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Discovery of phosphatidylcholines and sphingomyelins as biomarkers for ovarian endometriosis

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BACKGROUND: Current non-invasive diagnostic methods for endometriosis lack sensitivity and specificity. In search for new diagnostic biomarkers for ovarian endometriosis, we used a hypothesis-generating targeted metabolomics approach.

METHODS: In a case–control study, we collected plasma of study participants and analysed their metabolic profiles. We selected a group of 40 patients with ovarian endometriosis who underwent laparoscopic surgery and a control group of 52 healthy women who underwent sterilization at the University Clinical Centre Ljubljana, Slovenia. Over 140 targeted analytes included glycerophospholipids, sphingolipids and acylcarnitines. The analytes were quantified by electrospray ionization tandem mass spectrometry. For assessing the strength of association between the metabolite or metabolite ratios and the disease, we used crude and adjusted odds ratios. A stepwise logistic regression procedure was used for selecting the best combination of biomarkers.

RESULTS: Eight lipid metabolites were identified as endometriosis-associated biomarkers due to elevated levels in patients compared with controls. A model containing hydroxysphingomyelin SMOH C16:1 and the ratio between phosphatidylcholine PCaa C36:2 to ether-phospholipid PCae C34:2, adjusted for the effect of age and the BMI, resulted in a sensitivity of 90.0%, a specificity of 84.3% and a ratio of the positive likelihood ratio to the negative likelihood ratio of 48.3.

CONCLUSIONS: Our results suggest that endometriosis is associated with elevated levels of sphingomyelins and phosphatidylcholines, which might contribute to the suppression of apoptosis and affect lipid-associated signalling pathways. Our findings suggest novel potential routes for therapy by specifically blocking highly up-regulated isoforms of phosphpolipase A2 and lysophosphatidylcholine acyltransferase 4.

Key words: sphingomyeline / phosphatidylcholine / ether phospholipid / acylcarnitine / ovarian endometriosis

Introduction

Endometriosis is a frequent reproductive disorder, characterized by the presence of endometrial tissue outside the uterine cavity. The nature of this disease is heterogeneous and comprises ovarian, peritoneal as well as deep infiltrating endometriosis. While the estimated prevalence is 6–10% in the general female population, the frequency is 35–50% in women with pain, infertility or both (Giudice and Kao, 2004). The accepted standard for diagnosis is surgical visual inspection of the pelvic organs. As a result of this, it can take up to 12 years before affected women obtain a diagnosis and receive appropriate treatment (Hadfield *et al.*, 1996). By a reduction in the time to diagnosis, the cost-effectiveness of the management of endometriosis could be largely improved (D'Hooghe and Hummelshoj, 2006). However, although over 100 potential biomarkers of endometriosis have been reported (reviewed in May *et al.*, 2010), not a single one nor a panel of biomarkers has yet unequivocally been shown to be clinically useful (May *et al.*, 2010).

As the metabolome represents the end products of gene expression, it provides the closest link to the phenotypic responses in

tissue or biological fluid samples. Apart from the numerous studies on steroids and arachidonic acid metabolites on various indications, there were only a few studies on the association of individual small endogenous metabolites with endometriosis (Murphy et al., 1998; Dionyssopoulou et al., 2005; Mansour et al., 2009), and an evaluation of a larger group of metabolites has not yet been done. We adopted a hypothesis-generating targeted metabolomics approach, and analysed the plasma metabolomes of endometriosis patients by comparing them with healthy controls. Our panel of 148 metabolites included glycerophospholipids, sphingolipids and acylcarnitines. Based on our own data, which we complemented with findings of a published gene expression study (Borghese et al., 2008), we propose molecular mechanisms to explain the altered lipid balance in endometriosis patients. To our knowledge, this is the first report on using a metabolomics approach to search for biomarkers of endometriosis.

Table I Characteristics of the study participants.

Age category	Controls, $n = 52$		Patients, $n = 40$	
	Frequency	%	Frequency	%
<26 years	0	0.00	5	12.50
26–29.9 years	0	0.00	9	22.50
30–35.9 years	3	5.77	12	30.00
36–40.9 years	22	42.31	9	22.50
>41 years	27	51.92	5	12.50
Missing data	0	0	0	0
BMI category				
Underweight <18.5	0	0.00	8	20.00
Normal 18.6–24.9	23	44.23	29	72.50
Overweight 25–29.9	20	38.46	3	7.50
Obese >30	8	15.38	0	0.00
Missing data	1	1.92	0	0.00
Menstrual phase				
Proliferative	17	32.69	12	30.00
Late prol./early sec.	11	21.15	8	20.00
Secretory	21	40.38	20	50.00
Not determined[a]	2	3.85	0	0.00
Missing data	1	1.92	0	0.00
Ethnicity[b]				
Slovene	35	67.31	28	70.00
Slovene-foreign	1	1.92	7	17.50
Foreign	13	25.00	5	12.50
Missing data	3	5.77	0	0.00
Medication during the week before[c]				
No	33	63.46	26	65.00
Yes	16	30.77	14	35.00
Missing data	3	5.77	0	0.00
Concomitant diseases				
Adenomyosis	0	0.00	1	2.50
Myoma uteri or Uterus myomatosus	3	5.77	5	12.50
None	48	92.31	34	85.00
Missing data	1	1.92	0	0.00

[a]For two study participants, we could not determine the phase of the menstrual cycle as they were on oral contraception until the last week before the surgery.
[b]Origin of parents.
[c]Allowed medications had the half-lives up to 26 h, the only exception being levothyroxine taken by two patients and one control.

Materials and Methods

Study design and sample source

Patient enrolment took place from March 2008 to October 2009 at the Department of Obstetrics and Gynecology, University Clinical Centre Ljubljana, Slovenia. In a case–control study, we recruited 111 women from which we selected a group of patients with ovarian endometriosis who underwent laparoscopic surgery ($n = 40$) and a control group of healthy women who underwent sterilization ($n = 52$) and were surgically verified not to have endometriosis. On the day of surgery (prior to anaesthesia), we collected morning blood samples from fasting participants and interviewed them in order to obtain data on their ethnic origin, life style, gynaecological and clinical conditions.

