Early diagnosis biomarkers of prostate cancer by metabolomic analysis

(SUMMARY OF Ph.D. THESIS)

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AIM AND OBJECTIVES OF THE RESEARCH

The aim of the research was to identify early diagnosis biomarkers of prostate cancer by metabolomic analysis LC–ESI(+) QTOF-MS from human serum and urine samples.

The objectives of the research were represented by 3 independent experimental studies:
1. Untargeted metabolomic analysis realized by high performance liquid chromatography coupled with mass spectrometry - LC–ESI(+) QTOF-MS, used as rapid and global evaluation method of the biomarkers for early detection of prostate cancer in correlation with the concentration of prostate specific antigen (PSA).
2. Untargeted and targeted analysis used as qualitative and quantitative evaluation method of serum and urine metabolomic biomarkers, in order to differentiate patients with benign prostatic hyperplasia from patients with prostate cancer.
3. Biostatistic interpretation through comparative chemometry (Principal Component Analysis) of results obtained after untargeted and targeted analysis of serum and urine, in order to identify early diagnosis biomarkers of the prostate cancer.

Structure of the thesis:
The thesis is divided in two parts: the first one contains data from literature related to metabolomics – definitions and principles, the types of metabolomics: untargeted and targeted, study techniques used in metabolomics and metabolomic studies realized on serum samples and urine in prostate cancer (Chapter 1 and Chapter 2).
The second part is focused on personal contributions summarized in three experimental studies which include materials and methods used, as much as results and conclusions related.

The first part of the thesis is divided in two chapters:

**Chapter 1**:-summarizes the informations about metabolomics, general definitions and principles in this domain, the types of metabolomics: untargeted and targeted, as well as the analytical methods of study used in metabolomics.

**Chapter 2**: presents the literature studies run up to this moment in metabolomics from samples of blood an urine from patients with prostate cancer and description of clinically tests used in screening of prostate cancer: serum specific antigen.

The second part of the thesis is represented by personal contribution and includes 3 experimental studies:

**Chapter 3** presents the results obtained from untargeted LC–ESI(+)QTOF-MS metabolomic analysis applied on serum samples from normal patients and pathological patients.

**Chapter 4** shows the applicability of untargeted and targeted metabolomics, with identification of serum differentiation biomarkers of patients with benign prostatic hyperplasia and prostate cancer.

**Chapter 5** presents the comparative results obtained from analysis of urinary metabolomic profile, untargeted and targeted, differentiated for patients with benign prostatic hyperplasia and prostate cancer.

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PERSONAL CONTRIBUTION

**CHAPTER III. UNTARGETED LC–ESI(+) QTOF-MS METABOLOMIC ANALYSIS APPLIED ON SERUM SAMPLES FROM NORMAL PATIENTS AND WITH PROSTATE CANCER (STUDY I)**

The aim of the study was to use untargeted metabolomics by LC-QTOF-MS in order to identify and characterize small serum metabolites from patients with normal and pathological values of PSA and to evaluate possible correlations between PSA and selected biomarkers, for identification of metabolomic biomarkers relevant to early diagnosis of prostate cancer.

**III.1 MATERIALS AND METHODS**

**III.1.1 Patients**: a number of 100 patients test pattern were investigated by analysis of serum. 2 groups were formed based on PSA concentration. The first group of 50 patients (normal group) had values of PSA below 4 ng/ml and the second group had the PSA above 4 ng/ml.

**III.1.2 Blood Sample preparation**

Blood sera (100 samples) were collected by standard procedures, after clothing at room temperature for 30 minutes and centrifugation at 1000g for 15 minutes.

The PSA values were determined by the routine assay using electrochemiluminiscence and bioreaction with two mouse monoclonal antibodies, a
biotinylated monoclonal anti-PSA antibody 1.5 mg/l and monoclonal anti-PSA antibody labeled with ruthenium complex 1.0 mg/l.

