Lipid Markers of Breast Tissue for the Diagnosis of Regional Metastatic Lesion

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Research Article

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Abstract

The development of minimally invasive, non-traumatic and stable approaches for the diagnosis of metastatic lesions of regional lymph nodes upon breast cancer is of great urgency. Here we recorded lipid profiles of normal breast tissue and malignant tissue to reveal potential lipid markers of metastatic lesions of regional lymph nodes. Lipid identification was done using the Lipid Match package. The search for lipid markers was carried out using the Mann-Whitney test. Lipids for the construction of a diagnostic logistic regression were selected according to the Akaike information criterion. For normal breast tissue, a diagnostic model was obtained with the area under the curve (AUC) of 0.83; for tumor tissue, a model with AUC = 0.86 was obtained. The species PC 14:0_20:4, PE 18:1_20:1, PC P-16:0/20:4, PC P-16:0/20:4, PE P-16:0/22:4, SM d18:1/18 0, SM d18:1/22:0 were determined as markers for normal breast tissue. The species PC 18:2_22:6, PC O-18:0/20:2, SM d16:1/18:1, SM d22:0/20:2, SM d16:0/18:2 were determined as markers for tumor tissue. The high AUC values for the developed diagnostic model indicate the potential significance of the revealed marker species for the diagnosis of breast cancer metastasis and indicate the need for further research in this direction.

Introduction

For many years breast cancer (BC) remains the most frequent malignant tumor in women, with the highest mortality rate among cancers [1]. Surgery is the major approach to the treatment of BC patients. The number of surgical interventions has decreased over the past decades, both on the mammary gland and on the organs of regional metastasis: from complete lymphadenectomy to sentinel lymph node biopsy (SLNB). SLNB significantly reduced the number of both early and late postoperative complications. Nevertheless, the incidence of complications after SLNB remains rather high (up to 1/4 of cases) [2]. A complete abandonment of SLNB is proposed if no data on the metastatic process has been obtained at the stage of preoperative diagnosis.

The standard method for preoperative assessment of axillary lymph nodes in BC patients is ultrasound (US). The sensitivity and specificity of detecting metastases in regional lymph nodes upon BC using US are on average 85% and 90%, respectively, and are determined by the level of the instrumental base and the competence of the operator [3]. Attempts to improve these numbers using tomography (MRI and PET) have not been successful. The sensitivity and specificity of diagnostics using MRI is on average 88% and 90%. However, MRI diagnostics is contraindicated in patients with allergies, pacemakers, and renal failure. Also, the accuracy of the analysis strongly depends on randomly occurring image artifacts, due to which the sensitivity can drop down to 60% [4]. The disadvantages of PET include low diagnostic sensitivity of metastases to the axillary nodes [5]. Of high importance is the search for biomarkers of metastases in regional lymph nodes based on clinical, instrumental and molecular data, in particular, the woman's age, size and histological subtype of the primary tumor, lymphovascular invasion, HER2 status, and other factors [6,7].

The most common approach to the search for molecular markers of a malignant process is mass spectrometry (MS) and NMR analysis of the metabolome and proteome of tumor tissues and blood plasma. Lipids are biologically active compounds that regulate a number of important cellular processes, incuding proliferation, apoptosis, and angiogenesis [8]. Differences in the lipid profile of a tissue allow the identification of benign and malignant processes in the tissue [9–15]. High performance liquid chromatography combined with mass spectrometry (HPLC-MS) is regarded to be the most informative method for lipid analysis [16].

The aim of this study was to study the possibility of using HPLC-MS analysis of the primary tumor and surrounding tissues for the diagnosis of metastasis to regional lymph nodes upon breast cancer.

Results

The groups of patients with metastases and without metastases to axillary lymph nodes did not differ significantly in terms of age, size and location of the tumor focus, HER2 status (Table 1, Table 2). In the group without metastases, the nonspecific type (30.0%) and special histological variants of the tumor (35.0%) were statistically more frequent ($p < 0.046$). In the group with metastases, the most frequent histological variant was the mixed variant (41.7%). In the group of patients without metastases to regional lymph nodes, multifocal tumors with a high degree of malignancy (G3 = 55%) and proliferative activity (mean Ki67 level = 30.35%) were frequent.

