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Abstract

Objective: To characterize the lipid profile in vaginal discharge of women with vulvovaginal candidiasis, cytolytic vaginosis, or no vaginal infection or dysbiosis.

Design: Cross-sectional study.

Setting: Genital Infections Ambulatory, Department of Tocogynecology, University of Campinas, Campinas, São Paulo–Brazil.

Sample: Twenty-four women were included in this study: eight with vulvovaginal candidiasis, eight with cytolytic vaginosis and eight with no vaginal infections or dysbiosis (control group).

Methods: The lipid profile in vaginal discharge of the different study groups was determined by liquid chromatography-mass spectrometry and further analyzed with MetaboAnalyst 3.0 platform.

Main Outcome Measures: Vaginal lipids concentration and its correlation with vulvovaginal candidiasis and cytolytic vaginosis.

Results. PCA, PLS-DA and hierarchical clustering analyses indicated 38 potential lipid biomarkers for the different groups, correlating with oxidative stress, inflammation, apoptosis and integrity of the vaginal epithelial tissue. Among these, greater concentrations were found for Glycochenodeoxycholic acid-7-sulfate, O-adipoylcarnitine, 1-eicosyl-2-heptadecanoyl-glycero-3-phosphoserine, undecanoic acid, formyl dodecanoate and lipoic acid in the vulvovaginal candidiasis group; N–(tetradecanoyl)-sphinganine, DL-PPMP, 1-oleoyl-cyclic phosphatidic, palmitic acid and 5-aminopentanoic acid in the cytolytic vaginosis group; and 1-nonadecanoyl-glycero-3-phosphate, eicosadienoic acid, 1-stearoyl-cyclic-phosphatidic acid, 1-(9Z,12Z-heptadecadienoyl)-glycero-3-phosphate, formyl 9Z-tetradecenoate and 7Z,10Z-hexadecadienoic acid in the control group.

Conclusions: Lipids related to oxidative stress and apoptosis were found in higher concentrations in women with vulvovaginal candidiasis and cytolytic vaginosis, while lipids related to epithelial tissue integrity were more pronounced in the control group. Furthermore, in women with cytolytic vaginosis, we observed higher concentrations of lipids related to bacterial overgrowth.

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Guidelines

Nugent RP, Krohn MA, Hillier SL. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *J Clin Microbiol* 1991;**29**(2):297-301

Amsel R, Totten PA, Spiegel CA, Chen KC, Eschenbach D, Holmes KK. Nonspecific vaginitis. Diagnostic criteria and microbial and epidemiologic associations. *Am J Med* 1983;**74**(1):14-22.

Tautenhahn R, Patti GJ, Rinehart D, Siuzdak G. XCMS Online: a web-based platform to process untargeted metabolomic data. *Anal Chem* 2012;**84**(11):5035-9.

Xia J, Sinelnikov IV, Han B, Wishart DS. MetaboAnalyst 3.0--making metabolomics more meaningful. *Nucleic Acids Res* 2015;**43**(W1):W251-7.

http://www.hmdb.ca/metabolites.

http://www.lipidmaps.org/.

Materials

Chloroform by Contributed by users

Methanol View by Sigma Aldrich

Protocol

Define the groups

Step 1.

Define the groups by clinical and microbiological finds (gram stain).

Sample collection

Step 2.

Collect two samples of the vaginal wall by sterile Dacron swabs in a Falcon (15mL) tube and store immediately
-80ºC, until processing.

Lipid extraction

Step 3.

Randomize the samples and resuspend in 1 mL of 1:2 $CHCl_3$: MeOH solution (Sigma, Basel, Switzerland), followed by the addition of 0.33 mL of $CHCl_3$ and 0.33 mL of deionized water. The extraction was made in 15-mL glass tubes. The solution was then stirred for 5 minutes, followed by centrifugation at 13,000 rpm

for 5 minutes. The supernatant was discarded, and the bottom layer of the sample containing the lipid fraction was transferred to 1.5-mL glass tubes. All samples were dried using SpeedVac for 30 minutes at 30°C and kept frozen at -80ºC until the date of analysis.

Data acquisition

Step 4.

Lipid chromatographic separation was performed by ultra-high performance liquid chromatography (UHPLC) Agilent 1290 Infinity system (Agilent, Santa Clara, California, USA) and chromatographic elution was performed on Kinetex C18 column (4.6 mm x 50 mm x 2.6 µm) (Phenomenex, Torrance, CA, USA). For the positive ion mode, the aqueous mobile phase A solvent was 0.1% formic acid and phase B solvent was methanol; for the negative ion mode, phase A solvent was 5 mM Ammonium Acetate and phase B solvent was methanol. Before injection, the samples were randomized and inserted in the properly order. The mobile phase flow rate was 0.3 mL min^{-1} and the injection volume was 2 µL. The mobile phase gradient started at 5% of phase B changing linearly to 95% of phase B within 15 minutes and then returning to the initial composition, at which the gradient was kept constant for 5 minutes until the next run. This gradient profile was used for both positive and negative ion modes.

Mass spectrometry

Step 5.

Mass Spectrometry

To obtain the mass spectra of samples in positive and negative ion modes, a hybrid mass spectrometer with QTOF 6550 mass analyzer (Agilent, Santa Clara, California, USA) was used. The instrumental parameters of the electrospray ionization source used in this study for both positive and negative ion modes were: VCap of 3,000 V; 100 V shredder voltage, 65 V skimmer voltage, 750 V OCT 1 RF Vpp, 290°C Gas Temperature, 350°C Sheath Gas Temperature, 12 $L.min^{-1}$ Sheath Gas Flow. The mass spectra were acquired in centroid mode and the mass range used for acquisition was 50-1700 Da.

Data processing

Step 6.

The raw data obtained was converted to the mzData format using the MassHunter Qualitative software (Agilent, Santa Clara, California, USA), eliminating isotopic interference. After conversion, the files were imported into the XCMS online software for peak detection, alignment, retention time correction and other relevant pre-processing steps.

The data obtained from the online XCMS software was converted into an Excel table. Data normalization, scaling, hierarchical clustering into heatmaps and multivariate statistical analysis were performed in the MetaboAnalyst 3.0 platform. Metabolites with a value of $p < 0.05$ (ANOVA) were considered representative for further investigation. Exploratory multivariate data analysis was performed using the Principal Component Analysis (PCA) unsupervised method and the Partial Least Squares Discriminant Analysis (PLS-DA), allowing selection of molecules with a VIP index higher than 1.0 as potential biomarkers. Putative lipid identification of the selected biomarkers was performed by measurement of their exact mass, retention time and elution profile, and further matching of such compounds in METLIN, Human Metabolome Database (HMDB) and Lipid Maps databases.

Warnings

For the samples of the vaginal content use just the sterile swabs (without PBS or any conservative medium) and immediately stored in dry 10-mL tubes at -80ºC, until processing.