For blood collection and sample processing, we developed and implemented standard operating procedures. Blood samples of 4 ml were obtained by venipuncture from the median cubital vein using BD Vacutainer® tubes with K2 EDTA anticoagulant (Becton, Dickinson and Company, Franklin Lakes, NJ, USA, #368861). Samples were turned upside down for 8–10 times to allow for sufficient mixing with anticoagulant and placed immediately at +4°C. The time of the sample collection was logged. Samples were collected within 1 h and centrifuged at 1191*g* for 10 min at +4°C. Plasma was aspirated and aliquots of 80–100 μl were stored at −80°C in 1.8 ml cryotubes (Nalge Nunc International, Roskilde, Denmark, # 375418). The time from sample collection to freezing varied between 1 and 2 h. Aliquots used for measurements were transported on dry ice in a single batch to the measurement site.

Routine clinical biochemical parameters were measured at the Clinical Institute of Clinical Chemistry and Biochemistry of the University Clinical Centre Ljubljana, Slovenia. The study was approved by the National Medical Ethics Committee of the Republic of Slovenia, and all the participants signed their written informed consent before being enrolled in the study.

The diagnosis of endometriosis was confirmed histologically. Of the 40 patients, 14 (35%) had only ovarian endometriosis, 20 patients (50%) had also peritoneal endometriosis, and 6 patients (15%) had peritoneal and deep infiltrating endometriosis in addition to the ovarian endometriosis. The majority of our patients had stage III or IV endometriosis. Staging of endometriosis was done according to the American Society for Reproductive Medicine (1997) classification. Of the original 111 women, we excluded 19 for the following reasons: the absence of ovarian endometriosis (11 patients), pregnancy (1 control), menopause (1 patient), surgery did not take place (2 controls) and errors in the sampling procedure (2 patients and 2 controls). The majority of the participants were not on reproductive tract-related hormonal therapy or oral contraception in the last 3 months before the surgery (62% of controls and 75% of patients) and none of them were on hormonal therapy or on oral contraception in the last week before surgery. The two study groups were well balanced in terms of comorbidities (the presence of 'Uterus myomatosus or Myoma uteri') and medication type as well as in the proportion of participants who took/did not take any medication in the last week before surgery. The ethnic origins of the two study groups were similar. The

majority of the study participants were of Slovene origin (both parents of the participants were Slovene). The two groups differed in the age structure. The age of the patients ranged between 22 and 44 years (mean age 33.3 ± 6.06) and the age of the healthy controls was between 32 and 45 years (mean age 40.6 ± 3.1) (Table I). An inverse association between the adult BMI and the risk of endometriosis has been reported [reviewed in (Vitonis *et al.*, 2010)]. We observed the same tendency among our study participants; all participants classified into the underweight category were endometriosis patients and all of those classified into the obese category were healthy controls. The BMI of the patients ranged between 17.01 and 28.58 (mean 20.90 ± 2.72) and the BMI of the healthy controls was between 18.83 and 33.90 (mean 25.68 ± 4.05) (Table I).

For estimating the phases of the menstrual cycle, we used a proxy, calculated from the date of the last menstruation and corrected for an average length of the cycle. We classified the menstrual cycle phases of participants into proliferative, late proliferative/early secretory and secretory phases (Table I).

Metabolite measurements

Our targeted metabolomics approach was based on electrospray ionization mass spectrometry (ESI-MS/MS) measurements with the Absolute*IDQ*™ p150 kit (BIOCRATES Life Sciences AG, Innsbruck, Austria). The kit allows simultaneous quantification of 41 acylcarnitines (Cx:y), 92 glycerophospholipids [lysophosphatidylcholines (lyso PC) and phosphatidylcholines (PCx:y)], 15 sphingolipids (SMx:y), 14 amino acids and 1 hexose in a one-step analysis. The assay procedures as well as the metabolite nomenclature have been previously described in detail (Gieger *et al.*, 2008; Illig *et al.*, 2010; Römisch-Margl *et al.*, 2011). Quality assurance measures were done as already described (Illig *et al.*, 2010). Quantification of the metabolites in the sample is achieved by reference to appropriate internal standards. The method has been proved to conform with 21Code of Federal Regulations Part 11 Food and Drug Administration guidelines, which implies proof of reproducibility within a given error range.

Glycerophospholipids are distinguished with respect to the presence of ester (a) and ether (e) bonds in the glycerol moiety, where two letters (aa = diacyl, ae = acyl-alkyl) denote that the two glycerol positions are each bound to a fatty acid residue, while a single letter (a = acyl or e = alkyl) indicates the presence of a single fatty acid residue. Lipid side chain composition is described as C*x*:*y*, where *x* denotes the number of carbons in the side chain and *y* denotes the number of double bonds. The exact position of the double bonds and the configuration of the carbon atoms in different fatty acid side chains cannot be determined with this technology. Stereochemical differences are not always identifiable, neither are isobaric fragments, and therefore the assignment of metabolite names to individual masses can be ambiguous. For the metabolites, which are mentioned in the Results and Discussion sections, we provided identification numbers from the databases: PubChem (Bolton *et al.*, 2008), Lipidmaps (Fahy *et al.*, 2009), human metabolome database (Wishart *et al.*, 2007, 2009), chemical abstract service (US National Library of Medicine: ChemIDPlus Advanced) and KEGG (Kyoto Encyclopedia of Genes and Genomes, 2011) (Supplementary data, Table SI).

Statistical analysis

Measurements were performed on three 96-well plates. The sample positions on the plates were randomly assigned. For estimating intra-plate and plate-to-plate variability, we used several duplicates of reference samples for each plate. For calculating coefficients of variation among replicated measurements, we used the metaP-server (Kastenmüller *et al.*, 2011). We excluded 14 amino acids and hexose, as they were not in the focus of our interest, and 42 metabolites where most of the measurements were below the limit of detection or the coefficients of variation were over 0.25. In addition to 106 metabolites that passed the quality control, we also analysed all ratios between 5565 metabolite pairs [$(n^2-n)/2$ ratios for n metabolites].

We first assessed the strength of association between the metabolites and the disease with the non-parametric Mann–Whitney *U*-test, grouping participants according to their disease status (case and control). To take account of multiple testing, we applied the Benjamini–Hochberg false discovery rate correction procedure (Benjamini and Hochberg, 1995) for performing 106 tests at the significance level of 5%.