For metabolomics analysis, the serum samples were diluted with methanol for protein precipitation, in a ratio (1:5), then vortexed, ultrasonicated at 4°C for 5 minutes and centrifuged at 15 000g for 15 minutes. The supernatant was collected, filtered through 0.2 μm filters and kept in the deep freezer until analysis.

III.1.3 LC–ESI(+)-Q-TOF-MS analysis

Aliquots of 5 μl of each supernatant were subjected to liquid chromatography separation, using a Thermo Scientific HPLC UltiMate 3000 system equipped with a quaternary pump delivery system DionexUltiMate 3000 (UHPLC+ focused), Acclaim C-18 column (3 m, 2.1 X 50 mm), autosampler and Dionex Ultimate 3000 photodiode array detector. Also the column temperature was set at 40°C.

The detection was made by mass spectrometry using a BrukerDaltonicsMaXis 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 bars, the drying gas flow at 8 L/min, while the drying gas temperature was set at 180 °C. Before each run, a calibrant solution of sodium formate was injected. The control of the instrument and data processing were done using TofControl 3.2 and Data Analysis 4.1 (BrukerDaltonics) softwares. The mobile phases used were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The flow rate was set at 0.5 mL·min⁻¹. The gradient elution initial conditions were 1% B to 15% B, with a linear gradient from 0 to 3 min, followed by linear gradient to 50% B at 6 min, linear gradient to 95% B at 9 min, isocratic on 95% B for 6 min and then returned to initial conditions at 15 min and kept isocratic on 1% B for 5 min.

III.1.4 Statistical analysis

The Principal Component Analysis (PCA) method was used to discriminate between normal and pathological samples. PCA was performed using the Profile Analysis software (Bruker, Daltonics).

III.2 RESULTS AND DISCUSSIONS

III.2.1 Preliminary evaluation of patients PSA status

Table 1. Includes the mean values of PSA obtained for each age category of patients.

<table>
<thead>
<tr>
<th>PSA values (ng/ml)</th>
<th>0-4</th>
<th>4-10</th>
<th>10-100</th>
<th>100-1000</th>
<th>&gt;1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>50</td>
<td>24</td>
<td>15</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>Mean Age ± SD</td>
<td>54±2.35</td>
<td>63±3.65</td>
<td>71.66±3.8</td>
<td>60.12±3.02</td>
<td>62.33±4.30</td>
</tr>
<tr>
<td>Mean PSA ± SD</td>
<td>1.009±0.001</td>
<td>3.53±0.32</td>
<td>44.10±2.5</td>
<td>595.5±42.8</td>
<td>1133±144.3</td>
</tr>
</tbody>
</table>

PSA values from 0 to 4 ng/ml were considered normal, while the PSA values between 4-10 ng/ml were considered to characterize benign prostate hyperplasia or...
prostatitis (Hankey et al., 1999). The PSA values higher than 10 ng/ml were considered to be caused by prostate cancer, at different stages.

### III.2.2 LC-QTOF (ESI +) MS analysis

The comparative base peak chromatograms (BPC) of a typical normal vs pathological serum sample is shown in Fig. 1 A and B, respectively. A number of 14 main peaks were identified for normal serum and 20 main peaks for the pathological sample. Peak identification was made according to their retention times, the released ions of protonated molecules [M + H]+ and literature.

![Comparative base peak chromatograms of a typical normal serum with PSA<4ng/ml (A) and a pathologic serum with PSA=728 ng/ml(B)](image)

#### III.2.3 Biostatistics

As illustrated in Fig. 2, the PCA analysis generated comparisons of PC1 and PC2 accounted 97.5% of the total variance, considering the scores (based on score plots). The score plot shows the grouping of samples according to the grouping of PSA levels in normal and pathologic ones. A great variation was observed inside the pathological groups. There were no significant discriminations between groups of 4-10 and 10-100 PSA ranges, but slight discrimination against the most pathological group (100-1000), located at lower PC2 values. Furthermore the samples with PSA > 1000 ng/ml were considered outliers and excluded from the PCA model. The normal group presented high homogeneity since almost all samples were clustered together in two groups.
Figure 2. The score plot of the PCA analysis of MS data from serum samples: 50 normal PSA values and 50 pathological values (clustered in 4 groups, as presented in the figure).

The distribution of samples was made almost exclusively along PC1 axis. One can see that molecules having m/z values of 496.3558, 524.389, 520.358 and 522.374 are mainly responsible for the discrimination. These molecules are lysophosphatidyl choline derivatives like LPC(O-16:0/O-1:0), LPC(18:1/2:0) or PS(18:1(9Z)/0:0), LPC(18:2(9Z,12Z)/0:0 and their isomers and LPC(O-18:1(11Z)/2:0), respectively.

III.3.1 CONCLUSIONS

A cohort of 100 patients with different Prostate Specific antigen values were investigated by untargeted metabolomics. The serum small metabolite profile determined by LC-QTOF(ESI+)MS allowed the identification of specific biomarkers, for normal and four pathologic groups, having PSA values from 4 to >1000 ng/ml.

The major molecules identified in the samples were lysophospholipids, mainly Phosphatidyl choline derivatives, having m/z values from 496 to 524, like LPC(O-16:0/O-1:0), LPC(18:1/2:0) or PS(18:1(9Z)/0:0), LPC(18:2(9Z,12Z)/0:0 and their isomers and LPC(O-18:1(11Z)/2:0), respectively. Also, small molecules (free fatty acids and prostaglandin derivatives) were identified and are significantly different in pathologic vs normal serum samples. Generally the pathologic samples had increased concentrations of all the above mentioned molecules.

These results were published in Bulletin UASVM Food Science and Technology:

Ramona Maria MAXIM, Carmen SOCACIU, Anca Dana BUZOIANU, Raluca Maria POP, Florina ROMANCIUC, Untargeted LC-QTOF (ESI+) MS Analysis of Small Serum Metabolites Related to Prostate Cancer and Prostate Specific Antigen, Bulletin UASVM Food Science and Technology 71(2) / 71(2)/2014, 165-17.

CHAPTER IV. UNTARGETED AND TARGETED METABOLOMICS FOR IDENTIFICATION OF SERUM BIOMARKERS OF BENIGN PROSTATIC HYPERPLASIA AND PROSTATE CANCER (STUDY 2)
The aim of the study was to use untargeted and targeted metabolomics for the analysis of 133 serum samples for biomarkers identification to differentiate and compare patients with hyperplasia from patients with prostate cancer.

**IV.1 MATERIALS AND METHODS**

Patients and clinical parameters determined

In this study were included 4 groups of patients from which were collected serum samples. They were classified in: patients group diagnosed with benign prostatic hyperplasia (group IV), patient group with supposed prostate cancer, before biopsy (V-CB), patients with confirmed prostate cancer, before surgery (VI-CO) and the control group which include healthy patients (III-M).

For metabolomics analysis, the serum samples were diluted with methanol for protein precipitation, in a ratio (1:5), then vortexed, ultrasonicated at 4°C for 5 minutes and centrifuged at 15 000g for 15 minutes. The supernatant was collected, filtered through 0.2 μm filters and kept in the deep freezer until analysis.

**IV.1.2 LC–ESI(+)-Q-TOF-MS analysis**

Aliquots of 5 μl of each supernatant were subjected to liquid chromatography separation, using a Thermo Scientific HPLC UltiMate 3000 system equipped with a quaternary pump delivery system DionexUltiMate 3000 (UHPLC+ focused), Acclaim C-18 column (3 μm, 2.1 X 50 mm), autosampler and Dionex Ultimate 3000 photodiode array detector. The column temperature was set at 40°C.

The detection was made by mass spectrometry using a BrukerDaltonicsMaxis 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 bars, the drying gas flow at 8 L/min, the drying gas temperature at 180 °C. Before each run, a calibrant solution of sodium formate was injected. The control of the instrument and data processing were done using TofControl 3.2 and Data Analysis 4.1 (BrukerDaltonics) softwares. The mobile phases used were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The flow rate was set at 0.5 mL·min⁻¹. The gradient elution initial conditions were 1% B to 15% B, with a linear gradient from 0 to 3 min, followed by linear gradient to 50% B at 6 min, linear gradient to 95% B at 9 min, isocratic on 95% B for 6 min and then returned to initial conditions at 15 min and kept isocratic on 1% B for 5 min.

**IV.2 RESULTS AND DISCUSSIONS**

**IV.2.1 Untargeted metabolomic analysis. LC-MS fingerprint of serum samples**

Separation and identification of molecules from serum of the control patients in comparison with pathological patients: hyperplasia (group IV), cancer before biopsy (group V), cancer before surgery (group VI) was done taking the Base Peak Chromatograms and Dissect chromatograms for each study group.

From Dissect chromatograms and tables below, were identified major signals, common molecules or specific molecules for each retention time. Manual alignment was done for each study group dependent of m/z value, starting at minute 6 to 17. It was considered that at the minute 1, it was not possible the identification of the molecules (death volume of the column), while at the interval 1 to 6 minute, the separated molecules are insignificant, minor, it is just a washing step of the column.

Biostatistic analysis of the samples: Principal Component Analysis (PCA)
From the m/z values obtained it was possible to identify biomarkers and to established the homogeneity of each group of study (similarities)

IV.2.2 Targeted metabolomic analysis, with identification of quantitative differences of some biomolecules, between III-VI groups, based on RT values

To evaluate the quantitative differences between groups, it was taking in consideration significant retention time intervals (minutes), where were identified specific compounds, in addition the mean and standard deviation were calculated, as it showed in Tabel 2.

<table>
<thead>
<tr>
<th>RT intervals (min)</th>
<th>Compounds categories</th>
<th>Mean values ± standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>M</td>
</tr>
<tr>
<td>8.1 + 8.2</td>
<td>Testosterone</td>
<td>34896542.59±26470337.1</td>
</tr>
<tr>
<td>De la 8.2 la 9.9</td>
<td>Aa and peptides</td>
<td>56789817.72±61040561.42</td>
</tr>
<tr>
<td>De la 9.5 la 11.1</td>
<td>Lyzo PC and other lyso</td>
<td>109334969±134379209</td>
</tr>
<tr>
<td>11.1; 12.4 ; 12.5; 13.8;13.9+ 14 ;+ 14.2</td>
<td>DG + MG</td>
<td>125786092.2±87006755</td>
</tr>
<tr>
<td>De la 11.6 la 11.9</td>
<td>Prostaglandin es</td>
<td>100718008.7±68248000.5</td>
</tr>
<tr>
<td>12.8; 14.1 - 15.6</td>
<td>PC</td>
<td>45276788.03±66164509.02</td>
</tr>
</tbody>
</table>

The mean values were represented for each category of compounds. It was identified compounds such as ceramide, phosphatidicacolines PC (38:6), PC(39:2), PC(40:6) with increased values at the study group cancer before biopsy (CB). Ceramides and LPC (18:) can be utile biomarkers in diagnosis of benign prostatic hyperplasia.
IV.3 CONCLUSIONS

- M/z values were manually aligned depending on RT from Dissect chromatograms.
- It was represented by statistical analysis – untargeted PCA, the grouping of the samples based on m/z values and signals area, performing a qualitative analysis and showing the homogeneity of the III-VI groups.
- The molecules were separately identified, to retention times intervals and individual and the mean and standard deviation was calculated, the represented graphic to find specific biomarkers and their modifications depending on pathology.
- The results and their statistical significance were discussed, with relevance for diagnosis of benign prostatic hyperplasia and prostate cancer in different cases (before biopsy and before surgery).
- It was identified candidate biomarkers for early diagnosis differentiated H-CB-CO, molecules like lysophospholipids 18:0, PC 16:1, PC 18:1, PC 18:2, butenil-carnitines, galactosylceramide C18:1/24:1, tromboxanes and pregnenolones.

CHAPTER V. UNTARGETED AND TARGETED METABOLIC URINE PROFILE ANALYSIS, DIFFERENTIATED AT PATIENTS WITH BENIGN PROSTATIC HYPERPLASIA AND PROSTATE CANCER

The aim of the study was to use metabolomic urine analysis of patients with benign prostatic hyperplasia and prostate cancer to find specific biomarkers.

V.1 Patients analyzed

In this study a number of 39 urine samples provided from 4 groups of patients were analyzed: 9 samples from patients with benign prostatic hyperplasia (group IV-H), 12 samples from patients with cancer before biopsy (group V-CB), 8 samples from patients with prostate cancer confirmed (group VI-CO) and at last 10 samples from the control group, healthy patients (group III-MU).

V.1.2 Urine samples processing

The urine samples from these 4 studied groups were collected by standard procedure, in sterile recipients, with the informed consent of the patient. For the metabolomic analysis, urine samples, were diluted with methanol for protein precipitation, in a ratio (1:5), then vortexed, ultrasonicated at 4°C for 5 minutes and centrifuged at 3000rpm for 5 minutes. The supernatant was collected, filtered through 0.2 μm filters and kept in the deep freezer until metabolomic analysis.

V.1.3 LC-ESI(+)-Q-TOF-MS analysis

Aliquots of 5 1 of each supernatant were subjected to liquid chromatography separation, using a Thermo Scientific HPLC UltiMate 3000 system equipped with a quaternary pump delivery system DionexUltimate 3000 (UHPLC+ focused), Acclaim C-
V.2 RESULTS AND DISCUSSIONS

V.2.1 Determination of the specific LC-MS fingerprint of urine samples from the control patients with benign prostatic hyperplasia and prostate cancer (CB and CO groups)

It was realized the separation and identification of molecules from urine samples of the control group (M) in comparison with pathological patients: hyperplasia (H), cancer – surgery (CO), cancer biopsy (CB) and processing of the Base Peak Chromatograms and Dissect chromatograms for each study group.

From the comparative analysis of the chromatographic profiles of the urine samples of controls and patients groups: H, CB, CO, we conclude the following:

- The chromatographic profiles generated at control group are loaded with heterogen molecules in comparison with the pathological groups, which have more molecules (180-200 molecules).
- The samples were characterized with major molecules in the intervals: 4.5-4.8 min, 9.2-9.4 min, 10.1-10.2 min, 11.1-11.7, 12.5, 16-17.1 min
- m/z values range for this molecules is between m/z=114 and m/z 425. It was not identified more bigger molecules, also as minore molecules we can identify m/z values between 450-780.

V.2.2 Untargeted biostatistic analysis – Principal Component Analysis: evaluation of the homogenicity of the samples and differencies identification between the groups of patients and identification of the m/z values which differentiate the samples

Through the untargeted analysis of the samples, using the software Bruker-Profile Analysis, based on statistic buckets, it was identified groups well defined and isolated samples.

Using the software Unscrambler 10.X we can conclude:

1. The statistics, based on differences between m/z values, show a good homogenicity of the m/z values from MU group/ PCA analysis bidimensional and tridimensional emphasises which is the retention time intervals that differentiate the samples from the control group (M). The sample MU10 has significant different values related to the MU1-9 samples.
2. The statistics based on differences between the peak are quantitative, they show different values of the areas, highlighting the fact that quantitative differences are more important than qualitative regarding this group’s case. As far as this group is concerned, significant differences are showed for MU1 sample to the rest of the samples.

3. Statistics based on differences between m/z values for the group H shows a heterogenicity of the m/z values, the samples H4, H6, H10 being less significant to the rest of the samples.

4. PCA and PCR (Principal Component Regression) showed 3 subgroups in the case of the H group: samples H4, H6, H10 constitute a group with small values of the m/z, while the rest of the sample, with the exception of H15, which is more homogen. Cluster analysis shows a segregation, but it can be also considered homogen. Quantitative statistics, based on peak area differences, shows a good homogeneity, with values decreased in the case of 3 samples: H4, H6 and H10.

5. Statistics based on differences between m/z values, shows heterogenicity of m/z values, CB2, CB3, CB14 and CB 17 having significant bigger values than the rest of the samples. PCA bidimensional and tridimensional analysis shows which are the retention time intervals that differentiate the samples from the H group (those with RT=13.1, 14.4).

6. Cluster analysis shows a weak segregation of the samples, this can be considered homogeneity. Quantitative statistical analysis based on peak area differences shows a good homogeneity, except CB13 and CB14 samples.

7. Statistics based on differences between m/z values for the CO group shows a good homogeneity of the m/z values. Bidimensional and tridimensional PCA analysis show which are the retention times that discriminate the samples from the CO group. The samples can be considered homogen. Statistics based on peak area differences show a good homogeneity, except CO22 and CO28, which have values increased of the m/z.

V.2.3 Targeted metabolomic analysis: identification of the individuals molecules from urine, specific to the groups of patients III-IV

- Molecules 1,3,4 and 10 significantly increased CB and CO groups and can constitute good candidates for real biomarkers of evolution from hyperplasia to prostate cancer
- To highlight hyperplasia are utile data concerning the evolution of the molecules: pregnenolonesulphat, alpha-N-phenilacetyl-L-Glutamine (increasing) dihydrocortisol, tetrahydrocortison (increasing), MG(18:3/0:0/0:0), prostaglandine D2, E2 (increasing), L-tryptophan, prolinebetaine, indoxylsulphat and N-phenilacetylpyroglutamic, S-3-oxodecanoil cysteamine (decreasing)
- Evolution from CB and CO can be evidentiate by molecules like: linoleylcarnitine and MG(20:3/0:0/0:0)
- Hippuric acid and guanosine increase significantly at CO group, but not at CB group
Pregnenolonsulphate decrease significantly at CO and CB in comparison with M and H groups
Carnitine derivates increas in all the cases at CO and CB

V.3 CONCLUSIONS

1. After a manual alignment in Excell of m/z value depending of RT, it was identified those molecules which had majore peaks, repeatable in more samples, with superior areas. In this way, it was filtered, from a total of 150 molecules/samples, 23 molecules and finally 14 molecules identified in Human Metabolomic Database
2. It was performed targeted metabolomic analysis (PCA, PCR and Cluster analysis), to the 14 identified molecules in the 4 studied groups: M, H, CB and CO.
3. Molecules like hippuric acid, guanosine, N-phenilacetylpyroglutamic, S-3-oxodecanoil cysteamine, butenylcarnitine, serotinine, mevalonic acid and D-2-hydroxyglutaric increases significantly and can be considered real biomarkers of evolution from hyperplasia to prostate cancer
4. To evidentiate a pathologic process beginning with hyperplasia molecules like pregnenolonesulphate, alpha-N-phenilacetyl-L-Glutamine, dihidrocortisol and tetreahidrocortisol have to be followed.
5. Evolution from hyperplasia to CB and CO can be showed through linoleylcarnitine and MG(18:3/0:0/0:0), MG(0:0/18:3/0:0), prostaglandine D2, E2

ORIGINALITY AND INNOVATIVE CONTRIBUTIONS OF THE THESIS

For the first time in Romania was done a preliminary study of untargeted metabolomics of prostate cancer and identification of candidate biomarkers, in correlation with PSA and with applicability in clinical oncology.

The methodology applied in the 3 studies presented, includes original protocols together with adapted ones after standardized international methods, which were used also at preprocessing part of the samples and at LC-MS analysis and statistical interpretation of the dates.

We conclude that the results obtained can be an important phase in realizing a database for identification of prostate cancer, the first one of this type in Romania.