Serum Lipidomic Biomarkers from Patients with Prostate Pathology Identified by High Performance Liquid Chromatography Coupled with Mass Spectrometry

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Abstract
Lipidomics can offer an instant picture of the lipophilic metabolites from tissues and biofluids and can indicate the presence of different pathologies, such as hyperplasia or different types of cancers. Related to these pathologies, Prostate Serum Antigen (PSA), proved to have a low grade prediction for an accurate diagnosis. Meanwhile, untargeted or targeted metabolomics became a useful technology to discover new biomarkers for a better diagnostic. The aim of this study is to realize an adequate procedure based on liquid chromatography coupled with mass spectrometry (HPLC-MS) to determine the profile of lipids from blood serum, followed by adequate biostatistics. Blood samples, obtained from healthy men and patients with prostate benign hyperplasia, post-biopsy cancer and post-surgery cancer were processed for extraction of lipids with Bligh & Dyer method, and subjected to HPLC–ESI(+)QTOF-MS measurements. TofControl 3.2 and Data Analysis 4.2 software (Bruker Daltonics) were used for the control of the instrument and data processing. To process the matrix data, Profile Analysis 2.0 software was applied for alignment and advanced bucketing and then, the multivariate analysis (PCA and Cluster Analysis) using Unscrambler 10.1 software. The statistical unsupervised analysis based on PCA scores and loadings, showed a good discrimination between the two cancer groups of patients (after biopsy and after surgery) and for benign hyperplasia patients against controls, based on the comparison of peak areas. The molecules responsible for such discriminations were identified to be mainly represented by lysophosphatidylcholines. By Cluster Analysis, the dendograms showed good statistical clustering of samples, especially for cancer patients against controls and less clustered for hyperplasia. Finally, one can consider that molecules belonging to phospholipid family and diacyl / triacylglycerides or ceramides or carnitines can be considered potential biomarkers for hyperplasia and prostate cancer.

Keywords: lipidomics, prostate hyperplasia, cancer, mass spectrometry, biomarker

INTRODUCTION
Prostate cancer (PCa) became a common malignancy and the second leading cause of cancer deaths in men (Ferlay et al., 2013). Recent studies of metabolite profiling in the prostate tissue, in blood and urine of patients with PCa showed the potential of metabolomics to improve diagnosis and provide answers regarding tumor invasiveness (Osl et al., 2008; Lochov et al. 2010; Teahan et al. 2011; Zhang et al. 2014), a better detection and prediction being crucial for the patient survival.

Metabolic biomarkers are small molecules (with molecular weight under 5000 Da) specific to tissues, cells or biofluids, which can indicate the presence and evolution of a certain metabolic
pathway or pathology, or to reflect the effects of applied treatments (Mishra and Verma, 2010).

Few prostate cancer biomarkers for early stage detection are available, the most known being the Prostate Serum Antigen (PSA) an androgen regulated protein biomarker. By its low predictability, PSA cannot have a good prognostic value, can identify less then 70% of PCa, does not distinguish sufficiently between malignant and benign diseases (prostatitis and prostatic hypertrophy) (Vicini et al., 2005)

New reliable, predictive biomarkers are necessary for early and strength diagnosis beside the biopsy, as a gold standard (Verma and Banerjee 2015).

Lipidomics, a branch of metabolomics specifically focuses on lipids and their metabolites in physiologic or pathologic states (Han, 2009). These molecules are the structural component of the cell membrane embedded with various protein complexes with different functions in cell signaling as a substrate, product or co-factor in the biochemical reactions. Lipids are involved in a variety and complex physiological processes like signaling membrane, reserve energy and endocrine action (Feng and Prestwich, 2005; Wenk, 2005; Wolf and Quinn, 2008). Therefore, lipidomics can be used to improve the diagnostic and prognosis by small lipid metabolites which are expected to reflect the alterations in the cellular and tissular metabolism (Punglia 2003). Recent studies show the lipidomic profile changes in the etiology and prognosis of cancer pathology (Cvetkovic et al., 2009; Lo et al., 2007). Metabolomic fingerprints of lipids represents a signature yet established in cancer biology (DeBerardinis et al., 2008; Santos and Schulze 2012). Modified proteins by lipids and bioactive lipids assist network signaling of many cancer pathologies (Wymann and Schneider, 2008)

Lipidomic analysis defines the phenotype of cells or tissues as a response to the environment or genetic changes, the level of lipids being fundamental for a defined genetic function (Van Meer, 2005; Villas-Boas and Gombert, 2006). Lipidomic biomarkers can also identify compounds of cytotoxic, aterogenic, mutagenic and carcinogenic origin (Seppänen-Laakso and Oresic, 2009).

