Assessment of Essential and Non-essential Elements as Risk Evaluation Indices in Men with Prostate Cancer in Calabar South-South Nigeria

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Abstract
Background: Heavy metal (HM) toxicity has been described as a risk factor for the development of prostate disease in men and its assessment could predict susceptibility to prostate cancer (PCa).
The current study aimed to assess the levels of HM (selenium [Se], copper [Cu], chromium [Cr] and lead [Pb], iron [Fe], zinc [Zn], magnesium [Mg], and cobalt [Co]) in men with PCa.
Method: 90 men aged 40 to 75 years, including 30 men with PCa, 30 with benign prostatic hyperplasia (BPH), and 30 controls, were recruited in this case-control study. Prostate specific antigen (PSA) was estimated via enzyme linked immunosorbent assay and heavy metals with atomic absorption spectrophotometry. Body mass index (BMI) was also determined.
Results: The men with PCa had significantly higher BMI, PSA, Fe, and Pb and lower Mg, Zn, Cu, and Se compared to the controls. They also had higher PSA, Fe, and Co compared to the BPH ($P < 0.05$). Those with BPH had higher BMI, PSA, and Fe and lower Mg, Zn, Cu, Se, and Co compared to the controls ($P < 0.05$). Zn was positively correlated with Mg ($r = 0.937$, $P < 0.001$), Cu ($r = 0.548$, $P = 0.002$), Se ($r = 0.731$, $P < 0.001$), and Co ($r=0.733$, $P < 0.001$) only in the men with PCa. Levels of Cu, Mg, and Se were associated with the risk of BPH and PCa.
Conclusion: The men with prostate disease were found to have higher levels of lead and iron and lower magnesium, copper, selenium, and zinc, which necessitate assessment of these elements for early detection prostate cancer and monitoring the progression of the disease.
Keywords: Metals, Heavy, Prostate, Neoplasms
Introduction

In most developing countries, including Nigeria, prostate cancer (PCa) is ranked as the most prevalent malignancy and the leading cause of cancer death among men.\(^1\) Some of the prevailing factors associated with increased risk of prostate carcinogenesis include sexual and physical activities, smoking, genetic and environmental factors.\(^2,3\) Direct activation of pathways involved in carcinogenesis or induction of gene mutation in susceptible individuals has been described as some of the pathologic mechanisms employed by these factors in prostate tumour development and progression.\(^3\) Occupational and environmental exposure to trace and heavy metals through inhalation, dermal contact, and ingestion of metal contaminated water and food have been implicated in the initiation, metastasis, or inhibition of prostate carcinogenesis.\(^4\) Certain studies have provided evidence of positive associations between exposure to heavy metals and prostate carcinogenesis, while, others have reported an inverse or no association. Thus, investigating mineral excess or deficiency and perturbation in their homeostasis in elderly men with non-hyperplastic prostate glands may highlight the role of these disturbances in the initiation and promotion of prostate carcinogenesis.\(^4\) Certain metals serve as enzyme co-factors, which are essential for intracellular processes and are effective protectors against carcinogenesis in-vivo. Other metals have been shown to induce malignant transformation of cells and carcinogenesis in various tissues and organs.\(^5,6\) Heavy metals, as inorganic lead and hexavalent chromium, have been implicated in various steps in prostate carcinogenesis, including cell proliferation and migration\(^7\) while low magnesium and selenium levels has been linked to higher grade of PCa and increased risk and incidence of other malignancies.\(^8\)

Even though the relationship between metals and PCa has been extensively studied, the available findings are contradictory and conflicting. Disparity in these findings could be attributed to contributory role of genetic and environmental factors in the aetio-pathologic mechanisms of prostate cancer development and progression across diverse ethnic groups and races. Moreover, studies on the probable association of trace and heavy metals with the development and progress of prostate cancer are not commensurate with the increasing incidence of the disease in the study area. Considering the established association between heavy metals exposure and incidence of PCa, routine examination of heavy metal levels in men within the vulnerable age group and those occupationally exposed to heavy metals may be important in identifying individuals at increased risk of prostatic disease. It could be conducive to commencement of appropriate preventive measures and also monitoring treatment outcomes in those with prostatic disease. The levels of some heavy metals were estimated herein in sera of men with prostate cancer, benign prostatic hyperplasia, and a control group without any forms of prostatic disease in Calabar metropolis, Southern Nigeria.