Table 1
Demographic and clinical data of patients.

Characteristic	Metastases absence	Metastases presence	P-value
Age (years)	56 (10)	56 (11)	0.90
Size of tumor (sm)	2.1 (1.6; 2.9)	2.7 (2.1; 3.5)	0.07
Ki67 level	32.5 (15.8; 62.5)	22.0 (14.8; 32.8)	0.17

Table 2
Histological characteristic of tumor tissue

Characteristic	Metastases absence		Metastases presence		P-value
	Absolute number	Percentages of the total number	Absolute number	Percentages of the total number	
Location (quadrant):	7	35.0	11	45.8	0.23
• Top-outer	2	10.0	5	20.8	
• Bottom-outer	7	35.0	2	8.4	
• Top-inner	3	15.0	3	12.5	
• Bottom-inner	1	5.0	3	2.5	
• Center					
Histological type of tumor:	6	30.0	1	4.2	0.046
• No special	2	10.0	5	20.8	
• Lobular	3	15.0	10	41.7	
• Mixed type	7	35.0	8	33.3	
• Special types					
Maligant level:	2	10.0	2	8.3	0.10
• I	7	35.0	16	66.7	
• II	11	55.0	6	25.0	
• III					
Multifocality:	5	25.0	2	8.4	0.28
• presence	15	75.0	22	91.6	
• absence					
Estrogen receptors:	16	80.0	23	95.8	0.24
• presence	4	20.0	1	4.2	
• absence					
Progesteron receptors:	14	70.0	20	83.3	0.49
• presence	6	30.0	4	16.7	
• absence					

Characteristic	Metastases absence		Metastases presence		P-value
	Absolute number	Percentages of the total number	Absolute number	Percentages of the total number	
HER2/neu:	1	5.0	1	4.2	1.00
• presence	19	95.0	23	95.8	
• absence					

The analysis of normal breast tissue revealed 6 compounds in the positive ion detection mode and 12 compounds in the negative ion detection mode which were the most significant for the potential diagnosis of metastases to regional lymph nodes. The analysis of breast tumor tissue revealed 4 compounds in the positive ion detection mode and 5 compounds in the negative ion detection mode which were the most significant for the potential diagnosis of metastases to regional lymph nodes.

The best quality of diagnostics was demonstrated by the models obtained in the positive ion mode for normal tissues by the regression equation (Table 3) with the sensitivity of 81% and specificity of 78% and in the negative ion mode for tumor tissues by the regression equation (Table 4) with the sensitivity of 79% and the specificity of 81% (Fig. 1, Tables 3 and 4). The diagnostics of mammary gland by healthy tissue (Table 3) can be achieved with the predictive value of a positive result 78% and the predictive value of a negative result 80%. In the case of diagnostics by tumor tissue (Table 4), these corresponding values are 81% and 79%. The area under curve (AUC) values of 0.83 and 0.86 indicate a very good quality of the diagnostic models.

Table 3
The analysis of multiple logistic regression for a diagnostic model built for the diagnosis of metastasis to regional lymph nodes based on the examination of normal tissue. The table contains information on the β coefficients and the probability of zero coefficient p.

	β	p
Free coefficient	-1.2E1 (-1.9E1; -5.8E0)	< 0.001
PC 14:0_20:4	9.3E-4 (5.0E-4; − 1.5E-3)	< 0.001
PE 18:1_20:1	-2.0E-3 (-3.3E-3; -1.0E-4)	< 0.001
PC P-16:0/20:4	2.2E-5 (2.5E-6; 4.5E-5)	0.04
PE P-16:0/22:4	-1.3E-4 (-2.1E-4; -7.5E-5)	< 0.001
SM d18:1/18:0	1.1E-4 (5.7E-5; 1.8E-4)	< 0.001
SM d18:1/22:0	8.2E-5 (4.5E-5; 1.3E-4)	< 0.001

Table 4
The analysis of multiple logistic regression for a diagnostic model built for the diagnosis of lymph node metastasis based on the study of tumor tissue. The table contains information on the β coefficients and the probability of zero coefficient p