Analytical platforms based on Chromatography coupled with Mass Spectrometry (MS) are frequently used in Lipidomics to provide the sensitive and reproducible detection of hundreds metabolites in a single biofluid or tissue sample. Biofluids, such as blood serum and plasma are the most frequent materials utilized for clinical diagnosis, to measure the level of lipids used for prediction of cancer progression (Jelonek et al., 2013; Grahmanfar et al., 2015; Chawda et al., 2011).

Many protocols for serum- and plasma-based metabolic profiling apply gas chromatography-MS (GC-MS) and high performance liquid chromatography (HPLC) coupled with MS. Such protocols include biofluid collection, sample preparation, data acquisition, data pre-processing and quality assurance (Dunn et al., 2011).

The most suitable tool to investigate small lipid metabolites as potential biomarkers is HPLC-MS due to its high sensitivity, specificity, ease handling of samples and large number of metabolites detected (Gika et al., 2014). Two strategies can be applied: untargeted metabolomics which measures all the metabolites in a sample, screening a specific sample fingerprint and/or the targeted metabolomics that measure a limited number of metabolites and quantify their concentrations (Han and Gross, 2003; Dunn et al., 2011).

The aim of this study is to determine a lipidomic profile of lipids from the blood serum of patients with benign hyperplasia and prostate cancer, based on liquid chromatography coupled with mass spectrometry (HPLC-MS) and unsupervised biostatistics to identify the discriminations between the pathological and healthy state of lipidomic phenotypes.

MATERIALS AND METHODS

Human blood samples were collected according to a protocol approved by the Bioethical Commission Board from the University of Medicine and Pharmacy “Iuliu Hatieganu” Cluj-Napoca and after the patients’ written consent prior to be included in this study. All blood samples were collected from patients registered at the Municipal Clinical Hospital Cluj-Napoca, Romania (in 2015) previously diagnosed and histologically classified with benign prostatic hyperplasia (BPH) (n = 40, mean age 63.92±7.8 years old), prostate sample taken before biopsy (PCa-b) (n=51, mean age 65.67±7.5 years old), prostate sample taken after surgery (PCa-s) (n=38, mean age 66.05±5.5 years old), comparatively with control subjects (n = 12,
mean age 50±6.4 years old). The pathologic groups BPH, PCa-b, PCa-s were characterized previously by different clinical parameters, including PSA values and Gleason scores (data not shown). The blood serum was obtained by standard procedure after coagulation for 30 minutes at room temperature and centrifugation, 10 min at 2000 g.

Lipids were extracted according to Bligh and Dyer method (Bligh and Dyer, 1959). A volume of 0.1 ml serum was mixed with 0.2 ml methanol, then vortexed for 20 s, and 1.66 ml chloroform was added and vortexed for 20 s. Finally, a volume of 0.1 ml water was added to induce the phase separation and centrifuged at 8000 g for 10 min. The lipid-chloroform phase was collected and evaporated, then reconstituted in 500 µl of chromatographic eluent ACN/IPA/H2O (65:30:5, v/v/v) and ultrasonicated for 10 min. After filtration through 0.2 µm PTFE filters, the LC-MS analysis was performed.

Aliquots of 5 µl of each extract were subjected to HPLC-MS on a Bruker Daltonics MaXis Impact equipment with a Thermo Scientific HPLC UltiMate 3000 system including a quaternary pump delivery system Dionex UltiMate and MS detection. The separation was done on a C18 reverse-phase column [5µm, 2.1 x 100 mm], (Acclaim, Dionex) maintained at 55°C.

The mobile phases were: A: water:acetonitrile (60:40) containing 0.1% formic acid and 10 mM ammonium formate and B: isopropyl alcohol:acetonitrile (90:10) containing 0.1% formic acid and 10 mM ammonium formate. The gradient used: 75% A: 25% B followed by linear gradient to 50% A: 50% B at 4 min, then a gradient from 50% A to 3% A: 97% B at 19 min, isocratic on 3% A: 97% B for 4 min and then returned to the initial condition 75% A: 25% B at 24 min for 4 min. The flow rate was 0.260 ml/min.

Mass spectrometry was performed on a Bruker Daltonics MaXis Impact Q-TOF operating in positive ion mode (ESI+). The mass range was set between 50-1000 m/z. The nebulizing gas pressure was set at 2.8 bar, the drying gas flow at 12 L/min, the drying gas temperature at 300 °C. Before each chromatographic run, a calibrant solution of sodium formate was injected.

TofControl 3.2 and Data Analysis 4.2 (Bruker Daltonics) software were used for the control of the instrument and data processing.

