**Diagnostic potential of major and trace elements in the serum of bladder cancer patients**

**Short title: Major and trace elements in bladder cancer patients**

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**Keywords**

Major and trace elements, bladder cancer, diagnosis, serum, ICP-OES, ICP-MS

**Abbreviations:** BCa: bladder cancer; ICP-MS: inductively coupled plasma mass spectrometry; ICP-OES: inductively coupled plasma – optical emission spectrometry

**Abstract**

Major and trace elements may play a role in the diagnosis of diseases. In this study, we investigated the concentration of 26 major and trace elements in the serum by inductively coupled plasma (ICP) – optical emission spectrometry (OES) and ICP-sector field-mass spectrometry (sf-MS). We analyzed the serum from a discovery cohort of 9 bladder cancer (BCa) patients and 12 healthy controls as well as from a validation cohort of 30 BCa patients, 39 non-tumor bladder patients (with acute and chronic inflammation) and 18 healthy controls. Patients were recruited after written consent was obtained at one medical center. Serum was prepared from peripheral blood prior to surgical treatment.

Differences in the levels of major and trace elements were determined by a nonparametric Mann-Whitney test and Kruskal-Wallis statistics. In the discovery cohort, we measured significantly increased levels of mercury, potassium, lithium, magnesium, nickel, and phosphorus and significantly decreased levels of sodium and selenium in BCa patients compared with healthy controls.

These findings were reassessed in our validation cohort. We measured significantly increased levels of boron, calcium, cadmium, copper, chromium, lithium, potassium, magnesium, manganese, nickel, phosphorus, sulfur, strontium, titan, vanadium, and zinc and significantly decreased levels of cobalt.

When we studied the concordance for the discovery and validation cohorts, five elements were detected as significantly increased in BCa patients compared with healthy controls: lithium, potassium, magnesium, nickel, and phosphorus. Interestingly, the levels of these five elements were also significantly increased in non-tumor bladder patients compared with healthy controls. In addition, potassium and phosphorus were significantly increased in non-tumor bladder patients compared with BCa patients.

In summary, we suggest that detection of the elements lithium, potassium, magnesium, phosphorus and nickel in the serum, with a focus on potassium, could be a new and promising tool for the early diagnosis of BCa and non-tumor bladder diseases.

**Introduction**

Urothelial carcinoma of the bladder (bladder cancer; BCa) is the ninth most frequently diagnosed cancer worldwide, with 439,000 new cases and 165,000 deaths estimated for 2012 [1]. Conventional diagnosis for BCa is still based on morphologic and pathologic criteria such as histology, tumor stage and tumor grade [2] An improvement in diagnosis was achieved by the application of fluorescence endoscopy [3]. Molecular studies have focused on pathohistological classification and outcome prediction of BCa patients by gene expression analysis of coding genes or noncoding genes and characterization of chromosomal aberrations or mutational alterations [2,4][5-8]. A noninvasive approach in studying urologic cancers is the molecular analysis of a liquid biopsy, e.g., blood and its components, such as serum [9]. However, there are only a few reports using serum from BCa patients for biomarker studies. The utility of the serum CRP value for assessing the prognosis and therapeutic response of urological malignancies, including BCa, has been reported [10]. Serum DNA analysis for hypermethylation of a set of genes shows promise as an indicator of cancer progression and mortality [11]. In addition, the analyses of cell-free circulating DNA (cfDNA) and the molecular characterization of circulating tumor cells (CTCs) (RNA, DNA and protein levels) are promising for biomarker identification in BCa [12,13]. Furthermore, the analysis of bodily fluids for inorganic substances, i.e., major or trace elements, is another promising process to identify potential biomarkers for diagnosis, prognosis and prediction of cancers including BCa [reviewed in 14]. In this study, we analyzed the serum concentration of 26 major and trace elements in BCa patients by mass/optical emission spectrometry and compared them to those of healthy controls and non-tumor bladder patients. We detected five elements (lithium, magnesium, nickel, phosphorus and potassium) as increased in the serum of BCa patients in comparison to healthy controls. Our results suggest that these five elements could be potential diagnostic biomarkers for future routine diagnostics.