We determined the age and the BMI to be the most important potential confounders or effect modifiers. Both study groups were well balanced in terms of distribution between phases of the menstrual cycle categories. However, the phase of the menstrual cycle is usually taken into account in endometriosis studies. Therefore, we decided to check the influence of this variable as well. The normality of distributions was assessed with the Shapiro–Wilk test and most of the metabolite and ratio variables were natural logarithmic transformed for further analyses. For assessing the strength of association between the metabolite or metabolite ratios and the disease, we used the odds ratio (OR) measure (Edwards, 1963). Missing values were excluded from non-descriptive analyses.

With a logistic regression basic model, we first assessed crude associations ('crude OR') between single metabolite concentrations or metabolite ratios and the disease. For assessing the effect of potential confounders, we then extended the basic model by including the variables: age, BMI and phase of the menstrual cycle (multivariable logistic regression modelling). For every metabolite, we tested a number of different models including combinations of the three selected confounder variables as well as interactions between them. In the case of most metabolites, inclusion of the phase of the menstrual cycle did not show a significant confounding effect and did not improve the fit of the model. On the basis of the Akaike information criterion, we therefore selected the model that included age and BMI as the best one. The model, which included a particular metabolite or ratio, adjusted for the effects of age and BMI, was used for calculating the 'adjusted OR'.

As the selected metabolites belong to four classes of lipids (phosphatidylcholines, ether-phospholipids, acylcarnitines and sphongomylins) in four biosynthetic pathways, the correlation between the variables within the same lipid class is high. We have therefore considered the adjustment of the significance level to $P < 0.01$ as being sufficient and rejected the null hypotheses of the OR being equal to 1 where the *P*-values were <0.01.

As more biomarkers substantially increase the reliability of a diagnostic/prognostic test, we used a backward stepwise-regression selection procedure to assess the impact of selected multiple metabolite variables. The data were processed with Microsoft Excel 2003. For statistical analyses, we used R 2.11.1 (R Development Core Team, 2008), R-package Epicalc (Chongsuvivatwong, 2010) and SAS 9.1 (SAS Institute Inc: SAS/STAT User's Guide, Version 9.1. Cary, NC, USA: SAS Institute Inc; 2003).

Data mining

Owing to the neovascularization around and within endometriosis lesions (Taylor *et al.*, 2009), we postulated that many changes within ectopic endometrium could be reflected in the blood plasma of the patients. In addition to analysing plasma metabolites, we used the gene expression results of Borghese *et al.* (NCBI GEO database, accession number GSE12768) (Borghese *et al.*, 2008) to assist in the interpretation of our data. The design of the latter study differed from the present study as the control tissue originated from the same patients. However, as the focus of their and our study was advanced stage ovarian endometriosis, we considered this data set a suitable source for checking the expression of enzymes in potentially affected biochemical pathways.

Results

Metabolites ($n = 8$) elevated in the plasma of patients compared with controls

In this study, we quantified the plasma metabolites of endometriosis patients and healthy controls in a targeted metabolomics approach. In the first step, we looked at differences in concentrations of single metabolites between the two study groups. We calculated crude, as well as age- and BMI-adjusted, ORs with the corresponding 95% confidence intervals (CIs) for 106 metabolites, which passed measurement quality control. After correction for age and BMI, eight metabolites showed significant differences ($P < 0.01$) between patients and controls (Fig. 1). Among them were the hydroxysphingomyelins SMOH C16:1 and SMOH C22:2, the sphingomyelin SMC16:1 and five ether-phospholipids (acyl-alkyl-phosphatidylcolines): three unsaturated 2-acyl-1-(1-alkenyl)-sn-glycero-3-phosphocholines (plasmenycholines), PCae C32:2, PCae C34:2 and PCae C36:1 as well as two saturated 2-acyl-1-alkyl-sn-glycero-3-phosphocholines (plasmanylcholines), PCae C34:0 and PCae C30:0. The ORs higher than 1 indicate that all eight metabolites were elevated in the plasma of patients compared with controls (Table II). None of the eight CIs included 1 and the lower limits of the 95% CIs were reasonably higher than 1 for at least the first six metabolites; therefore, we can be confident that the observed differences are not due to chance.

After correction for multiple testing, univariate analysis showed significantly lower values in patients for three more metabolites: medium-chain acylcarnitine C8:1, phospholipid PCaa C38:4 and free carnitine C0 with respective *P*-values of 0.00032, 0.00085 and 0.00124. The result was confirmed by logistic regression (crude OR). In addition to these three metabolites, the crude OR showed that also phospholipid PCaa C38:3 was significantly lower (OR <1) and long-chain acylcarnitine C18 and ether-phospholipid PCae C42:3 were significantly higher (OR >1) in patients. However, the differences were no longer significant after adjustment for age and BMI (data not shown). Either age or BMI, or both (data not shown) showed strong confounding effects on associations of these metabolites with the disease.

Metabolite ratios ($n = 81$) showing significant differences between patients and controls

In addition to differences in single metabolite concentrations, we also analysed ratios between pairs of metabolite concentrations. These served primarily as sources of additional information on affected biochemical pathways. We calculated the crude, as well as the age- and BMI-adjusted, OR with corresponding 95% CIs for all 5565 metabolite pairs. After correction for age and BMI, 81 ratios showed significant differences between patients and controls ($P < 0.01$).

Being aware of limitations due to the study sample size, we decided to take only the nine most significantly different ratios into consideration for the further biomarker selection procedure (Fig. 2). Interestingly, we observed different metabolites such as diacyl-phosphatidylcholines and free carnitine (C0) in the numerator but only ether-phospholipids (acyl-alkyl-phosphatidylcholines) in the denominator (Table III). None of the nine CIs included one and the upper limits of the 95% CIs were reasonably lower than 1.

The ratio between long-chain acylcarnitine C16 and medium-chain acylcarnitine C8:1 does not belong to the nine most significantly different ratios (Fig. 3). However, it attracted our attention as it was one of the rare ratios which were elevated in patients. To see whether this represents a general tendency between long- and medium-chain acylcarnitines, we also examined the other ratio pairs of long-chain acylcarnitines over medium-chain acylcarnitine C8:1 (Table IV). They all turned out to be elevated in patients; however, after correction for age and BMI, the differences were no longer significant ($P > 0.01$). We calculated the Pearson correlation coefficients between these ratios and the number of leucocytes in blood, an indicator of inflammation. The correlation was positive for all 8 ratios and significant ($P < 0.01$) for 5 out of 8.