The algorithm Find Molecular Features (FMF) was applied on data in Data Analysis software and then Profile Analysis 2.0 (Bruker) for data pre-processing, alignment, bucketing (retention time range from 1’ to 28’ was used for bucket generation) and normalization (Sum of bucket values in analysis algorithm) was done to obtained proper matrix for biostatistics and bioinformatics analysis. Advance bucketing was done using the parameters obtained automatically from the time alignment. Only metabolites detected in more than 80% of the samples were selected to obtain the

Fig.1. Comparative image of Dissected chromatograms of serum samples from C (control), PCa-b (Biopsy cancer), PCa-s (Surgery cancer) and BPH.
matrix utilized for the unsupervised biostatistics and multivariate analysis, by Principal Component Analysis (PCA) and Cluster Analysis (CA). The Unscrambler X 10.1 (CAMO Software AS, Norway) software was also used, importing the matrix data and applying the multivariate analysis like PCA and CA. The distance method used for CA was Euclidean and the linkage method was Average.

RESULTS AND DISCUSSION
Chromatographic profile of serum lipids

Fig.1 shows the fingerprints of the blood serum lipid extracts, comparatively, from C (control), PCa-b (Biopsy cancer), PCa-s (Surgery cancer) and BPH patients.

The number of lipid molecules which were separated by HPLC ranged from 62 (BPH) to 123, separated in 23-25 min. Around six groups of molecules were separated, according to their polarity and molecular weight: very polar lipids and non-lipid molecules from min 1 to 5, polar lipids from min 6 to 9 (group 2), semipolar lipids from min 12-15 (group 3), neutral lipids from min 12 to 16 (group 4) and nonpolar lipids from min 20 to 23 (group 5). The identification of these lipids was done by comparing their ESI + m/z values with the values registered in Lipidomics Gateway (http://www.lipidmaps.org/) and Human Metabolomic Data Base (http://www.hmdb.ca/).

Nontargeted, unsupervised analysis of data by Principal Component Analysis

Using the Uncrambled software and the optimized parameters for data processing (see Materials and Methods), the statistics applied for data inputs was Principal Component Analysis (PCA). In Fig. 2 there are shown the PCA scores (upper graphics) and loadings (down graphics), to discriminate between biopsy cancer vs Control (a), surgery cancer vs Control (b), Hyperplasia vs Control (c).

Fig. 2a shows 2 subgroups of biopsy cancer patients, one group having a fingerprint close to controls (marked with red circles) and one subgroup significantly different. The loadings graphic shows that 3 molecules with m/z values of 282.281, 520.344 and 524.345 may be responsible for these differences.

Fig. 2b shows also 2 subgroups, but the second one less abundant, of surgery cancer patients, one group having a fingerprint close to controls (marked with red circles) and a secondary, less abundant subgroup significantly different. The loadings graphic shows that molecules with m/z 282.281, 520.344 and 524.345 may be responsible for these differences.

Fig. 2c shows 2 subgroups of hyperplasia patients, one group having a fingerprint close to controls (marked with red circles) and one subgroup significantly different. The loadings graphic shows that molecules with m/z...
values of 256.265, 381.300, 353.268, 520.344, 522.360 and 524.345 are responsible for these differences.

Fig. 2c shows also 2 subgroups, a first group less abundant, having a fingerprint close to controls (marked with red circles) and a secondary, less abundant subgroup significantly different. The loadings graphic shows that molecules with m/z values of 256.265, 282.281, 353.268, 520.344, 522.360 and 524.345 are responsible for these differences.

Finally, according to these data eleven molecules were selected as potential biomarkers, in all cases three similar molecules with m/z values of 520.344, 522.360 and 524.345 (corresponding to 3 successive C18-lysophosphatidyl cholines) were responsible for the discriminations between the pathologic and control groups. The molecules with m/z=282.281 were different in biopsy and

Fig. 3. Cluster dendogram based on average linkage method using Squared Euclidean distance of biopsy group (CB) and controls (M).

Fig. 4. Cluster dendogram based on average linkage method using Squared Euclidean distance of surgery group (CO) and controls (M).
hyperplasia groups, while molecules with m/z = 256.265, 381.300, 353.268 were significantly different in surgery cancer group vs controls.

**Nontargeted, supervised interpretation of data: Cluster Analysis**

The Hierarchial Cluster Analysis processed the data obtained from FMF matrix considering an advanced bucketing. According to Fig.3, for biopsy cancer (marked CB) two clustering groups were obtained, in accordance with the PCA data (Fig.2a). For controls, a good clustering was found also, excepting M10 and M11.

According to Fig.4, for surgery cancer group (marked CO) there were found more dispersed clustering groups, the CO samples being clustered in four subgroups.