**Material and Methods**

**Patients**

The use of patient serum samples for the molecular analyses was approved by the institutional ethical review board, and the study was conducted according to the standards set by the Declaration of Helsinki. The first patient set of 9 BCa patients and 12 healthy control probands was designated the discovery set. A second set of 30 BCa patients, 39 non-tumor bladder patients (with acute and chronic inflammation) and 18 healthy control probands was designated the validation cohort.

**Serum samples**

Serum was prepared from fresh blood samples collected in serum monovettes (Sarstedt, Nümbrecht, Germany). Serum vials were checked to exclude hemolysis and to avoid contamination with peripheral blood cells. Serum samples were stored at -80°C until used, and samples were sent on dry ice to Helmholtz Zentrum München (Research Unit, Analytical BioGeoChemistry/Prof. B. Michalke). The samples were thawed slowly at 4°C in a refrigerator before being diluted 1:10 with Milli-Q water; the diluted samples were used for element measurements.

**Sample analysis by ICP-OES**

An ICP-AES “Spectro Ciros Vision” system (SPECTRO Analytical Instruments GmbH & Co. KG, Kleve, Germany) was used for element determination in 1:10 diluted (Milli-Q water) samples. Sample introduction was carried out using a peristaltic pump connected to a Meinhard nebulizer with a cyclonic spray chamber. The spectral element lines measured were (nm) Al: 167.078, B: 249.773, Ba: 455.404, Ca: 183.801, Cu: 324.754, Fe: 259.941, K: 766.491, Li: 670.770, Mg: 279.079, Na: 589.592, P: 177.495, S: 180.731, Sr: 407.771, Ti: 334.941, and Zn: 213.856. The RF power was set to 1400 W, and the plasma gas was set to 13 L Ar/min; the nebulizer gas was approximately 0.6 L Ar/min after daily optimization. Although the element barium was analyzed, the concentrations were below the internal quantification limit of 11.2 µg/L in the majority of samples, and it was therefore not considered further.

**Sample analysis by ICP-sf-MS**

An ELEMENT 2, Thermo-Electron (Bremen, Germany) ICP-sf-MS instrument was employed for determination of elements that were below the level of detection from ICP-OES. 103Rh was administered to each sample at a concentration of 1 µg/L as an internal standard. Sample introduction was carried out using a peristaltic pump connected to a Seaspray nebulizer with a cyclonic spray chamber. The RF power was set to 1300 W, the plasma gas was 15 L Ar /min, whereas the nebulizer gas was approximately 0.9 L Ar/min after daily optimization. Measured element isotopes were 75As, 114Cd, 59Co, 52Cr, 202Hg, 55Mn, 98Mo, 60Ni, 208Pb, 120Sn, 77Se, 47Ti, and 51V.

**Quality control for element determinations**

The determination method had been validated previously by regular laboratory intercomparison studies and by regular analysis of adequately certified reference materials.

Routinely, after every ten measurements, three blank determinations and a control determination of a certified standard for all mentioned elements were performed. Calculation of results was carried out on a computerized lab-data management system, relating the sample measurements to calibration curves, blank determinations, and control standards.

**Statistical analysis**

The differences between the major and trace element levels in the serum of BCa and non-tumor bladder patients and healthy volunteers were estimated by a Mann-Whitney U test or a Kruskal-Wallis test and determined in ROC analyses. For establishing classification trees, we used recursive partition as implemented in the RPART package. Classification trees were pruned in order to minimize the relative misclassification error. All calculations were performed with the R statistical framework Ver. 3.2.1 (R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/).