Hydroxysphingomyelin C16:1 and the ratio between phosphatidylcholine C36:2 to ether-phospholipid C34:2 represent a potential biomarker combination

In a next step, we assessed the impact of multiple metabolites as well as ratios and selected the best combination of potential biomarkers for endometriosis diagnostics, using a backward stepwise-regression selection procedure. The model, containing hydroxysphingomyelin SMOH C16:1 and the ratio of phosphatidylcholine PCaa C36:2 to ether-phospholipid PCae C34:2, corrected for the effect of age and BMI, showed the best characteristics (Table V). By assuming a 10% prevalence of endometriosis in the women population, we ended up with a sensitivity of 90.0%, specificity of 84.3%, positive likelihood ratio (LR+) of 5.7, negative likelihood ratio (LR−) of 0.1 and a ratio of LR+ to LR− of 48.3 for the given combination of variables. The receiver operating characteristic (ROC) curve shows improving effects of adding separate variables to the model. The combination of all four variables results in a significantly better performance curve and allows very good discrimination between patients and healthy controls (Fig. 4).

Discussion

Quantitative tracing of metabolic responses to pathophysiological stimuli, or environmental or genetic modifications has already been successfully applied in human population studies (Illig *et al.*, 2010). However, this approach has not yet been employed for the identification of metabolite changes in endometriosis. In our study, we identified eight lipids as novel disease-associated biomarkers. Furthermore, the observation of 81 significantly different metabolite ratios revealed affected lipid pathways. A combination of two biomarkers (SMOH C16:1 and the ratio between PCaa C36:2 and PCae C34:2) shows a very good potential for use in diagnosis.

Sphingomyelins

Several sphingomyelins were present in higher concentrations in the plasma of patients (Table II). One possible explanation for this observation could be ongoing denervation followed by re-innervation processes in endometriosis (Quinn, 2004). Sphingomyelins are key components of the sphingomyelin cycle signal transduction pathway. Some metabolites of the cycle, i.e. ceramide and sphingosine, induce apoptosis, while sphingosine-1-phosphate (S-1P) promotes

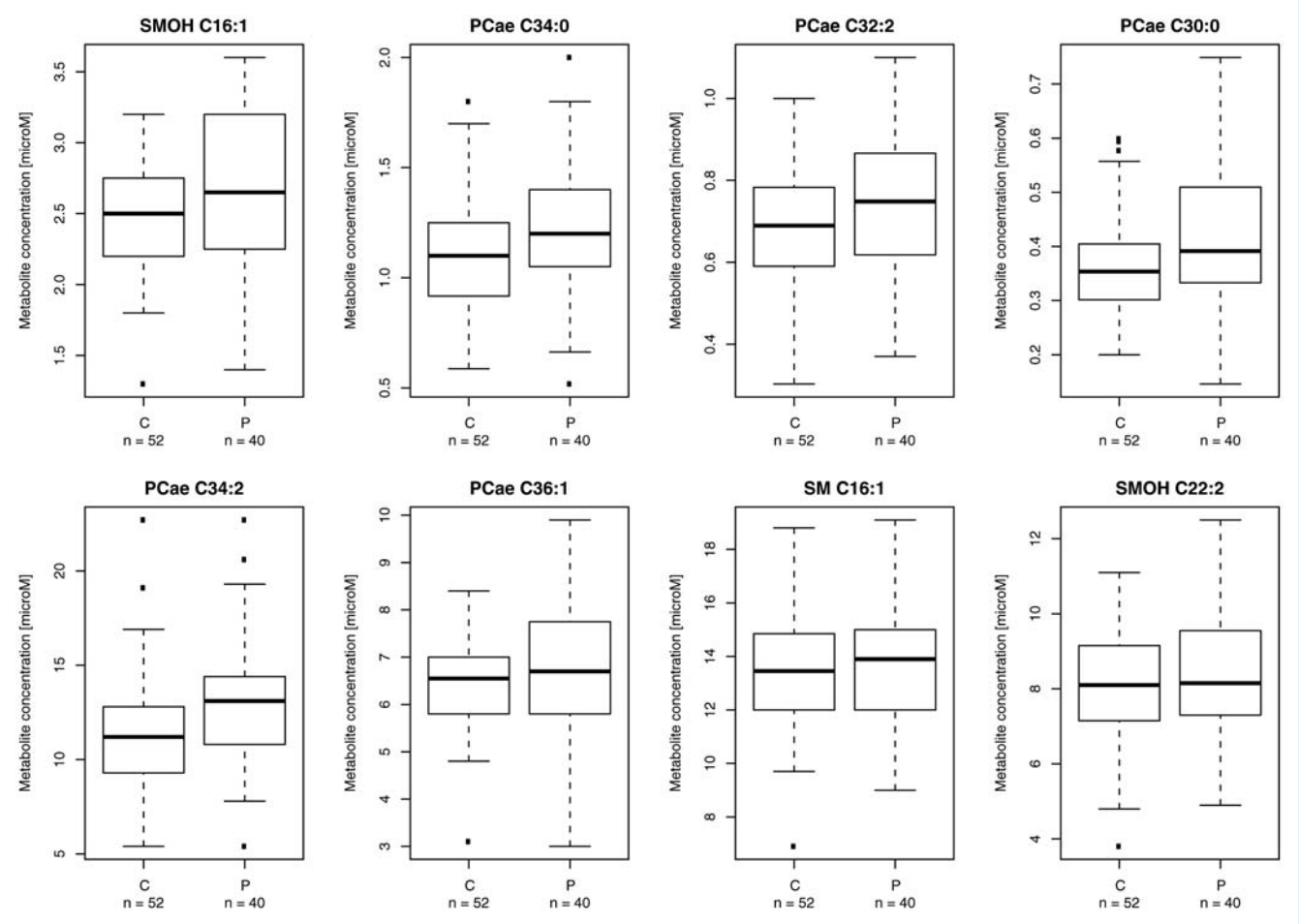

Figure 1 Box plot for eight metabolites. Box plots of quartile distributions of eight metabolites for patients (P) and healthy controls (C).