	B	p
Free coefficient	-3.0E0 (-6.1E0; -4.8E-1)	0.03
PC 18:2_22:6	-6.8E-4 (-1.1E-3; -3.9E-4)	< 0.001
PC O-18:0/20:2	3.8E-4 (1.9E-4; 6.7E-4)	0.002
SM d16:1/18:1	-1.1E-3 (-2.0E-3; -4.8E-4)	0.006
SM d22:0/20:2	1.7E-3 (9.1E-4; 2.7E-3)	< 0.001
SM d16:0/18:2	-2.7E-4 (-4.5E-4; -1.3E-4)	< 0.001

Lipids identified as diagnostic markers of lymph node metastasis belong to the classes of phosphotidylcholine (PC 14:0_20:4, PC 18:2_22:6, PC P-16:0/20:4, plasmanyl-PC O-18:0/20:2), phosphotidylethanolamines (PE 18:1_20:1, PE P-16:0/22:4), ester lipids (PC P-16:0/20:4, PE P-16:0/22:4, PC O-18:0/20:2) and sphingomyelins (SM d18:1/18:0, SM d18:1/22:0, SM d18:1/22:0, SM d22:0/20:2, SM d16:0/18:2) (Fig. 2).

From the above diagram, it can be seen that the level of sphingomyelins in normal and in tumor tissue alters due to the presence of metastases in the two opposite directions: the level grows in normal tissue and decreases in tumor tissue. In contrast, the level of essential lipids increases in the presence of metastases in both types of tissues.

Discussion

Sphingomyelins are involved in reactions that trigger the processes of apoptosis with the participation of sphingomyelases, which break down sphingolipids to ceramides. Ceramides induce the activation of protein phospholipase, which is responsible for the suppression of cell growth and cell division. [17,18] At the same time, the level of the SMPD3 gene, which is responsible for the expression of neutral sphingomyelase, is increased in tumor tissues compared with normal breast tissues [17]. In addition, in mice with a deactivated acid sphingomyelase gene which were injected with melanoma cells, metastasis was significantly less pronounced than in mice with a normal genome [19]. Roy et al. reported a lower level of sphingomyelins in metastatic bone cancer cells compared to primary neoplastic bone cancer cells [20]. Also Peng et al. in an article devoted to the comparison between the metabolomic profiles of two types of colon cancer cell lines reported a significantly higher level of sphingomyelins in the cancer cell line which was less prone to metastasis [21].

Essential phospholipids are known as lipid markers of neoplastic tissue damage [22]. A higher content of phosphotidylcholides and phosphotidylethanolamines with an ether bond was recorded for the cell lines with a high metastatic potential compared to the cell lines with a low metastatic potential [23].

Materials And Methods

The current study included 44 patients with breast cancer treated in the National Medical Research Center for Obstetrics, Gynecology and Perinatology named after Academician V.I. Kulakov of the Ministry of Healthcare of Russian Federation, Moscow. The exclusion criteria were neoadjuvant therapy and the presence of malignant neoplasms of other localization prior to the diagnosis of breast cancer. All experimental protocols and methods are approved by the Ethical Committee of the National Medical Research Center for Obstetrics, Gynecology and Perinatology named after Academician V.I. Kulakov of the Ministry of Healthcare of Russian Federation, Moscow. All clinical investigations are conducted according to the principles expressed in the Declaration of Helsinki. All the patients signed informed consent.

More than half of the patients (55%) had metastases to at least one lymph node. In patients with metastases to regional lymph nodes stage pT1N1M0 was diagnosed in 5 (21%) patients, stage pT2N1-3M0 was diagnosed in 18 (75%) patients, and stage pT3N3M0 was diagnosed in 1 (4%) patient. In the group of patients without metastases to regional lymph nodes, one half had the pT1N0M0 stage, and the other half had the pT2N0M0 stage.

Two samples of breast tissue were collected from each patient: a tumor site and a site of normal tissue away from the tumor. Histological verification was performed for each sample. The analysis of lipid composition of the tissue was carried out by HPLC-MS according to the previously developed protocol [9–13]. The dried lipid extract was redissolved in acetonitrile / isopropanol mixture (1/1) and separated on a Dionex UltiMate 3000 chromatograph (Thermo Scientific, Germany) with detection on a Maxis Impact qTOF mass spectrometer (Bruker Daltonics, Germany) both in the positive and negative ion detection modes. To verify chemical identification, tandem MS analysis with a scanning window of 5 Da was additionally carried out.