**Results**

**Analyses of major and trace elements in BCa patients, non-tumor bladder patients and healthy controls**

In this study, we analyzed the levels of 26 major and trace elements (major elements: Al, B, Ca, Cu, Fe, K, Li, Mg, Na, P, S, Sr, and Zn and trace elements: As, Cd, Co, Cr, Hg, Mn, Mo, Ni, Pb, Sn, Se, Ti, and V) in the serum of BCa patients by ICP emission spectrometry and mass spectrometry and compared them to those of healthy controls and/or non-tumor bladder patients (Suppl. Data).

At first, we performed a comparison of major and trace elements in the serum of BCa patients and healthy controls. We studied a discovery cohort with nine BCa patients and 12 healthy controls. Analyses by ICP-OES or ICP-sf-MS revealed six elements as significantly increased (mercury, potassium, lithium, magnesium, nickel, and phosphorus) and two elements as significantly decreased (sodium and selenium) (Suppl. Data).

Next, we studied a validation cohort that consisted of 30 BCa patients, 39 non-tumor bladder patients and 18 healthy controls. We detected 18 elements with significantly higher concentrations (aluminum, boron, calcium, cadmium, copper, chromium, potassium, lithium, magnesium, manganese, nickel, phosphorus, lead, sulfur, strontium, titan, vanadium, and zinc) and one element with significantly lower concentration (cobalt) when comparing BCa patients and healthy controls (Suppl. Data).

**Comparison of discovery and validation cohorts**

In a comparison of the discovery and validation cohorts, five elements (lithium, magnesium, nickel, phosphorus and potassium) appeared in both cohorts as significantly increased but none appeared as decreased in BCa patients compared to healthy controls. We chose this approach of using discovery and validation cohorts to control for the α-error while avoiding more conventional correction methods (α=0.0025; Table 2).

Altogether, two elements (magnesium and potassium; all P<0.001) showed the most pronounced changes in serum levels, and an area under the curve (AUC) of 0.9043 and 0.947, respectively, was calculated to differentiate BCa patients from healthy controls (Fig. 1A).

Since the differential diagnosis of bladder cancer encompasses patients with acute or chronic inflammatory diseases, we also included patients with non-tumor diseases (N=39) in the validation cohort of our analyses. In a purely exploratory approach, the comparison of non-tumor bladder patients with healthy controls again revealed five elements with significantly increased levels: lithium, magnesium, nickel, phosphorus and potassium (Table 2A). However, for potassium and phosphorus, the comparison of BCa patients with non-tumor bladder patients showed significantly higher levels of both elements in the serum of non-tumor bladder patients.

Next, we were interested in whether our levels determined by mass spectrometry were in or out of the range of references given for healthy probands in routine laboratory diagnostics (http://www.med4you.at/laborbefunde/referenzwerte/). The levels of magnesium for all three groups (BCa patients, non-tumor bladder patients, and healthy controls) in our study were mostly within the range for healthy probands as those determined in routine diagnostics, which makes a comparison impossible. However, the potassium levels for the BCa patients (mean: 452.8 mg/L; median: 333.0; range: 162-1200) and for the non-tumor bladder patients (mean: 560.9 mg/L; median: 503.0 mg/L, range: 185-1390 mg/L) were, for the most part, above those described in routine laboratory diagnostics (range: 148.59-203.33 mg/L) and those of the healthy controls in this study (mean: 159.05 mg/L; median: 161.5 mg/L; range: 92-222 mg/L; Table 2B). However, levels of potassium were not associated with tumor grade or tumor stage in BCa patients (data not shown).

**Model for differentiation between BCa patients, non-tumor bladder patients and healthy controls**

A classification tree was designed to differentiate between BCa, non-tumor bladder patients and healthy controls using threshold levels of potassium, magnesium and phosphorus. With this model, it was possible to identify 29/30 (96.6%) of healthy controls, 30/39 (76.9%) of BCa and 26/39 (66.6%) of non-tumor bladder patients correctly (Fig. 2). However, the model revealed that there are still difficulties in distinguishing between BCa and non-tumor bladder patients. To get an overview of all elements altered between BCa patients, non-tumor bladder patients and healthy controls, further statistical analysis was performed (Kruskal-Wallis test).

**Comparison of all patients and healthy probands adjusted to the α-level**

For considering differences between all samples, the Bonferroni-adjusted α-level was applied as α=0.00192 (P=0.05/26 elements). Only chromium was increased but calcium, potassium, selenium and zinc were decreased in the serum of BCa patients compared with non-tumor bladder patients. The comparison of serum of BCa patients *vs.* healthy controls revealed increased levels of boron, calcium, chromium, copper, lithium, magnesium, nickel, phosphorus, potassium, sulfur, strontium, and zinc, but no elements were decreased. Increases of boron, calcium, copper, magnesium, phosphorus, potassium, sulfur, selenium, strontium and zinc were detected in the serum of non-tumor bladder patients compared with healthy controls, whereas levels of cobalt, molybdenum and tin were significantly decreased (Tab. 2C).

**Discussion**

There are several studies that describe a relationship between the amounts of major and/or trace elements in the environment, e.g., in drinking water, and an increased risk of cancers including BCa [reviewed in 14]. An increased risk of cancer mortality (including BCa) in relation to the vicinity of the production of cement, lime, plaster, and magnesium oxide has been reported as well [15]. Recently, a study analyzed the amounts of heavy metal and trace elements in BCa tissue, adjacent non-tumor tissue and cadaveric controls. The BCa tissue had higher concentrations of cadmium, calcium, chromium, lead, phosphorous, potassium, magnesium, nickel, selenium, strontium, and zinc than cadaveric controls. The boron level was higher in the cadaveric control than the BCa tissue and the adjacent non-cancerous tissue. The authors suggest that high concentrations of cadmium, lead, chromium, nickel, and zinc in the cancerous tissues together with arsenic in the adjacent non-cancerous tissues of BCa have a pathogenic role in BCa [16].

However, only a few elements have been studied in the serum of BCa patients thus far. A decreased level of selenium, considered as an essential trace element, was associated with an increase in BCa risk [reviewed in 14]. In addition, significantly increased amounts of cadmium, nickel and cobalt, but significantly decreased serum levels of manganese and zinc, were detected in BCa patients compared to healthy controls [17]. In line with this finding, BCa patients demonstrated significantly lower levels of zinc but higher levels of copper in the serum compared to controls [18], and the Cu/Zn ratio was significantly higher in the serum of BCa patients than in control patients [19]. In an Asian BCa cohort, serum copper levels were significantly higher, whereas calcium and selenium serum levels were significantly lower than those of the control group [20].

In our study, we also observed increased levels of potassium, magnesium, lithium, phosphorus and nickel (discovery and validation cohorts), decreased levels of selenium (only in discovery cohort) and increased levels of boron, cadmium, zinc and manganese in the BCa patients (only in validation cohort; Suppl. data). Comparing our data with those from the literature, we found that all but nickel were described for the first time in this study as significantly changed in the serum of BCa patients. Since three elements (K, Mg, P) are major elements, they could be easily and affordably measured as part of routine diagnostics. However, only potassium levels in the serum of BCa patients are, for the most part, clearly above those described in routine laboratory diagnostics for healthy probands, i.e., are highly detectable by the applied clinical diagnostics. Therefore, we suggest evaluating the levels of potassium for the early diagnosis of BCa in well-known risk groups of BCa as a screening tool.

Furthermore, we were interested in whether serum levels of elements also differed in the comparison of healthy controls to non-tumor bladder patients since the latter group is a more relevant control group than healthy controls in a urologic clinic. All five elements (Li, K, Mg, Ni, and P) showed significantly increased serum levels in non-tumor bladder patients compared to healthy controls. Interestingly, the mean levels of the four elements excluding lithium were even higher in non-tumor bladder patients than in BCa patients with significant differences for potassium and phosphorus.