Table II ORs for metabolites which are elevated in endometriosis.

	Metabolite	Crude OR	Adjusted OR	95% CI lower (adj. OR)	95% CI upper (adj. OR)	*P*-value (adj. OR)
1	SMOH C16:1	2.38E + 00	1.70E + 01	2.90E + 00	9.93E + 01	1.69E−03
2	PCae C34:0	4.48E + 00	7.87E + 01	4.69E + 00	1.32E + 03	2.41E−03
3	PCae C32:2	1.55E + 01	1.44E + 03	1.06E + 01	1.95E + 05	3.68E−03
4	PCae C30:0	5.63E + 00	3.84E + 01	3.25E + 00	4.53E + 02	3.76E−03
5	PCae C34:2	6.96E + 00	4.94E + 01	3.39E + 00	7.20E + 02	4.32E−03
6	PCae C36:1	1.22E + 00	2.41E + 00	1.28E + 00	4.54E + 00	6.65E−03
7	SM C16:1	9.98E−01	1.73E + 00	1.15E + 00	2.59E + 00	8.48E−03
8	SMOH C22:2	1.10E + 00	2.00E + 00	1.18E + 00	3.39E + 00	9.77E−03

cell survival in response to apoptotic stimuli (Cuvillier, 2002). With regard to the sphingomyelin signal transduction pathway, the data set from Borghese *et al.* (2008) showed 2.3 to 3.4-fold up-regulation of the genes encoding enzymes that catalyse conversions from sphingomyelin to S-1P (ENPP7, ACER1 and SPHK1) in one out of the two pooled diseased tissue sample sets. In addition, the genes encoding enzymes that catalyse the conversion of S-1P back to sphingosine (SGPP1 and PPAP2A) were down-regulated in both pooled sets, with 0.4 to 0.5-fold changes, respectively (Borghese *et al.*, 2008).

A continuous denervation-re-innervation in ectopic endometrium may relate to elevated levels of sphingomyelins, and the changed expression of the genes encoding sphingomyelin cycle enzymes would balance this effect with a higher net conversion of sphingomyelins and their metabolites to S-1P. A suppression of apoptosis in

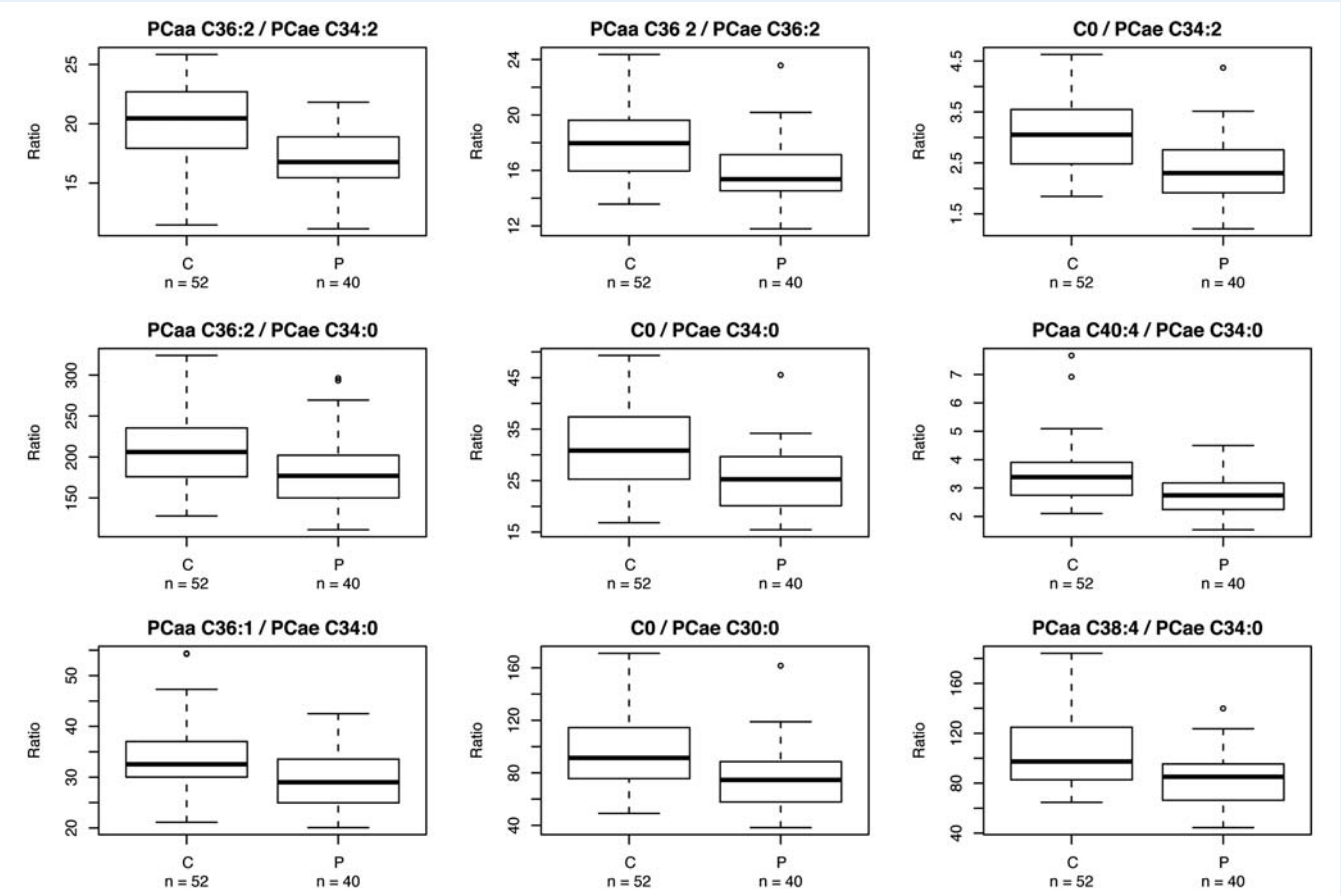

Figure 2 Box plot for nine selected metabolite ratios. Box plots of quartile distributions of nine selected metabolite ratios for patients (P) and healthy controls (C).

Table III ORs for the nine most significantly different ratios.

