Diagnosis of Urological Cancer by $^1$H NMR based Metabonomics Urinalysis: a Pilot Study

ABSTRACT

Aims: The most prevalent urological malignancies are prostate cancer (PC), bladder cancer (BC) and renal cancer (RC). The diagnosis of each of these diseases is conducted, in most cases, invasively and each procedure may lead to complications. The method of metabonomic spectrometry by nuclear magnetic resonance of hydrogen ($^1$H NMR) provides pathways of diagnostic information that can identify pathologies without invasive procedures. The possibility of using this method for the diagnosis of those cancers by a single sample of urine has not been described yet.

Study design: prospective, observational.

Place and duration of the study: Department of Urology and Department Fundamental Chemistry of Universidade Federal de Pernambuco (UFPE), between July of 2015 to February of 2016.

Methodology: A sample of 3ml of urine was collected from 25 volunteers distributed into 4 groups: a control group (07 volunteers), a PC (08 volunteers), a BC (05 volunteers), and an RC (05 volunteers). All samples underwent $^1$H MRI to generate spectra. A multivariate statistics analysis for the development of metabonomic models and comparison analysis groups was performed. Results: These models showed a slight separation between the control group and each of the three groups of patients with oncological diseases. For the elaboration of the definitive models it was necessary to incorporate the volunteers of the BC and RC into one group (BC/RC). The metabonomic method when compared to control group, shown sensitivity of 90.9%, specificity of 100%, 100% PPV and NPV of 85.7% for CB/CR and sensitivity, specificity, PPV and NPV of 100% for the PC.

Conclusion: This pilot study demonstrates that the method is feasible with easy execution, showing simplicity besides being not invasive and allowing the diagnosis of oncological diseases with a single urine collection.

Keywords: metabonomics, nuclear magnetic resonance, prostate cancer, bladder cancer, kidney cancer, diagnosis.

1. INTRODUCTION:
The most common urological malignancies are prostate cancer (PC), bladder cancer (BC) and renal cancer (RC)(1). These cancers have aggressive treatments and are related to a significant loss of quality of life of patients. The diagnosis of each of these diseases is conducted, in most cases invasively, and each procedure includes complications (2-7).

For these reasons, the search for non-invasive diagnostic methods is being studied. Nuclear magnetic resonance of proton ($^1$H NMR) spectra of the metabonomics routes can provide information for pathology diagnosis without the need for invasive procedures(8).

This methodology is based on the principle of homeostasis. That is, if a living being is exposed to stress, disease or some chemical agent, the response is in an attempt to neutralize this external action, changing the concentration and endogenous metabolite flow, breaking the balance, and triggering extracellular environment adjustments in order to maintain stability. It is this change in
the metabolic profile that can be evaluated by MRI spectroscopy (8-10), expecting these spectra to
identify a particular profile for each specific aggression.

Recently, there are many reports of studies that use metabonomics/metabolomics strategies
for clinical diagnosis of several diseases, as liver, lung, kidney, breast, ovarian and colorectal cancers
or not malignant conditions (11-17)

The possibility of using this method for the diagnosis of those cancers by a single urine
sample has not been described yet.

2. MATERIALS AND METHODS:

This study was submitted, approved by the ethical committee of the institution and
conducted in accordance with the Declaration of Helsinki. The patients were selected from the
outpatient clinic of the Department of Urology. All patients were approached, and voluntarily decided
to participate signing the free and informed consent. (protocol number: 33003214.3.0000.5208).

For the major study the total number of volunteers calculated was 120, 30 in each the
experimental group and 30 in the control. This number was established as follow: the margin of error
(5%), the confidence level (95%). The expected accuracy for determining the results taken as true
based on previous research using metabonomics, whose expected ratio for positivity was 94%(18).
The calculation was based on a normal distribution, according to the formula described below.

\[ C = Z \times Z \times (P(1-P)) / (D \times D) \]

Where P refers to the proportion expected; D, the semi amplitude of the confidence interval
(CI); and Z [standardized standard curve] is equal to 1.96, thus the 95% CI(19).

The number of volunteers for this pilot study was established as a fraction of 20% of the total
number of participants. In this case, the number of participants in the pilot would be at least 24(19).
The volunteers were allocated into four groups: Control (without any malignances); PC (with prostate
cancer); BC (with Bladder cancer) and RC (with renal cancer)

Volunteers for the control group were selected by the same criteria as for the study group and
had no history of urological disease. Volunteers with PC had all been diagnosed by transrectal
prostatic biopsy and had not yet started any treatment.