After comparing all samples with each other using the Bonferroni-adjusted α-level, our results from the comparison of the discovery and validation study provided, as expected, even more support for potassium, magnesium, phosphorus and sulfur. In particular, chromium increased in BCa patients compared to healthy controls or non-tumor bladder patients, whereas chromium levels were not different between non-tumor bladder patients and healthy controls. This finding supports *in vitro* results that chromium can induce chromosome instability in human urothelial cells, which suggests a chromium/chromate-induced mechanism of bladder cancer [21]. In addition, it was shown that drinking water contaminated with chromium chlorination (and arsenic) byproducts increases the risk of BCa [22] and that BCa tissue had a higher concentration of cadmium than the cadaveric control [16]. However, why is potassium increased in the serum of BCa patients and non-tumor patients? Our non-tumor bladder patients exhibit mostly acute or chronic inflammatory diseases. It is known that patients with interstitial cystitis/painful bladder syndrome have an impaired Na+/K+-ATPase function in the epithelial bladder cells. Usually, the Na+/K+-ATPase transports controlled Na+ out of the cells and transports K+ into the cells. The Na+/K+-ATPase is also a potential regulator of tight junction formation and function, i.e., tight junctions function as urothelial barriers between urine and the underlying bladder. When the Na+/K+-ATPase function is disturbed, urine and, in this way, mostly K+, can pass the barriers. An increased potassium leakage causes clinical symptoms in patients with interstitial cystitis/painful bladder syndrome [reviewed in 23]. In addition, hyperkalemia and hyperphosphatemia, i.e., increased levels of potassium and phosphate, can be part of the tumor lysis syndrome that may occur as response to chemotherapy but also spontaneously [24].

Our study was not without shortcomings. We are far from knowing completely what the BCa and the non-tumor bladder patients take as prescribed and non-prescribed drugs. These patients are at an age when comorbidities often occur, e.g., cardiovascular, autoimmune, and diabetic diseases. Therefore, we cannot exclude treatment/drug effects on the serum levels of major and trace elements in BCa patients and non-tumor bladder patients. For example, hyperkalemia can result from general use of diuretics or from antibiotics prescribed for bladder inflammation. Furthermore, potassium infusions for treating kidney insufficiency or myasthenia of the bladder can increase potassium levels. On the other hand, insulin treatment is known to affect potassium levels negatively [25].

To summarize, the serum levels of five elements were significantly different between BCa patients or non-tumor bladder patients and healthy controls, i.e., lithium, magnesium, nickel, phosphorus and potassium levels were increased. Potassium and nickel levels in BCa patients and non-tumor bladder patients were predominantly outside of the reference range of healthy probands determined by routine laboratory diagnostics. We suggest that serum analyses for the five elements, with a focus on potassium as the major element, have the potential to be diagnostic markers for the early detection of BCa patients and non-tumor bladder patients in populations at risk of bladder diseases.

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**Conflicts of interest**

All authors declare to have no conflicts of interest.

**Table 1. Clinicopathological data of the BCa patients**

|  |  |  |
| --- | --- | --- |
|  | **Discovery****cohort** | **Validation****cohort** |
| **N** | 9 | 30 |
| **gender** |  |  |
| female | 2 | 6 |
| male | 7 | 24 |
| **tumor stage** |  |
| pTa | 2 | 1 |
| pTis | 0 | 1 |
| pT1 | 1 | 6 |
| pT2 | 4 | 16 |
| pT3 | 2 | 3 |
| pT4 | 0 | 2 |
| n.d. | 0 | 1 |
| **tumor grade** |  |
| G1 | 2 | 0 |
| G2 | 1 | 2 |
| G3 | 6 | 25 |
| n.d. | 0 | 3 |
| low grade | 2 | 1 |
| high grade | 7 | 26 |
| n.d. | 0 | 3 |