The resulting .d files were converted into ms2 files, which contained information on the ion fragmentation spectra at each time point (those .d files that contained tandem MS data were transformed), and MzXml, which contained full-MS data at each time point of chromatographic analysis. The free software msConvert (Proteowizard, 3.0.9987) was used for file conversion. The MxXml were then processed in MzMine to isolate the ion peaks and normalize them to the total ion current. Tandem MS files were used to identify lipids by means of LipidMatch scripts. Times and masses of ions from a table generated by MzMine were correlated with the tandem MS data of corresponding ions at a given time point. To evaluate the relevane of the ion fragmentation spectrum to the lipid fragmentation spectrum, a library of characteristic fragments included in the package [24] was used. Lipid nomenclature is consistent with LipidMaps [25].

Statistical analysis was done using scripts in the R language (3.3.3) in the RStudio (1.383 GNU) environment [26,27]. The clinical data of the patients and the histological characteristics of tissues related to the numerical characteristics were verified for normality using the Shapiro-Wilk test ($p > 0.05$). The presence of statistically significant differences for normally distributed variables was determined using the Student's t-test with an accepted critical value of $p < 0.05$. Values outside the normal distribution were tested by the nonparametric Mann-Whitney test for statistically significant differences with an accepted critical value of $p < 0.05$. To assess the differences in factorial histological characteristics of tissues in patients with and without metastasis, the Pearson chi-square criterion of agreement was used with the accepted critical value $p < 0.05$. The identified lipids were tested for significant differences in the level in the presence and absence of metastases separately for tumor tissues and for tissues of normal mammary gland by the nonparametric Mann-Whitney test with the accepted critical value $p < 0.05$.

Categorical data were described using the absolute number and percentages of the total number of patients in the group. Quantitative normally distributed data were described using the arithmetic mean value (M) and standard deviation (SD) as $M \pm SD$. Quantitative data with a distribution other than normal were presented as the median (Me) and quartiles Q1 and Q3 as Me (Q1; Q3).

Lipids with levels that statistically significantly changed in the group were used to create a diagnostic model based on logistic regression. The optimization of logistic regression was carried out by the stepwise addition of variables and verification of the Akaike information criterion [28]. Lipid levels in tissues were used as variables. The diagnosis for the presence / absence of metastases was used as response variables. To assess the quality of a potential diagnostic model based on logistic regression, N logistic regressions were constructed based on N different samples containing (N – 1) object, followed by a test on an object not participating in the construction of regression, where N is the number of all objects in a pair of clinical groups. Sensitivity and specificity were evaluated as the number of true positives / total number of patients with metastases and the number of true negative results / total number of patients without metastases, respectively. The predictive value of positive and negative results was evaluated as the number of true positives / the number of positives and true negative results / the number of negative results.

Conclusion

Metastatic lesions of regional lymph nodes are associated with alterations in the lipid composition of tumor and normal breast tissues. Moreover, the alterations in the level of sphingomyelin differ for normal and tumor tissue, which may indicate a disturbance in the metabolic pathways associated with apoptosis. Thus, there exists a potential possibility of using the lipid profile of normal and tumor breast tissues in order to predict metastatic lesions of regional lymph nodes in breast cancer patients.

Declarations

Author Contributions: Conceptualization, V.V.C., N.L.S., V.V.R., and V.E.F.; data curation, M.V.R., A.O.T., V.V.K. and K.C.; formal analysis, M.V.R., A.O.T., and K.C.; investigation, V.V.C., N.L.S., V.V.K.; methodology, V.V.C., N.L.S., A.O.T. and M.V.R.; project administration, V.E.F., and V.V.R.; resources, V.V.R., V.V.K. and V.E.F.; software, V.V.C., A.O.T., and K.C.; supervision, V.V.C., N.L.S. and V.E.F.; validation, N.L.S., A.O.T. and K.C.; writing—original draft, V.V.C., A.O.T., and N.L.S.; writing—review and editing, K.C., V.V.R., V.V.K. and V.E.F.. All authors have read and agreed to the published version of the manuscript.