Volunteers with BC who had undergone immunotherapy with bacillus Calmette-Guérin or
mitomycin C, as well as other specific treatments were excluded from the study.
Volunteers with RC were those who had a confirmed diagnosis of clear cell carcinoma by pathological examination in the postoperative period. Those volunteers in RC group diagnosed with urothelial carcinoma were excluded.

All volunteers ages were submitted to normality test and one-way ANOVA using GraphPad prism® 4.0 statistical package, for comparison among groups.

The samples were obtained from urination. Volunteers provided 3ml of urine for analysis that were cooled for storage and sent to the Fundamental Chemistry Department (DQF) to undergo to $^1$H NMR. The spectra were obtained using a mixture of 400 µL of natural urine and 200 µL of buffer solution of phosphates ($\text{Na}_2\text{HPO}_4$/NaH$_2$PO$_4$ 0.2 mol L$^{-1}$) to avoid pH variation.

After homogenization, the solution was transferred to a NMR tube with an inner diameter of 5 mm and introduced into a spectrometer (VNMRS400), operating at 400 MHz to $^1$H nucleus. It was used a pulse sequence with pre-saturation to suppress the water signal in the spectrum. The following parameters were used: spectral window equal to 6.4 kHz, acquisition time equal to 2.56 s, 90° radiofrequency (RF) pulse, saturation delay equal to 2.0 s and 64 repetitions. All spectra were processed using line broadening equal to 0.3 Hz and the signal at 3.06 ppm, assigned to methyl group of creatinine, was used as the chemical shift reference.

The baseline and phase distortions were adjusted manually. $^1$H NMR spectra (δ 0.0 to 10.0 ppm) were automatically reduced to 154 parts (bins) of equal length (δ 0.05 ppm), excluding the region between δ 4.2 and 6.4 ppm, to eliminate the signals from water and urea. Binned data were used to build a matrix where in the lines are the samples and in the columns are the variables (20-22). In addition, the spectra were normalized by sum.

Due to the complexity, the multivariate statistical technique was used to resize the data without loss of results, being unsupervised or supervised methods.

Principal Components Analysis (PCA) is an unsupervised method where the original data matrix is projected into a subspace defined by linear combinations with maximum variance. The first major component obtained is the axis that describes the maximum possible variance in the original multidimensional space and the second is the orthogonal component. The junction of these axes provide the best representations possible in terms of biochemistry.

The Partial least squares discriminant analysis (PLS-DA), its orthogonal variant (OPLS-DA) and linear discriminant analysis (LDA) are supervised methods which make it possible to analyze the
spectra in a simple, fast and reproducible. PCA, PLS-DA and OPLS-DA were performed using
MetaboAnalyst Platform (online), while LDA was performed using Statistica® 10.0 software (23-25).
The selection of variables to build the LDA models was performed using Wilks’ Lambda. For Prostate
Cancer Model were used four variables, while for BC/RC Model were used five variables. The models
were validated by leave-one-out-cross validation (LOOCV)(26).

Once the inferences were created, metabonomic models calculated for sensitivity, specificity,
and positive predictive value (PPV) and negative predictive value (NPV) were built.

3. RESULTS AND DISCUSSION:

A total of 25 volunteers from the outpatient clinic were distributed into the following groups: a
control group with healthy volunteers (07); Prostate cancer (PC) with 08 volunteers; Bladder cancer
(BC) with 05 volunteers and Renal Cancer (RC) with 05 volunteers.

The control group consists of men aged between 60 and 67 years (mean: 62.4 SD: 2.1
years); PC with men aged between 61 and 69 years (mean: 65.2; SD: 3.0 years); BC with 02 men
aged between 70 and 76 years and three women aged between 57 and 75 years (total group mean:
69.4; SD: 7.5 years); and RC with one man and 35 years of age and four women aged between 54
and 64 years (total group mean: 53.0; SD: 10.9 years); BC/RC group (total group mean: 61.2 SD:
12.6 years). All results assume a normal curve and there were no statistical differences among
groups after the ANOVA procedure, considering p<0.05.

In the exploratory analysis (PCA), there was no separation of the spectra in any of the cases.
The samples were then subjected to the supervised methods PLS-DA, OPLS-DA and LDA. A
separation between groups BC and RC was observed. Due to the small sample size, it was not
possible to build a LDA based metabonomic model for each group, so the two groups were treated
together. In this case, samples from the BC and RC groups were pooled for analysis as if they were a
single group (BC/RC), thus creating an arrangement for analysis and comparison with the control
group. The split in the PC group allowed for the creation of a specific model, despite not having the
ideal sample size.

