the Student test of tissue types under investigation.

Lipid ID	p-value		
	Pelvic *vs* ovarian	Pelvic *vs* endometrium	Ovarian *vs* endometrium
PE O-20:0	0.06	0.07	0.00
SM 34:1	0.98	0.00	0.01
TG 41:2	0.79	0.00	0.00
DG 44:9	0.03	0.91	0.01
PC 32:1	0.62	0.01	0.02
PC O-36:3	0.06	0.02	0.21
PC 38:7	0.97	0.05	0.05
PC 38:6	0.47	0.01	0.00
PC 40:8	0.71	0.07	0.02
PC 40:7	0.92	0.02	0.03
PC 40:6	0.14	0.01	0.00
TG 49:4	0.09	0.16	0.00
PC 40:9	0.44	0.00	0.01
TG 52:3	0.03	0.04	0.18
PC O-42:1	0.03	0.05	0.28

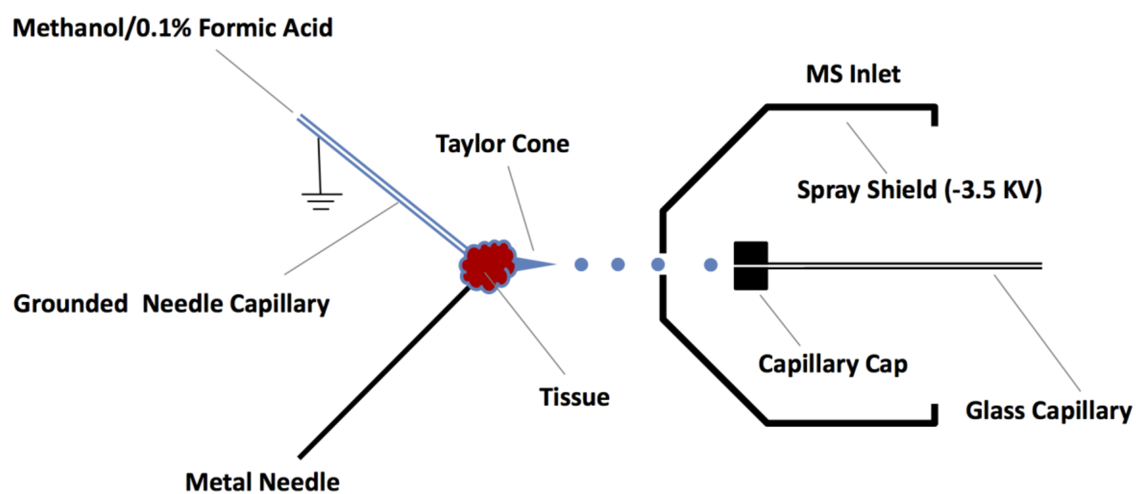
474

475

476

Accepted Manuscript

ACCEPTED MANUSCRIPT



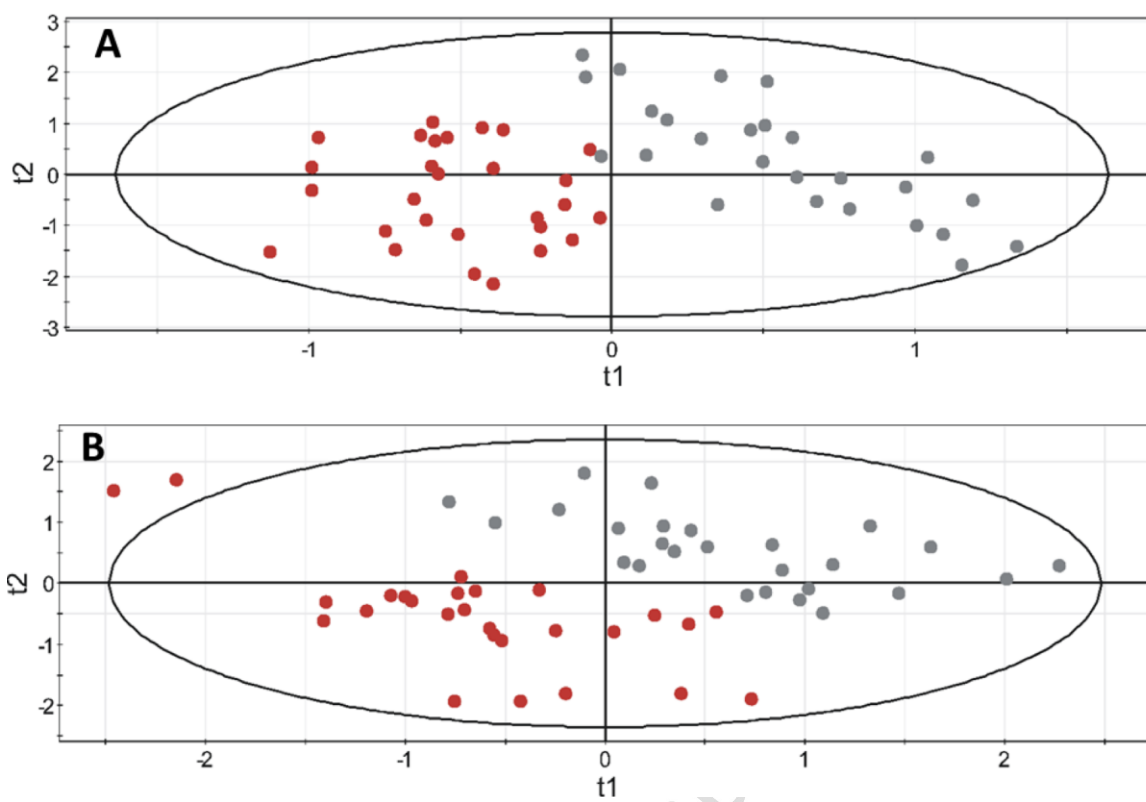
477

478 **Figure 1.tiff**

479

Accepted Manuscript

ACCEPTED MANUSCRIPT



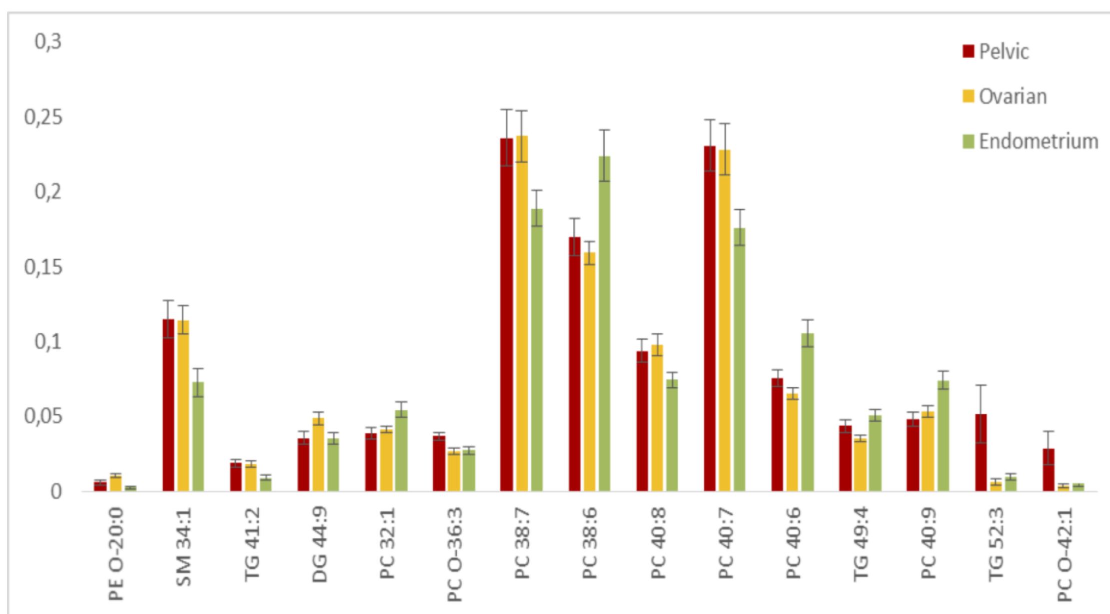
480

481 **Figure 2.tiff**

482

Accepted Manuscript

ACCEPTED MANUSCRIPT



483

484 **Figure 3.tiff**

Accepted Manuscript