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Figures

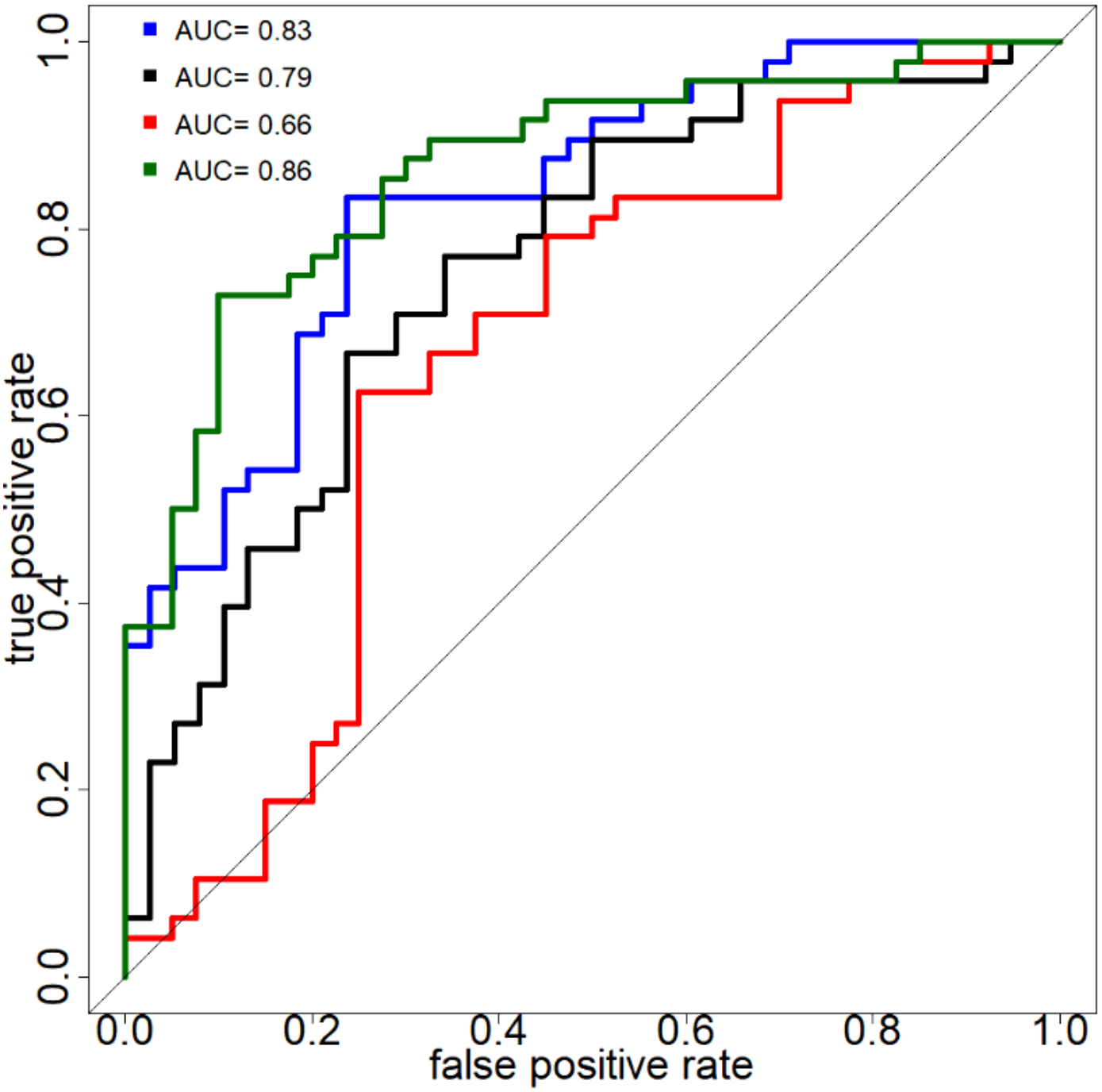

Figure 1

ROC curves constructed to analyze the predictive ability of diagnostic models of metastasis to regional lymph nodes based on the study of normal tissues (blue line, black line) and tumor tissues (red line, green line) analyzed in the positive ion detection mode (blue line, red line) and in the negative ion detection

mode (black line, green line) based on logistic regression using the relative intensities of the selected biomarker compounds as variables.