Two models were created: (1) Bladder Cancer and Renal Cancer (BC/RC) versus Healthy;
and (2) Prostate Cancer (PC) versus Healthy.

In this study, the variables are the integration areas under each signal. We defined that the
spectrum will be divided into regions (bins) equals to 0.05 ppm between δ 0.00 and 10.0 ppm,
excluding the region between δ 4.20 and 6.40 ppm. These signals can be attributed to metabolites presents into sample. The attribution was made using HMDB platform online (27).

When PC model is considered, the discriminatory bins are: δ 2.05, 2.50, 2.75 and 7.40 ppm. These signals can be attributed to citrate (δ 2.50 and 2.75 ppm) and hippurate (δ 7.40 ppm). We did not able of attribute the signal at δ 2.05 ppm. Already there are reports in the literature associating citrate and hippurate to diagnosis of prostate cancer (28), however, the results here presented were obtained in a simpler way.

To BC/RC model, the discriminatory bins are: δ 2.30, 2.50, 4.20, 4.30 and 7.40 ppm. Again, citrate and hippurate were identified in this group. Beyond them, we attributed also the signals at δ 2.30 ppm to acetone, δ 4.20 ppm to gluconate and δ 4.30 ppm to trigonelline.

For the BC/RC group, 17 urine samples were used, as follows: 7 healthy, 5 volunteers diagnosed with bladder cancer and 5 diagnosed with renal cancer. Below are presented the results of

OPLS-DA and LDA models, represented separately

Figures 1A and B. Scores plot: Metabonomics models for discrimination between bladder or renal cancer and healthy samples. (A) Score plot of OPLS-DA and (B) Classifications of urine samples by LDA formalism. Samples provided by healthy volunteers in the control group (healthy) and patients diagnosed with bladder or renal cancer (disease).

In order to obtain an alternative model for the classification of the samples studied, a statistical model based on the LDA was built. The results indicate that metabonomic model built obtained 100% PPV, NPV of 85.7%, 100% specificity and 90.9% sensitivity. These results are reported in Table 1.
Table 1 shows the PPV and NPV for samples together after cross analysis of variance, showing a clear separation between the control group and the BC/RC group.

<table>
<thead>
<tr>
<th>Classification Matrix - LDA</th>
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<tbody>
<tr>
<td>Class</td>
</tr>
<tr>
<td>Control</td>
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<tr>
<td>BC/RC</td>
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<tr>
<td>Total</td>
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<th>LDA with LOOCV (5 variables)</th>
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<tbody>
<tr>
<td>Class</td>
</tr>
<tr>
<td>Control</td>
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<tr>
<td>BC/RC</td>
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<tr>
<td>TOTAL</td>
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**Table 1.** Matrix of Leave-one-out cross-validation (LOOCV) obtained from LDA. Control vs BC/RC.

For PC group the model was constructed from 15 samples of urine, taken from 7 healthy volunteers and 9 diagnosed with prostate cancer. Similar to the above analysis, we built statistical models using the following arrangements: PCA, PLS-DA, and DA-OPLS LDA, with pre-processing and normalization by the total.

Figures 2 A and B discriminate among the groups without intersecting the areas, showing the R2Y (91.4%) and Q2 (15.8%) values. The results from the LDA indicate that the metabonomic model built correctly classified all samples. Thus, the values of PPV, NPV, specificity and sensitivity were equal to 100%. These results are shown in Table 2.

**Figures 2A and B.** Scores plot of OPLS-DA and (B) Classifications of urine samples by LDA formalism.

Metabonomics models for discrimination between prostate cancer and healthy samples. (A) Score plot of OPLS-DA and (B) Classifications of urine samples by LDA formalism.
<table>
<thead>
<tr>
<th>Class</th>
<th>Correct percentage</th>
<th>Control</th>
<th>PC</th>
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<tbody>
<tr>
<td>Control</td>
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<td>0</td>
</tr>
<tr>
<td>PC</td>
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<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>100%</td>
<td>7</td>
<td>8</td>
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LDA with LOOCV (4 variables)

<table>
<thead>
<tr>
<th>Class</th>
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<th>PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
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<tr>
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</tr>
<tr>
<td>TOTAL</td>
<td>100%</td>
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Table 2: Matrix of Leave-one-out cross-validation (LOOCV) obtained from LDA. Control vs PC.