	Ratio	Crude OR	Adjusted OR	95% CI lower (adj. OR)	95% CI upper (adj. OR)	*P*-value (adj. OR)
1	PCaa C36:2/PCae C34:2	6.83E−01	6.12E−01	4.63E−01	8.08E−01	5.40E−04
2	PCaa C36:2/PCae C36:2	9.28E−04	7.13E−05	2.41E−07	2.11E−02	1.01E−03
3	C0/PCae C34:2	1.28E−02	7.85E−03	4.14E−04	1.49E−01	1.24E−03
4	PCaa C36:2/PCae C34:0	4.67E−02	3.80E−03	1.26E−04	1.14E−01	1.33E−03
5	C0/PCae C34:0	4.01E−02	9.14E−03	4.86E−04	1.72E−01	1.72E−03
6	PCaa C40:4/PCae C34:0	3.24E−02	6.25E−03	2.58E−04	1.51E−01	1.79E−03
7	PCaa C36:1/PCae C34:0	4.31E−02	3.99E−03	1.21E−04	1.32E−01	1.97E−03
8	C0/PCae C30:0	4.34E−02	2.04E−02	1.67E−03	2.49E−01	2.31E−03
9	PCaa C38:4/PCae C34:0	3.00E−02	4.07E−03	1.16E−04	1.42E−01	2.40E−03

endometriosis patients could partially be a physiological consequence of this process. A study, involving paired specimens of eutopic and ectopic endometrial tissue from women with endometriosis and eutopic endometrium from controls, showed that eutopic and ectopic endometrial tissue from women with endometriosis was significantly less susceptible to spontaneous apoptosis than tissue from controls (Gebel *et al.*, 1998).

Ether-phospholipids

Several plasmenylcholines and plasmanylcholines were elevated in patients (Table II). Elevated plasmenylcholines might be a result of a higher levels of phospholipases D (EC:3.1.4.4) in ectopic endometrium (Borghese *et al.*, 2008), favouring the degradation of plasmenylethanolamine to plasmenic acid and its downstream products

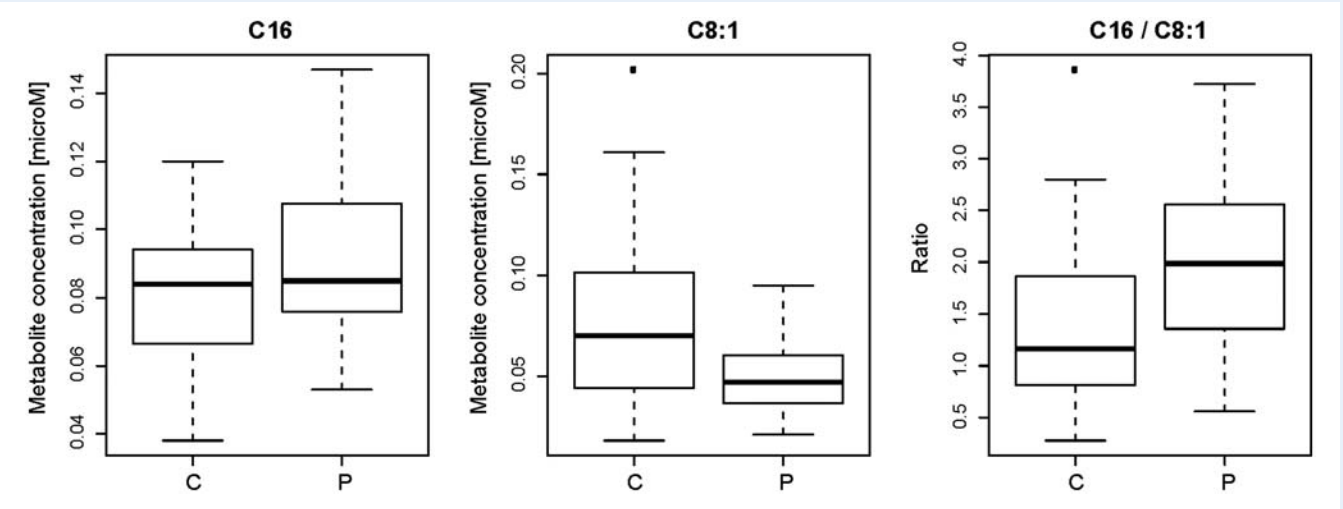

Figure 3 Box plot for acylcarnitines C16, C8:1 and their ratio. Box plots of quartile distributions for long-chain acylcarnitine C16, medium-chain acylcarnitine C8:1 and their ratio for patients (P) and healthy controls (C).

Table IV ORs for the ratios between long-chain acylcarnitines and the medium-chain acylcarnitine, C8:1, which are elevated in endometriosis.

	Ratios	Crude OR	Adjusted OR	95% CI lower (adj. OR)	95% CI upper (adj. OR)	*P*-value (adj. OR)	*r*	Corr. test *P*-values
1	C16/C8:1	6.93E + 00	9.29E + 00	1.83E + 00	4.71E + 01	7.12E−03	0.29	5.93E−03
2	C14/C8:1	6.14E + 00	7.99E + 00	1.60E + 00	3.99E + 01	1.14E−02	0.40	9.00E−05
3	C18/C8:1	6.80E + 00	5.69E + 00	1.47E + 00	2.21E + 01	1.20E−02	0.18	7.81E−02
4	C14:1 OH/C8:1	6.80E + 00	6.69E + 00	1.41E + 00	3.18E + 01	1.69E−02	0.29	6.23E−03
5	C16:2/C8:1	3.82E + 00	4.78E + 00	1.32E + 00	1.74E + 01	1.74E−02	0.39	1.10E−04
6	C18:2/C8:1	4.78E + 00	4.42E + 00	1.20E + 00	1.62E + 01	2.51E−02	0.14	1.73E−01
7	C18:1/C8:1	5.79E + 00	4.66E + 00	1.16E + 00	1.87E + 01	2.97E−02	0.23	3.27E−02
8	C12/C8:1	4.47E + 00	3.62E + 00	1.02E + 00	1.28E + 01	4.62E−02	0.37	3.20E−04

The Pearson correlation coefficient between these ratios and the number of leucocytes in blood is designated as 'r'.