According to Fig.5, for hyperplasia group (marked H) there were found more dispersed clustering groups, spread among the controls. The significance of this clustering is that not so many statistical significant differences can be found between the hyperplasia and control groups.

**Identification of specific lipid biomarkers of the pathologic groups**

Using the untargeted biostatistics processed by Profile Analysis, there were identified

**Fig. 5.** Cluster dendogram based on average linkage method using Squared Euclidean distance of Hyperplasia group (H) and controls (M).

**Tab. 1.** Tentative assignment of main molecules corresponding specifically to pathologic groups (for abbreviations, see the text).

<table>
<thead>
<tr>
<th>m/z</th>
<th>Tentative assignment</th>
<th>Specific to</th>
</tr>
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<tbody>
<tr>
<td>316.322</td>
<td>Eicosanoic acid or decanoylcarnitine</td>
<td>PCa-s</td>
</tr>
<tr>
<td>369.352</td>
<td>Tetracosanoic acid</td>
<td>PCa-b, BPH</td>
</tr>
<tr>
<td>415.211</td>
<td>Ascorbyl palmitate</td>
<td>PCa-s, PCa-b, BPH</td>
</tr>
<tr>
<td>432.240</td>
<td>N-stearoyl phenylalanine</td>
<td>PCa-b</td>
</tr>
<tr>
<td>607.570</td>
<td>DG(16:0/0:0/18:2n6)</td>
<td>PCa-b</td>
</tr>
<tr>
<td>666.627</td>
<td>PS(0-16:0/12:0)</td>
<td>PCa-b, BPH</td>
</tr>
<tr>
<td>582.582</td>
<td>Ceramide(d14:1/22:0(2OH)) or Ceramide (d16:1/20:0(2OH))</td>
<td>PCa-s</td>
</tr>
<tr>
<td>806.573</td>
<td>PC(16:0/22:6) or PC(18:1/20:5) or PC(18:2/20:4)</td>
<td>PCa-s</td>
</tr>
<tr>
<td>801.561</td>
<td>TG(16:1/14:0/18:2) or TG(16:0/14:0/18:3)</td>
<td>BPH</td>
</tr>
<tr>
<td>760.589</td>
<td>PC(16:0/18:1) or PC(16:1/18:0)</td>
<td>BPH</td>
</tr>
<tr>
<td>810.604</td>
<td>PC(18:0/20:4) or PC(18:1/20:3) or PC(18:2/20:2)</td>
<td>BPH</td>
</tr>
</tbody>
</table>
the specific molecules, related to a specific pathology, based on the peak areas and m/z values (Table 1). The molecules with m/z values of 316.322 (corresponding to eicosanoic acid or decanoylcarnitine), 582.582 (ceramides) and 806.573 (corresponding to PC(16:0/22:6) or PC(18:1/20:5) or PC(18:2/20:4) are specific to surgery cancer (PCa-s). The molecules with m/z values of 432.240 (N-stearoyl phenylalanine) and 607.570 (TG(16:0/0:0/18:2n6)) are specific to biopsy cancer (PCa-b). The molecules with m/z values of 801.561 (TG(16:1/14:0/18:2), TG(16:0/14:0/18:3)), 760.589 (PC(16:0/18:1) or PC(16:1/18:0), 810.604 (PC(18:0/20:4) or PC(18:1/20:3) or PC(18:2/20:2) are specific to hyperplasia (BPH). The molecules 369.352 (Tetracosanoic acid) and 666.627 (PS(0-16:0/12:0) are specific to groups of biopsy cancer (PCa-b) and hyperplasia (BPH). The molecule with m/z= 415.211 (Ascorbyl palmitate) is common to all groups.

CONCLUSION

By an advanced technique HPLC coupled with Mass Spectrometry and an optimized data processing and analysis we were able to separate and identify hundreds if lipid molecules in blood serum, as candidates for prostate pathologies (hyperplasia and cancer).

The statistical unsupervised analysis based on PCA scores and loadings, using the Uncrambled software, showed a good discrimination between the two cancer groups of patients (after biopsy and after surgery) and for benign hyperplasia patients against controls, based on the comparison of peak areas. The molecules responsible for such discriminations were identified to be mainly represented by lysophosphatidylcholines. By Cluster Analysis, the dendograms showed good statistical clustering of samples, especially for cancer patients against controls and less clustered for hyperplasia.

Using a Profile Analysis software, the identification of lipid biomarkers for specific pathologic groups was possible. Molecules belonging to phospholipid family and diacyl / triacylglycerides or ceramides or carnitines can be considered potential biomarkers for hyperplasia and prostate cancer.

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REFERENCES


