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Urinary metabolite and lipid alterations in Colombian Hispanic women with breast cancer: A pilot study

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ABSTRACT

Metabolic biomarkers for breast cancer (BC) prognosis and diagnosis are required, given the increment of BC incidence rates in developing countries and its prevalence in women worldwide. Human urine represents a useful resource of metabolites for biomarker discovery, because it could reflect metabolic alterations caused by a particular pathological state. Furthermore, urine analysis is readily available, it is non-invasive and allows in-time monitoring. Therefore, in present study, a metabolic- and lipid fingerprinting of urine was performed using an analytical multiplatform approach. The study was conducted in order to identify alterated metabolites which can be helpful in the understanding of metabolic alterations driven by BC as well as their potential usage as biomarkers. Urine samples collected from healthy controls and BC subjects were analyzed using LC–MS and GC–MS. Subsequently, significantly altered metabolites were determined by employing univariate and multivariate statistical analyses. An overall decrease of intermediates of the tricarboxylic acid cycle and metabolites belonging to amino acids and nucleotides were observed, along with an increment of lipid-related compounds. Receiver operating characteristic analysis evaluated the combination of dimethylheptanoylcarnitine and succinic acid as potential urinary markers, achieving a sensitivity of 93% and a specificity of 86%. The present analytical multiplatform approach enabled a wide coverage of urine metabolites that revealed significant alterations in BC samples, demonstrating its usefulness for biomarker discovery in selected populations.

1. Introduction

Metabolic fingerprinting (MF) is a useful strategy in the field of metabolomics for the exploration of the metabolome of a living organism in a particular physiological state [1]. This approach allows the identification of metabolic alterations related to a disease, providing potential markers for its diagnosis, prognosis, and treatment [2]. A wide range of compounds (metabolites) can be analyzed in the metabolome, ranging from inorganic species to nucleotides, carbohydrates, lipids, organic and amino acids. Therefore, different analytical platforms, such as mass spectrometry (MS) and nuclear magnetic resonance (NMR) have been employed for this task, allowing a comprehensive study of complex biological matrices, since each platform generates only a particular analytical window [3]. Given the variety of metabolites in a sample, a vast amount of data is collected and processed in order to generate a data matrix on which chemometrics and variable selection could be performed [4].

In recent years, lipids have been analyzed separately from other metabolites, this because of their diversity, complexity and

Abbreviations: AUC, area under the curve; BC, breast cancer; BCP, breast cancer patient; BMI, body mass index; BSTFA, *N,O*-Bis(trimethylsilyl)trifluoroacetamide; CP, control patient; CV, coefficient of variation; DG, diacylglycerol; ESI, electrospray ionization; FDR, false discovery rate; GC, gas chromatography; GC, MS- gas chromatography mass spectrometry; HPLC, high performance liquid chromatography; JK, jackknife; LC, liquid chromatography; LC, MS- liquid chromatography mass spectrometry; LF, lipid fingerprinting; MF, metabolic fingerprinting; MFE, molecular feature extraction; MTBE, methyl *tert*-butyl ether; MVA, multivariate analysis; NMR, nuclear magnetic resonance; OPLS, DA- orthogonal partial least squares-discriminant analysis; PA, phosphatidic acid; PC, phosphatidylcholine; PCA, principal component analysis; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; QTOF/MS, quadrupole time-of-flight mass spectrometry detection; QC, quality control; RFE, recursive feature extraction; ROC, receiver operating characteristic; TCA, tricarboxylic acid; TMCS, trimethylchlorosilane; tRNA, transfer ribonucleic acid; UVA, univariate analysis; VIP, variable importance in projection.

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biological relevance in various diseases. Lipid fingerprinting (LF), along with MF, have been useful approaches in the identification of metabolic alterations driven by specific pathologies [5]. For instance, in cancer research, LF and MF could elucidate new therapeutic targets and biomarkers to provide us with screening tests for the early detection and disease progression [6].

Breast cancer (BC) is the most frequently occurring cancer type and is the leading cause of cancer-related death in women around the world [7]. In recent years, incidence rates have increased in Colombia and other developing countries, due to the lack of a proper coverage of BC screening and diagnosis tests [8]. The precariousness of public health programs and difficult access to rural areas in these countries have caused restricted access to physical examination, mammography and specialized medical instrumentation required for the early detection of BC. Therefore, biological fluid analysis represents a promising resource of biological information to provide a minimally invasive and simple functional screening of this physiopathological state.

Biomarker discovery uses mainly serum and urine samples, this because of their ease of sampling, handling, storage and analysis [9]. Several serum-based BC studies have been published recently. Asiago et al. generated a prediction model for BC recurrence based on NMR and GC–MS metabolite profiling, based on the metabolites choline, 3-hydroxybutyrate and nonanedioic acid [10]. Oakman et al. reported a discrimination between early and metastatic BC by NMR using a panel of amino acids and lipids [11]. Wei et al. carried out a prediction of the response to chemotherapy using a combination of three amino acids detected by NMR with linolenic acid detected by LC–MS [12]. Tenori et al. reported a correct differentiation between early and metastatic disease in 83.7% of the studied cases using a metabolite panel including lactate and glucose analyzed by NMR [13]. However, only a few studies have been reported addressing urinary metabolic and lipid alterations in BC [14,15], even though urine sampling is painless, non-invasive and can be performed as long as needed for monitoring. Slupsky et al. reported a down-regulation of several metabolites using NMR, including amino acids (alanine, isoleucine, leucine, asparagine, valine), organic acids (lactate, succinate, hippurate) and monodisaccharides (glucose and sucrose) [16]. Nam et al. proposed four potential biomarkers using urine from BC patients. The analysis of GC–MS data identified the down-regulation of homovanillate, 4-hydroxyphenylacetate and 5-Hydroxyindoleacetate, along with the up-regulation of urea [17].

In the present study, alterations in metabolites representative for BC were identified from urine samples, using an analytical multiplatform approach applying a metabolic- and lipid fingerprinting. GC–MS and LC–MS were performed in order to achieve a comprehensive insight into the BC metabolome, and exploring the viability of urine analysis to establish the state of this pathology. To our best and current knowledge, this is the first metabolic urine study performed in a Hispanic origin population addressing BC.

2. Experimental

2.1. Characterization of studied subjects and sample collection

The present study was approved by the ethics committee from the Universidad de Los Andes and the Liga contra el Cancer- Seccional Bogotá (Colombia), written informed consent was signed by all subjects. Urine samples from sixty women were collected with the following characterization of individual groups: breast cancer patient (BCP) group consisted of 31 women diagnosed with BC, mostly invasive ductal carcinoma between stage I and III, with an average of 51 ± 9 years and a body mass index (BMI) mean value of $27 \pm 3\ kg/m^2$. The control patient (CP) group consisted of 29 healthy women with an average age of 50 ± 7 years and a BMI with a mean value of $25 \pm 3\ kg/m^2$. All participants were non-smokers, and at the moment of the study not taking hormonal contraception. In the morning after overnight fasting, the first-pass urine was collected in a suitable urine cups. After collection, the samples were centrifuged for 10 min at 3000 x g (room temperature). Sodium azide (0.05% w/v) was added to prevent bacterial growth and the urine samples were fractionated into 200 μL aliquots and stored at −80 °C until analysis.

2.2. Urine metabolic fingerprinting

2.2.1. Gas chromatography-mass spectrometry (GC–MS)

Urine sample preparation for GC–MS analysis was performed according to Garcia et al. [18]. First, 200 μL of urine were incubated with 50 μL urease (30 IU) at 37 °C for 30 min. Subsequently, urease and other proteins were precipitated with 800 μL of methanol, vortex-mixed for 5 min and centrifuged for 15 min at 15,400 × *g* and 4 °C. An aliquot of 200 μL was evaporated to dryness in a speed vacuum concentrator (LABCONCO, USA) at 30 °C. Thereafter, urinary metabolites were derivatized with 20 μL of *o*-Methoxyamine hydrochloride (15 mg/mL) in pyridine for 16 h followed by addition of 20 μL of *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) at 70 °C for 1 h. Before GC–MS analysis, 100 μL of the internal standard (methyl stearate in heptane C18:0, 10 ppm) were added to each sample. 2 μL of the derivatized urine samples were injected into an HP 6890 Series GC system equipped with an HP 6890 autosampler and an Agilent Mass Selective Detector 5973 (Agilent Technologies, Palo Alto, CA, USA). Derivatized samples were separated onto a Zebron ZB-5MSi capillary GC column (30 m × 0.25 mm; 0.25 μm) at a constant gas flow of 1.0 mL/min. The injector temperature was set at 250 °C and the split ratio was 1:10. The temperature gradient program applied was initially held at 60 °C for 1 min, then increased to 320 °C at a rate of 10 °C/min. The mass spectrometer was operated in full scan mode (*m/z* 50–600) using the following settings: electron ionization source (70 eV), scan rate (1.38 scan/s), transfer line (280 °C), filament source (230 °C) and the quadrupole temperature (150 °C).

2.2.2. Liquid chromatography-quadrupole time of flight mass spectrometry (LC-QTOF/MS)

Urine sample preparation for metabolic fingerprinting was performed using the protocol published by Want et al. [19]. First, 50 μL of urine were centrifuged at 17000 x g for 10 min at 4 °C, and the supernatant was mixed with 100 μL of water and vortex-mixed for 1 min. Subsequently, 100 μL of sample were transferred into LC vials and kept in an autosampler at 4 °C. 10 μL of the sample extract were analyzed using an HPLC 1200 series system coupled to a Q-TOF 6520 (Agilent Technologies, Santa Clara, CA, USA). LC separation was performed on a C18 column (Zorbax Extend-C18 Rapid Resolution 50 × 2.1 mm, 1.8 μm; Agilent Technologies) using a mobile phase that consisted of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B) at a flow rate of 0.5 mL/min and 40 °C. Gradient elution started from 1%B to 9%B at 2 min, then increased to 20%B at 5 min, then to 45%B at 8 min, finally to 99%B at 9.5 min the gradient was kept at 99% for 1.5 min and thereafter returned back to starting conditions in 1 min and finally an 8 min of re-equilibration time was applied [20]. The mass spectrometer was operated in as well positive as in negative electrospray ionization (ESI) mode in separate runs, employing a full scan mode in the mass range from 50 to 1100 *m/z* and a scan rate of 1.0 scan/s. The mass spectrometer source conditions consisted of a capillary voltage of 3500 V, a nebulizer gas flow rate of 11 L/min, a pressure of 50 psi and a source temperature of 325 °C. A constant mass correction was performed during all analysis using two reference masses: *m/z* 121.0509 ($C_5H_4N_4$) and *m/z* 922.0098 ($C_{18}H_{18}O_6N_3P_3F_{24}$) for the