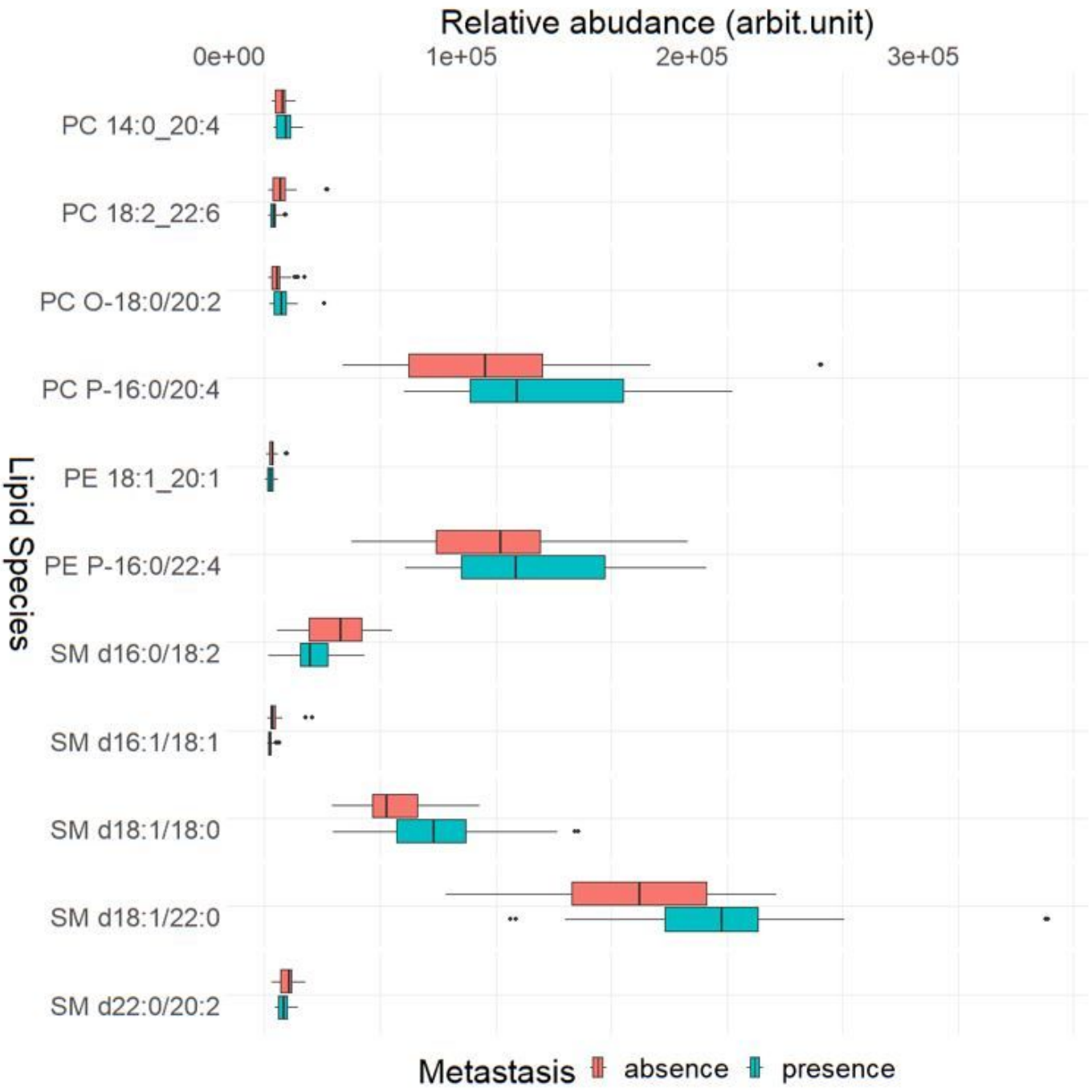

Figure 2

Box plot for diagnostic lipid markers, obtained by Mann-Whitney test and Akaike information criteria (in arbitrary units). The diagram shows Q1−1.5 * IQR, Q1, Me, Q3, Q3 + 1.5 * IQR and outcomes.