2-acyl-l-(1-alkenyl)-glycerols (AEGs) and plasmenylcholines (Fig. 5). Ether-phospholipids and their metabolites play roles in protein kinase C (PKC) signalling cascades (Nagan and Zoeller, 2001). The observed elevated plasmenylcholines might be downstream products of physiologically active AEGs. Ether-linked diradylglycerols (2-acyl-1-alkyl-glycerols and AEGs) increase in many cell types following receptor stimulation (Daniel *et al.*, 1986; Agwu *et al.*, 1989; Billah *et al.*, 1989; Hii *et al.*, 1989) and inhibit some calcium-independent PKC isoforms in cell-free systems (Mandal *et al.*, 1997).

Elevated plasmanylcholines in patients might reflect a higher demand for the platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) or PAF-like molecules in neutrophils and macrophages (Bussolino and Camussi, 1995; Nagan and Zoeller, 2001). The PAF is a mediator of inflammation and angiogenesis, which upon binding to its receptor, triggers a release of mediators or cytokines (Prescott *et al.*, 2000) and pro-angiogenic factors such as vascular endothelial growth factor (Biancone *et al.*, 2003; Ko *et al.*, 2006). The expression of different phospholipases A2, which convert plasmanylcholines to lyso PAF, as well as lyso PC acyltransferase enzymes 4 (LPCAT, EC:2.3.1.51), which convert lyso PAF to PAF, seems to favour an elevated PAF synthesis in ectopic endometrium (Borghese *et al.*, 2008) (Fig. 5). In addition, we recently observed a more than 2000-fold up-regulation of *PLA2G2A* at the mRNA level in ovarian ectopic endometrium when compared with the control endometrium (Vouk *et al.*, 2011). As many of the actions of PAF can be mimicked by structurally related phospholipids, the rate of degradation by PAF acetylhydrolases represents a primary regulatory mechanism (Prescott *et al.*, 2000). Expression of all three genes encoding enzymes (or their subunits) that act as PAF acetylhydrolases (EC:3.1.1.47) is down-regulated in the ectopic endometrium of patients with 0.4–0.6-fold changes, respectively (Borghese *et al.*, 2008). Our data thus suggest that specific inhibition of the enzyme LPCAT4 and the five up-regulated phosphpolipase A2 isoforms in endometriosis (Fig. 5) may provide novel routes for therapy.

Table V Final combination of the best potential biomarkers of endometriosis.

	Crude OR	Adjusted OR	95% CI lower (adj. OR)	95% CI upper (adj. OR)	*P*-value (adj. OR)
SM OH C16:1	2.38E + 00	9.46E + 00	1.41E + 00	6.37E + 01	2.09E−02
PC aa C36:2/PC ae C34:2	6.83E−01	6.48E−01	4.79E−01	8.77E−01	4.89E−03
Age	7.29E−01	7.08E−01	5.83E−01	8.60E−01	4.97E−04
BMI	8.54E−05	1.03E−03	5.10E−06	2.08E−01	1.11E−02

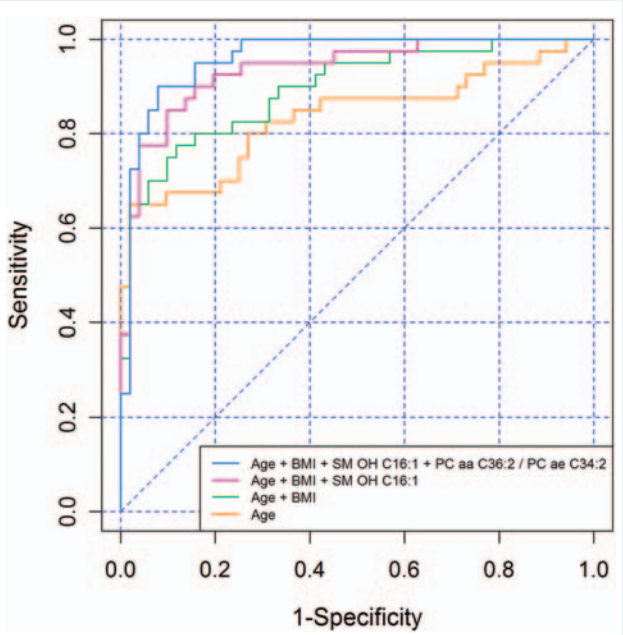

Figure 4 ROC curve. ROC curve shows improving effects of successive addition of separate variables to the model for differentiation between endometriosis patients and healthy controls.

Acylcarnitines

The ratio between long-chain acylcarnitine C16 and medium-chain acylcarnitine C8:1 is elevated in endometriosis patients (Fig. 3). We hypothesize that excessive heat generation due to inflammation may affect the efficiency of local mitochondrial membrane-bound enzymes involved in the β-oxidation process. Major endogenous pyrogens interleukin (IL)-1, IL-6 and tumour necrosis factor-α are elevated in plasma or/and peritoneal fluid of patients with endometriosis (Kharfi and Akoum, 2002; Velasco *et al.*, 2010; Mier-Cabrera *et al.*, 2011). Vasopressin, which induces a reduction in body temperature, is elevated in the plasma of women with dysmenorrhoea (Mechsner *et al.*, 2010). The membrane-bound enzymes and complexes such as very-long-chain acyl-CoA dehydrogenase, mitochondrial trifunctional protein and carnitine palmitoyl transferase II might work less efficiently when the temperature is elevated, in contrast to medium-chain acyl-CoA dehydrogenase , which is present in the mitochondrial matrix (Li *et al.*, 2010). As a result, one would expect more of the long-chain substrates and less of the medium-chain products, as seen in our study.

Our hypothesis is supported by a recent study that examined acylcarnitine profiles in normal skin fibroblasts and fibroblasts of patients with various fatty acid oxidation disorders at 37°C and after exposure to 41°C (Li *et al.*, 2010). In all cell lines, the levels of medium-chain acylcarnitines were lower and in most of the cell lines the levels of long-chain acylcarnitines were higher at 41°C (Li *et al.*, 2010). In our case, long-chain acylcarnitines were slightly, however not significantly, elevated in the plasma of patients. All ratios of long-chain acylcarnitine over C8:1 (Table IV) were elevated in endometriosis patients, however, after correction for age and BMI, the differences were no longer significant. On the other hand, the correlation between these ratios and the number of leucocytes, which signifies a presence of inflammation, was positive for all and was significant for 5 out of 8 tested ratios, which corroborates our hypothesis.

Conclusion

This is the first report on using a metabolomics approach for identification of biomarkers in endometriosis. Our results reveal that endometriosis is associated with elevated levels of sphingomyelins and ether-phospholipids, which might contribute to suppression of apoptosis and affect lipid-associated signalling pathways. Validation of these candidate biomarkers would require enlarging the existing study groups and testing in another study population. Determination of sensitivity also requires additional testing to determine whether the candidate biomarkers are able to discriminate between patients with minimal and mild endometriosis and healthy controls. Checking specificity and discrimination of ovarian endometriosis from other diseases requires the inclusion of additional control groups such as patients with benign non-endometric ovarian cysts. Our findings point towards novel routes for therapy by specifically blocking up-regulated enzymes that convert plasmanylcholines to PAF in ectopic endometrium.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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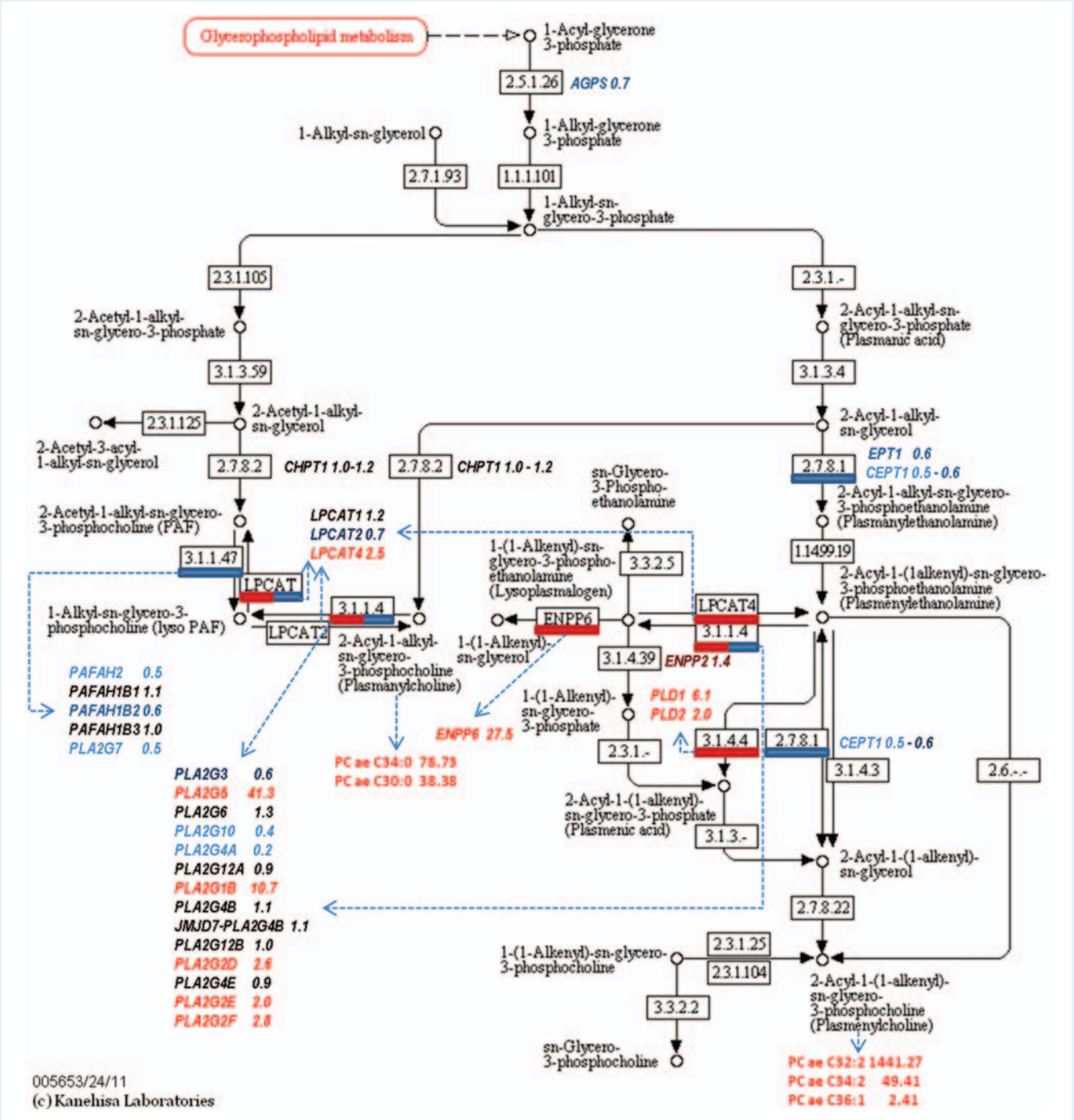

Figure 5 Comparison of metabolic pathways and mRNA levels in endometriosis. KEGG diagram (Kyoto Encyclopedia of Genes and Genomes, 2011) of ether lipid metabolism supplemented by data on ether-phospholipids increased in endometriosis (our results) as well as changed mRNA levels of the corresponding genes (the later were derived from data of Borghese *et al.* (2008). Elevated ether-phospholipids are marked in red. Ether lipid species and their adjusted ORs (our data, shown in bold) as well as enzyme names and fold changes in mRNA levels (Borghese *et al.*, written in italics) are added to the right side or connected with blue arrows to the appropriate metabolite or enzyme box. Up-regulation is coloured in red, down-regulation in blue.

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Authors' roles

K.V. designed the study, carried out and analysed the experiments and wrote the manuscript. N.H. was in charge of sample collection, participated in critical discussions and contributed in writing the manuscript. M.R.-P. was responsible for the selection of participants and participated in critical discussions. G.H. provided bio-informatic support and critical input on the interpretation of data. H.S. provided statistical support and critical input on the interpretation of data. J.O. contributed clinical biochemical parameters with their interpretation and participated in critical discussions. G.M. participated in the interpretation of results and contributed towards writing the manuscript. C.P. was in charge of metabolomic measurements and the interpretation of results, and contributed towards writing the manuscript. T.L.-R. initiated the project and was responsible for the study, and participated in the interpretation of results and contributed towards writing the manuscript, J.A. participated in the interpretation of results and preparing figures, and contributed towards writing the manuscript. All authors approved the final version of the manuscript.

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Conflict of interest

The authors have nothing to disclose.

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