Materials and Methods

Study design

This case-control study was carried out in the Department of Urology, University of Calabar Teaching Hospital (UCTH), between April to September 2018. Written informed consent was obtained from all the subjects prior to their recruitment in the study. The UCTH Health Research Ethical Committee (UCTH/HREC/33/45) approved the study protocol. This work was carried with strict adherence to the ethical principles of medical research involving human subjects as outlined in the Helsinki declaration in 1975 and the subsequent revisions.
Subject selection
A total of 90 participants aged 40 years, including 30 prostate cancer patients, 30 with benign prostatic hyperplasia, and 30 apparently healthy age matched men without any forms of prostate disease were recruited into the study. The prostate cancer patients herein were those newly diagnosed with prostate cancer via biopsy and preoperative blood samples. Benign prostatic hyperplasia subjects were those newly diagnosed with the disease while the controls were apparently healthy men with no history of prostate cancer among the first-degree relatives and who has not been diagnosed with prostate cancer, benign prostatic hyperplasia, or any other chronic organs or systemic illnesses. The exclusion criteria were those below 40 years of age, food supplement users, lifetime smokers, and those with a history of alcohol addiction, illicit drug abuse, and those with any forms of chronic organ, systemic illness, or chronic medication were excluded from the study. Socio-demographic information and medical history were obtained via a semi-structured interview and an administered questionnaire. Anthropometric measurements, including weight, height, waist, and hip circumferences, were performed to determine the body mass index (BMI) and waist to hip ratio. Estimation of the prostate specific antigen (PSA) level was used as an effective tumour biomarker for detection of prostate cancer.

Sample collection
Whole blood samples (5mL) were collected aseptically from all the subjects into dipotassium ethylene diamine tetraacetic acid (K₂EDTA) and stored at 4°C. The wet acid digestion method was utilized for extraction of heavy metals from blood samples. The samples were mixed with concentrated HNO₃ and heated in a water bath to a colourless solution, allowed to cool, and diluted with deionized water. Subsequently, HM analysis was carried out.  

Laboratory methods
Determination of PSA
PSA was analyzed via a solid phase enzyme-linked immunosorbenent assay using the PSA ELISA Kit manufactured by Phoenix Pharmaceuticals Inc. USA. A rabbit anti-PSA antibody directed against intact PSA was immobilized on microtitre well and another monoclonal anti-PSA antibody conjugated to horseradish peroxidase (HRP) was in the antibody-enzyme conjugate solution. The PSA molecules in the samples reacted simultaneously with the antibodies and became sandwiched between the enzyme linked antibodies and the solid phase. Unbound labeled antibodies were washed out after incubation and a solution of tetramethylbenzidine (TMB) was added. The solution was incubated for colour development. Colour reaction was terminated and absorbance of the colour formed was read at 450nm. The absorbance of sample was directly proportional to the concentration of PSA in the sample.  

Estimation of heavy metals via atomic absorption spectrophotometry (AAS)
The heavy metals (Se, Cu, Cr, Pb, Fe, Zn, Mg, Co) were analysed with atomic absorption spectrometry (model 2380 Perkin Elmer Inc. Norwalk, CT, USA). In AAS, the sample is atomized and a beam of electromagnetic radiation emitted from a light source pass through the vapourised sample. Some of the radiation is absorbed by the atoms in the sample; the amount of light absorbed is proportional to the concentration of the element in the sample.  

Statistical analysis
The results were expressed as mean ± standard deviation. Descriptive and inferential statistical analyses were performed via Statistical Package for Social Sciences (SPSS version 20.0, IBM, USA). Analysis of variance (ANOVA), multiple regression, and Pearson’s
correlation were used to determine the variations, relationships, and associations among the variables in the groups, respectively. A $P<0.05$ was considered to be statistically significant.

**Results**

Table 1 represents the comparison of the mean age, BMI, PSA, iron, magnesium, zinc, copper, chromium, lead, selenium, and cobalt in the men with prostate cancer (PCa), benign prostatic hyperplasia (BPH), and the controls. Significant variations were observed on the BMI, PSA, iron, magnesium, zinc, copper, selenium, and cobalt levels of the three groups ($P < 0.05$). No significant variations were observed between the chromium and lead levels of the three groups ($P > 0.05$). Significantly higher BMI, PSA, Fe, and Pb and lower Mg, Zn, Cu, and Se were found in the PCa compared to the controls. Higher PSA, Fe, and Co was also found in the PCa compared to the BPH ($P < 0.05$). Those with BPH had higher BMI, PSA, and Fe and lower Mg, Zn, Cu, Se, and Co compared to the controls ($P < 0.05$).

The relationship between essential and non-essential elements with the incidence of BPH and PCa is depicted in table 2. The relationship between essential and non-essential elements and the incidence of BPH and PCa indicated that the model was statistically significant (chi = 149.22, $R^2 = 0.911$, $P < 0.001$, df = 16) with 91.1% probability of having BPH and PCa correctly predicted at 91.1%. There were significant associations between Mg (odds ratio (OR) OR = 1.000, $P < 0.001$), Cu (OR = 1.013, $P < 0.001$), Se (OR = 1.963, $P < 0.001$), and Pb (OR = 1.909, $P = 0.018$) with occurrence of BPH and between Mg (OR = 1.000, $P < 0.001$), Cu (OR = 1.013, $P < 0.001$), Se (OR = 1.975, $P < 0.001$), and Fe (OR = 1.138, $P = 0.009$) with occurrence of PCa.

The correlation of essential elements among men with prostate cancer was shown in table 3. Significant positive correlations were observed between Zn and Mg ($r = 0.937$, $P < 0.001$), Zn and Cu ($r = 0.548$, $P = 0.002$), Zn and Se ($r = 0.731$, $P < 0.001$), and Zn and Co ($r = 0.733$, $P < 0.001$) only in the subjects with prostate cancer.

**Discussion**

Perturbations in the homeostasis of essential and non-essential elements have been implicated in prostate carcinogenesis. The levels of some heavy metals were assessed in the men with benign prostatic hyperplasia and prostate cancer to determine their possible use as risk evaluation indices and disease prognosis.

Our study demonstrated higher BMI and PSA levels in PCa and BPH subjects compared to their control counterparts. PSA is a serine protease whose physiological function involves proteolysis and liquefaction of the gel protein of the seminal fluid: herein, PSA was synthesised in the epithelial cells of the prostate and localized in the prostate gland. It was synthesised in normal prostatic tissue, benign prostatic hypertrophy, and PCa of all grades and stages. Its level in normal prostate tissue is tightly regulated such that only a minute proportion ($<4.0\text{ng/l}$) leaks into the general circulation. Disruption of prostate architecture, as seen in prostate tumour, which may result in increased expression of PSA during the development and progression of the tumour, may be responsible for higher PSA levels ($52.98\pm71.08\text{ng/l}$) in men with PCa. Elevation of PSA in men with PCa may also be attributed to increased synthesis of PSA and increased release into the serum arising from disruption of capillary and ductal tissue barriers. Other benign conditions, such as prostatitis and BPH, have also been associated with elevated PSA levels, but not as high as the levels observed in prostate cancer. In-vivo studies have shown that high PSA expression and serum levels are assigned to higher tumour mass and *vice-versa*.
while elevations in PSA levels could be used to predict clinical diseases by 10 years or more.\textsuperscript{12,14} In this work, the men with BPH and PCa had higher BMI compared to the controls. High BMI, which is defined as overweightness/obesity, has been directly associated with the risk of aggressive or fatal prostate cancer.\textsuperscript{15} Obesity has been shown to predispose and increase the risk of BPH and PCa development. Obesity/overweightness-associated mechanisms that promote BPH and PCa development may include intra-abdominal pressure, inflammation and oxidative stress, peripheral aromatization of androgens in the adipose tissue, low testosterone levels, insulin resistance, and altered adipokine status.\textsuperscript{16} Men with higher BMI and WC have been shown to have an increased risk of high grade PCa (10% increase in the risk of BMI for every 5kg/m\textsuperscript{2} increase and 13% of WC for every 10cm increase),\textsuperscript{17} compared to those with BMI <25kg/m\textsuperscript{2}.\textsuperscript{18}

Lower Se levels were observed in the subjects with BPH and PCa compared to the controls. Lower Se levels have also been previously demonstrated in men with BPH and PCa,\textsuperscript{19} which has been linked to higher incidence of PCa and enhanced tumour progression.\textsuperscript{20} Reduction in the risk of advanced PCa by down to 50% has been shown to be induced by high baseline Se levels. Anticancer activity of selenium has been attributed to its antioxidant and detoxification property, cell cycle modulation, enhanced immune surveillance, and inhibition of angiogenesis and tumour metastasis.\textsuperscript{21}

The men with PCa and BPH herein had lower zinc levels compared to the controls. The highest concentration of zinc in humans is localized in normal prostate tissue.\textsuperscript{2} Malignant prostate tissues has been shown to have a defective ability to accumulate Zn when compared to normal tissues.\textsuperscript{22} Loss of unique ability to retain high levels of zinc may explain lower zinc levels in men with PCa. Zinc is an antioxidant metal; lower zinc levels in PCa may also be attributed to their consumption in neutralization of increased ROS generation associated with malignant conditions.\textsuperscript{23} Anti-carcinogenic activity of zinc has been attributed to its role in structural stabilization of RNA and DNA, inhibition of growth and proliferation of normal prostate cells, and suppression of angiogenesis and metastasis in malignant prostate tissue.\textsuperscript{3, 24}

Lower Mg levels were observed in the participants with BPH and PCa compared to the controls. Mg deficiency has been linked with chronic inflammation, oxidative stress, and genomic instability, which may promote tumorigenesis.\textsuperscript{25} Thus, lower Mg levels in men with BPH and PCa may be attributed to chronic inflammation and oxidative stress associated with these conditions. The associations between lower Mg levels and high grade PCa have been also documented.\textsuperscript{25} However, in this study, higher Mg levels were demonstrated in serum and tissue samples from the individuals with PCa compared to BPH cases and controls in another study.\textsuperscript{3}

The participants with prostate cancer and BPH recorded lower Cu levels compared to their control counterparts. Our findings are consistent with the reports of a previous study demonstrating lower Cu levels in the BPH and PCa compared to the controls.\textsuperscript{26} Copper plays an important role in carcinogenesis by regulation of redox balance through its antioxidant activity.\textsuperscript{4, 27} The findings of lower Cu levels in the PCa and BPH patients compared to the controls may be an indication of increased utilization of Cu in maintaining redox equilibrium in a condition of increased ROS generation and oxidative stress resulting from PCa and BPH. Contrary to our findings, higher Cu levels were demonstrated in the patients with PCa compared to the controls, which was related to cancer progression.\textsuperscript{20, 28}

Higher Co levels were observed in the controls compared to the BPH. Lower
Co level has also been reported in BPH by a previous study. Studies on the association of Co with development and progression of PCa are scarce. However, Co has been implicated in cancer development in animal studies. The proposed mechanisms of Co-induced gene mutations and carcinogenesis include DNA breaks and inhibition of DNA repair as observed in experimental animals. However, no evidence is yet available in humans.

Higher levels of Pb were observed in the men with PCa compared to the controls studied. Inorganic Pb and Cr VI have been classified as probable carcinogens in humans. Lead’s involvement in carcinogenesis has been shown through its role in oxidative injury to DNA binding proteins, tumour suppressor proteins, and inhibition of DNA synthesis and repair. Higher lead levels have also been reported in BPH and PCA compared to controls.

The subjects with PCa herein also had higher levels of Fe compared to the BPH and controls, which was higher in the BPH compared to the controls. Dysregulation in iron metabolism characterized by the changes in differential expression of proteins that control iron entry, cellular iron distribution, and iron exit from prostate cells have been reported in PCa. Increased iron sequestration and intracellular iron release in cancer cells may be responsible for higher Fe levels seen in PCa. Certain studies have shown that increased Fe or iron overload could promote development of PCA through induction of ROS, leading to peroxidation of biomolecules, promotion of oncogenic activation, inhibition of tumour suppression, DNA strand breaks, mutagenesis, and cell proliferation. Iron levels in the malignant prostate tissues have been found to be higher than those in benign prostate tissue, supporting our findings of higher Fe levels in the PCa compared to the BPH and controls. The role of iron in prostate carcinogenesis still remains unclear.

Zinc is positively correlated with Cu, Mg, Se, and Co in subjects with prostate cancer. The interactions between metals and minerals have been implicated in human diseases. Studies have described the relationship between each of Zn, Cu, Mg, Se, and Co and prostate cancer, neglecting the complex interactions that can occur between these elements. These interactions are of importance on account of the complex relation between different metabolic pathways. They are also important for biological cellular capacity to compensate a metabolic pathway if there is a shortage of another pathway. These metals share similar chemical and biochemical properties and are therefore bound to have a certain level of metabolic interactions, which may be synergistic or antagonistic depending on the concentration and redox state of the metal. Lower Zn and Se with higher Fe levels have been reported in PCa compared to controls.

In the current paper, the risk of BPH and PCa were found to be associated with levels of Cu, Mg, and Se. In consonance with our findings, Cu, Zn, Se, Fe, and Mn have also been associated with the risk of prostate cancer in other studies; however, the mechanisms of causality are still uncertain.

Conclusion

The obtained findings herein revealed that the occurrence of prostate disease may be associated with the changes in the levels of certain essential and non-essential elements characterized by higher levels of Pb and Fe and lower Mg, Cu, Se, and Zn. This suggests that assessment of these elements could be conducive to early detection of men at risk of development of prostate cancer and monitoring the progression of the disease. Anti-carcinogenic activity of zinc may be synergistically associated with copper, cobalt, magnesium, and selenium.
Acknowledgment
The authors are grateful to Dr. Onyekere Glem of Urology Clinic University of Calabar Teaching Hospital for his assistance in recruiting the prostate cancer and benign prostate hyperplasia patients.

Conflict of Interest
None declared.
References


Karimi G, Shahar S, Homayouni N, Rajikan R, Abu Bakar NF, Othman MS. Association between trace element and heavy metal levels in hair and nail with


Table 1. Comparison of the mean age, BMI, PSA, and essential and non-essential elements in the prostate cancer (PCa), benign prostate hyperplasia (BPH), and control groups

<table>
<thead>
<tr>
<th>Index</th>
<th>PCa n=30</th>
<th>BPH n=30</th>
<th>Controls n=30</th>
<th>H-value df=2</th>
<th>P-value</th>
<th>C vs. BPH P-value</th>
<th>C vs. PCa P-value</th>
<th>BPH vs. PCa P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>66.60±10.19</td>
<td>62.76±9.75</td>
<td>61.93±9.29</td>
<td>3.568</td>
<td>0.168</td>
<td>0.841</td>
<td>0.149</td>
<td>0.073</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.63±2.519</td>
<td>25.88±3.27</td>
<td>24.12±1.60</td>
<td>13.495</td>
<td>&lt;0.001</td>
<td>0.043&lt;</td>
<td>&lt;0.001</td>
<td>&lt;0.001&lt;</td>
</tr>
<tr>
<td>PSA (ng/l)</td>
<td>52.98±71.08</td>
<td>3.66±3.34</td>
<td>1.48±0.99</td>
<td>56.593</td>
<td>&lt;0.001</td>
<td>0.041</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fe (µg/dl)</td>
<td>166.09±15.03</td>
<td>133.60±17.25</td>
<td>124.84±14.84</td>
<td>50.728</td>
<td>&lt;0.001</td>
<td>&lt;0.001&lt;</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mg (mg/dl)</td>
<td>9.96±1.56</td>
<td>9.43±1.39</td>
<td>11.67±1.03</td>
<td>32.743</td>
<td>&lt;0.001</td>
<td>&lt;0.001&lt;</td>
<td>&lt;0.001</td>
<td>0.160</td>
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<tr>
<td>Zn (µg/dl)</td>
<td>117.30±30.72</td>
<td>108.64±27.55</td>
<td>137.05±31.62</td>
<td>13.000</td>
<td>0.002&lt;</td>
<td>0.001&lt;</td>
<td>0.015</td>
<td>0.240</td>
</tr>
<tr>
<td>Cu (µg/dl)</td>
<td>100.81±26.67</td>
<td>101.73±31.12</td>
<td>154.89±20.81</td>
<td>43.629</td>
<td>&lt;0.001</td>
<td>&lt;0.001&lt;</td>
<td>&lt;0.001</td>
<td>0.929</td>
</tr>
<tr>
<td>Cr (µg/dl)</td>
<td>0.20±0.02</td>
<td>0.20±0.03</td>
<td>0.21±0.03</td>
<td>1.874</td>
<td>0.392</td>
<td>0.778</td>
<td>0.224</td>
<td>0.257</td>
</tr>
<tr>
<td>Pb (µg/dl)</td>
<td>12.47±3.81</td>
<td>11.30±3.46</td>
<td>10.40±2.54</td>
<td>4.857</td>
<td>0.088</td>
<td>0.203</td>
<td>0.022&lt;</td>
<td>0.506</td>
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<tr>
<td>Se (µg/dl)</td>
<td>94.17±14.57</td>
<td>87.42±13.87</td>
<td>106.47±9.64</td>
<td>26.299</td>
<td>&lt;0.001</td>
<td>&lt;0.001&lt;</td>
<td>0.001&lt;</td>
<td>0.057</td>
</tr>
<tr>
<td>Co (µg/dl)</td>
<td>0.19±0.03</td>
<td>0.18±0.03</td>
<td>0.20±0.03</td>
<td>6.785</td>
<td>0.034&lt;</td>
<td>0.016&lt;</td>
<td>0.656</td>
<td>0.043&lt;</td>
</tr>
</tbody>
</table>

Data presented as mean±SD, *= indicates significant variations among the groups at P < 0.05, a=values from Kruskal Wallis test, c=values from Mann-Whitney U test, BMI=body mass index, PSA=prostate specific antigen
Table 2. Relationship between age, BMI, PSA, and essential and non-essential elements with the incidence of BPH and PCa

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Controls vs. BPH</th>
<th>Controls vs. PCa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Df = 16</td>
<td>Chi=149.22, R^2=0.911, P &lt; 0.001</td>
<td>Chi=149.22, R^2=0.911, P&lt;0.001</td>
</tr>
<tr>
<td>Beta</td>
<td>OR</td>
<td>P-value</td>
</tr>
<tr>
<td>Intercept</td>
<td>-45416.40</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Fe</td>
<td>-0.059</td>
<td>0.943</td>
</tr>
<tr>
<td>Mg</td>
<td>8375.209</td>
<td>1.000</td>
</tr>
<tr>
<td>Zn</td>
<td>0.298</td>
<td>1.347</td>
</tr>
<tr>
<td>Cu</td>
<td>-421.684</td>
<td>1.013</td>
</tr>
<tr>
<td>Cr</td>
<td>1.138</td>
<td>3.120</td>
</tr>
<tr>
<td>Pb</td>
<td>0.647</td>
<td>1.909</td>
</tr>
<tr>
<td>Se</td>
<td>108.896</td>
<td>1.963</td>
</tr>
<tr>
<td>Co</td>
<td>168.140</td>
<td>1.053</td>
</tr>
</tbody>
</table>

Relationship determined using multiple logistic regression with the controls as the reference, BMI=body mass index, PSA=prostate specific antigen, BPH=benign prostate hyperplasia, PCa=prostate cancer; OR=Odds ratio, * = indicates significant differences among the groups at P < 0.05.
Table 3. Correlation among essential elements in men with prostate cancer

<table>
<thead>
<tr>
<th>Variables</th>
<th>r</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn</td>
<td>0.937</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Mg</td>
<td>0.548</td>
<td>0.002*</td>
</tr>
<tr>
<td>Cu</td>
<td>0.731</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Se</td>
<td>0.733</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Co</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* = indicates significant correlations among the variables at $P < 0.05$