n.d.-not determined

**Table 2A Serum concentrations for BCa patients, non-tumor bladder patients and healthy controls**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|   |   | **Li (µg/L)** | **K (mg/L)** | **Mg (mg/L)**  | **Ni (µg/L)** | **P (mg/L)** |
| **BCa** | N | 39 | 39 | 39 | 39 | 39 |
|  | mean | 2.88 | 452.8 | 18.29 | 18.59 | 155.80 |
|  | median | 0 | 333.0 | 18.0 | 8.25 | 134.0 |
|  | range | 0-18.1 | 162-1200 | 12.7-26.5 | 0-263 | 101-338 |
|  |  |  |  |  |  |  |
| **Non-tumor****bladder**  | N | 39 | 39 | 39 | 39 | 39 |
| mean | 2.65 | 560.9 | 19.29 | 18.90 | 161.2 |
|  | median | 0 | 503.0 | 19.80 | 4.26 | 157.0 |
|  | range | 0-24.2 | 185-1390 | 13-24.8 | 0.06-46.0 | 112-263 |
|  |  |  |  |  |  |  |
| **Healthy** **control** | N | 30 | 30 | 30 | 30 | 30 |
| mean | 0 | 159.05 | 14.84 | 3.11 | 121.0 |
|  | median | 0 | 161.5 | 14.75 | 0.48 | 115.0 |
|  | range | 0 | 92-222 | 12.5-17.3 | 0-27.0 | 85.9-173 |
|  |  |  |  |  |  |  |
|  | reference range1 | n.a. | 148.59-203.33 | 17.75-25.77 | 0.2-0.5 | [27 – 45]2 |
|  | referencerange3 | n.a. | 3.8-5.2 | 0.73-1.06 | n.a. | 0.84-1.45 |

n.a.-not applicable

1reference range (in mg/L or g/L): range for healthy probands given by routine laboratory diagnostics

2reference range is only given for phosphate but not for phosphorus

3reference range (in mmol/L): range for healthy probands given by routine laboratory diagnostics

**Table 2B Comparison of BCa patients, non-tumor bladder patients and healthy controls by their serum levels of selected major elements and trace elements**

|  |  |  |  |
| --- | --- | --- | --- |
| Element | Non-tumor bladder/Healthy control P-value | BCa/ Healthy control/ P-value | BCa/Non-tumor bladder/P-value |
| Li (µg/L) | **5.4e-03** | **3.1e-04** | 0.300 |
| K (mg/L) | **3.3e-12** | **2.5e-10** | **0.032** |
| Mg (mg/L) | **3.9e-10** | **1.2e-08** | 0.061 |
| Ni (µg/L) | **2.1e-03** | **1.8.e-04** | 0.064 |
| P (mg/L) | **7.1e-08** | **2.7e-04** | **0.022** |

BCa (N=39); non-tumor bladder (N=39); healthy control (N=30)

**Table 2C Comparison of BCa patients, non-tumor bladder patients and healthy controls by their serum levels of all major elements and trace elements**

|  |  |  |  |
| --- | --- | --- | --- |
|  | Non-tumor bladder/Healthy control  | BCa/ Healthy control | BCa/Non-tumor bladder/ |
| Increased levels | B, Ca, Cu, K, Mg, P, S, Se, Sr, Zn | B, Ca, Cr, Cu, K, Li, Mg, Ni, P, S, Sr, Zn | Cr |
|  |  |  |  |
| Decreased levels | Co, Mo, Sn | none | Ca, K, Se, Zn |

BCa (N=39); non-tumor bladder (N=39); healthy control (N=30)

The Bonferroni-adjusted α-level is 0.00192: (P=0.05/26 elements)



**Fig. 1 ROC analysis**: Comparison of BCa patients and healthy controls by their serum levels of potassium and magnesium

The ROC analyses calculated AUC-values for K and Mg of 0.947 and 0.9043, respectively. The optimal threshold for K (at 182.5 mg/L) gives a sensitivity of 0.9487 and a specificity of 0.8000; the optimal threshold for Mg (at 16.25 mg/L) gives a sensitivity of 0.8205 and a specificity of 0.9333.



**Fig. 2 Classification tree**: Model to distinguish between BCa patients, non-tumor bladder patients and healthy controls

**Suppl. Table** Levels of major and trace elements in the serum of BCa patients, non-tumor bladder patients and healthy controls