The NMR spectra of biofluids can be evaluated quantitatively in relation to the physical shape of the spectrum and qualitatively in relation to the chemical structure of the substances composing the spectrum.

The aim of the qualitative evaluation is not so much the quantification of a substance but rather an analysis of the profile changes of the endogenous metabolites, producing a pattern for a particular change, which can then be diagnosed or classified, enabling a "fingerprint" of a particular disease or condition (29).

With respect to the composition of the sample, it must be taken into consideration that the selection of volunteers was not homogeneous for gender or age. The control group was composed exclusively of men perfectly matched for the PC group; but the match was weak for the BC and RC groups. The absence of women in the control group generates a confounding factor, especially for the metabolism inherent in women. Furthermore, there was a discrepancy in ages, most markedly in the RC group.

Therefore, a better distribution of volunteers with a larger control sample, including women and volunteers from other age groups, is needed.

To equalize the samples it was decided to create a single model, merging the BC and RC groups (BC/RC).

The results presented by the BC/RC spectra, although not a comparison with the other groups, indicate a promising way to discover the presence of malignant disease, as the control group was known to be healthy and the BC/RC group, although composed of volunteers with different diseases, was composed entirely of people with a malignant urological disease.

The sensitivity profile of 90.9%, specificity of 100%, 100% PPV and NPV of 85.7% for the model demonstrates its ability to diagnose these pathologies. Thus it reveals positive way to validate
the diagnosis method for renal and bladder cancer. Direct biopsy has values above 90% for bladder
and kidney cancer, but it is a result from a direct evaluation of tissue using an invasive method (30).

In the specific case of kidney cancer, biopsy guided by imaging, particularly CT, has a
sensitivity 97.7%, specificity of 100%, PPV and NPV of 100%(30), but is invasive. The values found in
the metabonomic model are close to those observed with biopsy, but with the great advantage of
resulting from a non-invasive method. There are no reliable publications for non-invasive diagnostic
methods for cancer of the kidney.

For the diagnosis of bladder cancer using non-invasive methods, such as urinary cytology,
positivity depends on the tumor differentiation grade, with a sensitivity ranging between 21-53%,
specificity between 81-95%, VPP between 71-90% and VPN between 57-67%(5). Other non-invasive
methods, for example, gene transcription of urinary sediment, have demonstrated sensitivity of 81.5%
and specificity of 91.3%(31). Goodison et al., using a complex statistical model, using an intersection
of 8 biomarkers with sensitivity 90% and specificity of 97% for the diagnosis of bladder cancer (32),
similar to the results found in this study.

For PC the values found were 100% sensitivity, specificity, PPV and NPV, which is higher
than all values from transrectal biopsy, which were sensitivity 86.4%, specificity 60.7%, PPV 63.3%
and NPV of 85% (3). This makes it a promising method for the diagnosis of this pathology, mainly
because it is completely noninvasive.

When compared to other non-invasive methods, Mengual et al. in 2016, found sensitivity of
77% and specificity of 67% with the gene transcription method of urinary sediments (33). These were
lower than values found in the present study.

Aggio et al. used a gas chromatography technique that achieved satisfactory results for
bladder and prostate cancers. Further, they included a group that had samples of these two types of
cancer, achieving sensitivity and specificity respectively of 96% and 100% for BC, sensitivity of 95%
and specificity of 96% for PC and 78% and 98% (both cancer group)(34). These results are similar to
those found in this study, mainly by trying to detect more than one pathology and achieving a high
degree of sensitivity and specificity.

Besides, to obtain 1H NMR spectra of biofluids as urine and serum, practically it is not
necessary great interventions in the sample preparation, as extraction or derivatization, and the
spectra are obtained in minutes. However, when are used chromatography based metabolomics,
there are necessary various interventions in the samples and the runs are performed in long periods, compared with NMR experiments(35).

Limitations of this study are mainly due to the number, homogeneity of the sample and the composition of the groups, especially the BC and RC groups. Despite this, the metabonomics seems to be an acceptable model for diagnosis of urologic cancer, being better in some cases than other invasive procedures, but further studies with larger samples are needed.

4. CONCLUSION:

In the present study metabonomics has shown to be a promising non-invasive method in the diagnosis of urological tumors in urine samples of patients with prostate, bladder and kidney cancer.

ETHICAL APPROVAL

This study was submitted, approved by the ethical committee of the institution and conducted in accordance with the Declaration of Helsinki. All participants signed the free and informed consent.

(protocol number: 33003214.3.0000.5208).

REFERENCES:


