



Metal- and redox homeostasis in prostate cancer with vitamin D₃ supplementation



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ABSTRACT

Vitamin D₃ supplementation has a beneficial effect on cancerous patients, although it can influence the redox- and metal homeostasis. The aim of our investigation was to demonstrate the effect of vitamin D₃ consumption on the redox- and metal homeostasis in prostate cancer, because of the recommended daily dose increased from 200 IU to 2000 IU in recent years in Hungary. Forty-three volunteers were involved in the study. The grouping was applied according to the clinical routine laboratory parameters (vitamin D₃) and the tumor markers (PSA, fPFA). Patients were divided into 5 groups: (A) patient control (N = 8), (B) patient control with vitamin D₃ treatment (N = 9), (C) high-risk prostate cancer group (N = 6), (D) high-risk prostate cancer group with vitamin D₃ treatment (N = 8) and (E) vitamin D₃ treated cancerous group with androgen deprivation therapy (N = 11). The element concentrations were determined with ICP-OES. Among the redox parameters, free radical scavenging capacity and H-donating ability were determined with luminometry and spectrometry. Vitamin D₃ treatment caused differences in the metal- and redox homeostasis in either patient control and cancerous groups. The concentration of Fe, Cr, and Pb significantly increased in the erythrocytes of prostate cancer patients. According to the higher scavenging capacity by vitamin D₃ treatment, it seems that vitamin D₃ helps to equilibrate the redox homeostasis that could affect the outcome of cancer positively. However, the tendency in the metal element status does not give a clear explanation of cancer's outcome, but the accumulation of Pb by vitamin D₃ supplementation needs to be taken into more serious consideration in set terms of occupational diseases.

Abbreviations: Al, aluminium; ALT, alanine transaminase; AP-1, activated protein-1; AR, androgen receptor; AST, aspartate transaminase; B, boron; Ba, barium; BMI, body mass index; Ca, calcium; CDK4, cyclin dependent kinase; CEA, carcinoembryonic antigen; Chol, cholesterol; CHR, reticulocyte haemoglobin content; CKI, cyclin dependent kinase inhibitor; Co, cobalt; Cr, chromium; CREA, creatinine; CRP, c-reactive protein; Cu, copper; DNA, deoxyribonucleic acid; DV, declared value; EGF, epidermal growth factor; Fe, iron; fPSA, free prostate specific antigen; G1, gap 1 phase; G2, gap 2 phase; GADD45α, growth arrest and DNA-damage-inducible protein alpha; GGT, gamma-glutamyl transferase; GOT, glutamic-oxaloacetic transaminase; GPT, glutamic-pyruvic transaminase; GSK-3, glycogen synthase kinase; H₂O₂, hydrogen peroxide; HCHO, formaldehyde; HCl, hydrogen chloride; HGB, haemoglobin; HIF-1, hypoxia induced factor; HNO₃, nitric acid; ICP-OES, inductively coupled plasma optical emission spectrophotometry; IKEB, Intézet Kutatási Etikai Bizottság = Instructional Research Ethics Committee; IL-2, -6, -10, interleukin-2, -6, -10; INK4, inhibitors of CDK4; IU, international unit; LDH, lactate dehydrogenase; Li, lithium; LMWC, low molecular weight chromium; M, metaphase checkpoint; MCV, mean corpuscular volume; Mg, magnesium; Mn, manganese; MV, measured values; NADH, nicotinamide adenine dinucleotide; NFAT, nuclear factor of activated cells family; NF-κB, nuclear factor-kappaB; Ni, nickel; P, phosphorus; p21waf1, cyclin-dependent kinase inhibitor protein 1; p27kip1, multifunctional cyclin-dependent kinase inhibitor; p53, tumor suppressor p53; Pb, lead; PDGF, platelet-derived growth factor; PSA, prostate specific antigen; R, recovery; RLU, as relative light unit; RNA, ribonucleic acid; RXR, retinoid X receptor; S, sulfur; Sr, strontium; Ti, titanium; TNF-α, tumour necrosis factor alpha; TSC, total scavenger capacity; TUKEB, Tudományos és Kutatásaitikai Bizottság = Scientific and Research Ethics Committee; UA, uric acid; V, vanadium; VDR, vitamin D₃ receptor; VEGF, vascular endothelial growth factor; WBC, white blood cell; Zn, zinc

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1. Introduction

In the literature, there are a few investigations about the effect of vitamin D₃ against oxidative stress, but they show positive results, because vitamin D₃ increases the SOD activity, reverses the alteration in the oxidative and nitrosative stress parameters [1]. Epidemiological studies indicate that vitamin D₃ (1 α ,25(OH)₂D₃) deficiency is an important factor in various human cancer type [2–4]. In Europe, prostate cancer is one of the most commonly diagnosed malignant diseases among men after non-melanoma skin cancer, colorectal and lung cancer whose mortality increases with age. Besides genetic diversity and adverse environmental aspects, vitamin D₃ deficiency plays an important role in the incidence of prostate cancer. Clinical researches and molecular studies have already established that vitamin D₃ inhibits cell proliferation on androgen receptor (AR)-positive and AR-negative in the prostate tumor cells. Antiproliferative effect of vitamin D₃ is mediated by the vitamin D receptor (VDR), as calcitriol (1 α ,25(OH)₂D) binds to VDR, and it interacts with the retinoid X receptor (RXR) [5,6]. Several forms of cholecalciferol could be bound to the vitamin D₃ receptor, but 1,25-dihydroxycholecalciferol is biologically more potent due to its 1000 times higher affinity to VDR than that of 25-hydroxycholecalciferol [7]. Nevertheless, a hormonally active form of vitamin D₃ (25-dihydroxycholecalciferol) activates the apoptotic pathways through the expression of p53 tumor suppressor protein, modulates cell cycle by inhibition of the G₁–S transition and inhibits cell differentiation in tumors through the signal transduction processes [8–10]. In vitro studies on human prostate cancer cell lines and mouse models have demonstrated that vitamin D₃ and its derivatives can inhibit the growth of cancer cells, by inducing apoptosis and preventing cell proliferation [11–14]. Also, vitamin D secosteroids can activate cyclin-dependent kinase inhibitors, e.g. INK4 proteins, p21waf1, and p27kip1 [15].

With these findings, it is highly considered that the treatment of vitamin D₃ and its analogs could be used as alternative therapy in prostate cancer.

There are still not enough indicators available to diagnose prostate cancer at an early stage and differentiate the patients who require prostatectomy or oncological treatment for healing. Even the level of prostate-specific antigen (PSA), whose height is the most common sign of the disease occurrence, can stay within the normal range or give a false negative result, especially at the early stage. Several studies were carried out to find a specific biomarker of the redox system to determine the disease severity. Some studies in human tumor cell lines refer to the increase of interleukins (IL-6, IL-10), tumor necrosis factor alpha (TNF- α), VEGF signal protein, and the decrease of IL-2 in metastasis [16,17]. In one of our previous studies, the levels of cytokines and growth factors were lower at the early stage prostate cancer patients than the one in the controls. Furthermore, remarkable differences were found in the results of bound formaldehyde (HCHO), Zn-protoporphyrin and free protoporphyrin in erythrocytes of taxane-treated metastatic, histologically negative and positive patients compared to the healthy controls [18]. These findings are not only important in the process of DNA hypomethylation because it could lead to high mutation rate in most cases [19], but they are also in strong connection with the redox status [20].

On the other hand, it was also demonstrated that not only the metal elements could assist the absorption of vitamin D₃ but conversely, the uptake of micro and macronutrients (e.g., Ca, Mg, Cu, Zn, Fe, Se), and certain transition metals might be facilitated by vitamin D₃ in the gastrointestinal tract in healthy individuals [21]. Even the absorption of heavy metals can be assisted by vitamin D₃. For that reason, despite the beneficial effects of vitamin D₃, heavy metal accumulation needs to be taken into account, since the absorption of non-essential metal elements could be affected by the vitamin D₃ intake in human [22]. It was also demonstrated that the metal element status alters in the case of prostate cancer with special emphasis on the Pb level [23], but there is no study

for investigating the metal element status in the function of vitamin D₃ supplementation in prostate cancer.

Vitamin D₃ deficiency could be a risk factor for prostate cancer in the Hungarian man population since the vitamin D₃ is lower than the optimal level in 50% of the Hungarian population even in the summer period [24]. There is no information available in the literature about that how vitamin D₃ treatment effects on the metal element and redox homeostasis in prostate cancer patients. Therefore, in the current study our aim was to perform a comprehensive investigation on how vitamin D₃ supplementation in patient control and prostate cancer patients influences the metal element- and redox homeostasis after a three-year treatment.

2. Materials and methods

2.1. Materials

Vitamin D₃ was a general consumer product on sale in the pharmacy. Each gelatine capsule contained 3000–3300 IU (75–82.5 μ g) cholecalciferol (vitamin D₃). Standard solutions, nitric acid, and hydrogen chloride were purchased from Reanal (Budapest). 1,1-diphenyl-2-picrylhydrazyl, stable radical, hydrogen peroxide, luminol, microperoxidase were obtained from Sigma (St. Luis). Spectro multi-element standard solutions were used for ICP (CPAchem; Stara Zagora, Bulgaria). The CHR hemoglobin reagent solution was purchased from Reagents Ltd., Hungary.

2.2. Patients

42 volunteers with a mean age of 62.1 \pm 15.9 years were examined, of which 25 were the outpatients of the Medical Centre of Dunakeszi and 17 from the Department of Urology and Urooncological Centre at the Semmelweis University, Budapest Hungary. The grouping was executed by the treating physician according to cancer's progressivity (PSA, digital rectal examination, biopsy) and the vitamin D₃ treatment. Harmful habits and other comorbidities of some patients (smoking, alcohol consumption, diabetes mellitus, another type of cancers) were considered. Conforming to the latter, two patients had to be excluded from the investigation. Patients with vitamin D₃ treated groups received vitamin D₃ treatment for three years continuously under strict physician control.

The diet of the patients didn't alter from the conventional Hungarian dietary, the intake of vitamin D₃ was 2.5 μ g/day in men and 1.9 μ g/day in women of 35–64 years old, while Ca was 759 mg/day in men and 690 mg/day in women; Cu was 1.18 mg/day in men and 0.94 mg/day in women; and Zn was 9.65 mg/day in men and 7.31 mg/day in women [24]. According to their state, they were following a normal diet without taking any food supplements. The patients didn't work under extreme conditions and they weren't exposed to any metal toxicity (foundries, battery recycling, etc.). Blood samples were collected in spring (March–April) after the three-year treatment. The patients were divided into 5 groups according to the Table 1:

Table 1
PSA level (μ g/L) in different patients' groups after three-year treatment.

Patients	PSA (μ g/L)
Patient control group (N = 8)	1.11 \pm 0.45
Patient control group with vitamin D ₃ treatment (N = 9)	0.75 \pm 0.40
High-risk prostate cancer group (N = 6)	13.0 \pm 5.4 [†]
High-risk prostate cancer group with vitamin D ₃ (N = 8)	5.68 \pm 1.11 ^{***}
Vitamin D ₃ treated cancerous group with androgen deprivation therapy (N = 11)	1.40 \pm 2.44 ^{**}

[†] significant difference to the patient control $p < 0.01$.

^{**} significant difference to the high-risk prostate cancer without vitamin D₃ treatment $p < 0.01$.

a patient control group (N = 8 PSA < 2 µg/L); a patient control group with vitamin D₃ treatment (N = 9; PSA < 2 µg/L); a high risk prostate cancer group (N = 6, PSA > 9 µg/L); a high risk prostate cancer group with vitamin D₃ treatment (N = 8; PSA > 4.1 µg/L) and a cancerous group with vitamin D₃ treatment who went under androgen deprivation therapy (N = 11, PSA < 9 µg/L).

The prognosis of the prostate cancer was determined according to the PSA values, the digital rectal examination, and the biopsy results. Into the patient control group those patients were classified whose PSA value was under 2 µg/L, their digital rectal examination didn't show any inflammation or benign enlargement on the prostate and who therefore weren't suggested to have further biopsy examination.

Into the high-risk prostate cancer group those patients were classified whose PSA value was higher than 2, even higher than 4.1 µg/L, furthermore, their digital rectal examination showed some inflammatory signs or enlargement on the prostate. They were also sent to biopsy but the results came out negative.

Patients, whose cancer condition was rated with a Gleason score, according to the anamnesis, were declared as the cancerous group. They were 1–2 stadium prostate cancer patients, their tumor didn't spread aggressively, and it didn't break through the tissue of the prostate. In their case, androgen deprivation therapy (Firmagon, Eligard, Bicalutam, Tamsulosin, Soladex, Differelin) was sufficient therefore chemotherapy, or radiotherapy was not necessarily needed.

2.3. Vitamin D₃ dosage

The 25-OH-D₃ level in blood was adjusted by a therapist to about 100–150 nmol/L (40–60 ng/mL). Each patient received individually 1000–9000 IU vitamin D₃ in a daily intake. After determining the 25-hydroxyvitamin D level in the blood, the patients continuously received the vitamin D₃ treatment for three years before this study started. The ordered amount was determined according to the estimated BMI value and their health condition. The occasionally occurring vitamin D₃ resistance was also taken into account. The most accurate adjustment of vitamin D₃ level was achieved in the high-risk prostate cancer group (Table 2), due to the fresh diagnosis and the patient's better compliance with the vitamin D₃ treatment. In most cases, the routine parameters were normalized during the treatment, but unfortunately for 10–15% of the patients, there was no improvement, which can be due to the low VDR activity. In their case, Mg supplementation and a higher dose of vitamin D₃ helped to bring vitamin D₃ in their blood to a normal level. Further medical interventions would be needed in the cases of receptor disorders or vitamin D₃ resistance. According to the body weight, medical treatment or other diseases, the vitamin D₃ treatment was sustainable with daily 1000 IU and rarely 9000 IU. Before the determination of the proper vitamin D₃ dosage for each patient, the cholecalciferol level was measured in blood that was within 10–50 ng/mL (25–125 nmol/L). The study was accomplished in accordance with the ethical permissions, which was obtained from the Hungarian Medical Research Council (Permission number: TUKEB 167/1997. 15/2004) and the Institutional Review Board of the Semmelweis University (Permission number: IKEB 3944/2004.). According to the physiological

Table 2
D₃ level (nmol/L) in patients' group after three-year treatment.

Patients	D ₃ vitamin level (nmol/L)
Patient control group (N = 8)	74.3 ± 12.3
Patient control group with vitamin D ₃ treatment (N = 9)	99.2 ± 31.5
High-risk prostate cancer group (N = 6)	36.6 ± 13.4
High-risk prostate cancer group with vitamin D ₃ (N = 8)	116 ± 24
Vitamin D ₃ treated cancerous group with androgen deprivation therapy (N = 11)	133 ± 80

data under 5 ng/ml (12.5 nmol/L) vitamin D₃ level is considered to be deficient, between 10–20 ng/mL (25–50 nmol/L) low and the normal range is between 28 and 80 ng/mL (70–200 nmol/L) [25,26]. The vitamin D₃ level was within the normal range in the vitamin D₃ treated groups and the patient control group, but in the high-risk prostate cancer group (N = 6) without vitamin D₃ treatment it was in the low range (Table 2).

2.4. Preparation of blood samples

After three-year treatment blood samples were collected in 3.2% sodium citrate anticoagulant Vacutainer tubes (Greiner Bio-One, Hungary of Vacutainer, USA) and were stored at 4 °C. They were prepared with standard routine laboratory methods. The erythrocytes were separated from plasma and buffy coat by centrifugation at 2500 rpm for 10 min. The separation of the blood was started within 1.5 h after blood collection. The buffy coat suspension was removed from the blood to avoid its bias effect while activating free radical reactions. Subsequently, the erythrocyte was washed with isotonic saline solution twice, and 20 µl of the raw fraction was standardized to 1% hemoglobin with CHR Haemoglobin reagent. For hemoglobin determination, the absorbance was measured at 540 nm on a Hitachi U-2000 spectrophotometer. After blood separation and hemoglobin standardization process the redox parameter measurements and the metal element analysis were started.

2.5. Measurement of routine laboratory parameters

The laboratory parameters (hematology, immunochemical parameters, tumor markers) were measured by Synlab Hungary Kft, after the three-year treatment when the blood was taken. The monitoring of the vitamin D₃ treatment was accomplished by the measurement of 25-(OH) D₃ vitamin/cholecalciferol in sera among the laboratory parameters. The determination of routine laboratory parameters was performed with automatized laboratory instruments in ISO 9001:2008 environment. The following laboratory parameters were measured in blood: glutamic-oxaloacetic transaminase/aspartate transaminase (AST/GOT), glutamic-pyruvic transaminase/alanine transaminase (ALT/GPT), gamma-glutamyl transferase (GGT), uric acid (UA), cholesterol (Chol), creatinine (CREA), lactate dehydrogenase (LDH), haemoglobin (HGB), mean corpuscular volume (MCV), C-reactive protein (CRP), prostate-specific antigen (PSA), free prostate specific antigen (fPSA), white blood cell (WBC). Carcinoembryonic antigen (CEA) was measured from serum with LIA-Mat immuno-luminometry (Budapest) kits.

Most of the laboratory parameters were within the normal range, and there were no significant differences between the groups, which can be due to the adequate medical treatment. Therefore these data were omitted from the results part. There was no remarkable difference in the cancerous groups in the liver parameters (GGT, GOT, GPT), creatinine and MVC parameters. Cholesterol and leukocyte were 14% and 20% higher in the high-risk cancerous group without vitamin D₃ treatment, and triglyceride was 33% higher in the vitamin D₃ treated high-risk cancerous group compared to the control. The level of CRP and CEA should give information about inflammation or the presence of tumor in the body. Despite that in our result, the value of CRP and CEA didn't exceed the normal level, and neither was much difference between groups which can be due to the patients' appropriate treatment.

The PSA level should be considered abnormal above 4.1 (µg/L). In the high-risk prostate cancer group who didn't receive either vitamin D₃ or androgen deprivation therapy, the PSA level was significantly high compared to the vitamin D₃ treated group of which PSA value was closer to the normal range but still high (Table 1). In these groups, the digital rectal examination showed the mutation in the prostate size and density, but the biopsy examination came out with negative results.

2.6. Measurement of redox parameters

The hydrogen-donating ability in plasma was estimated in the presence of 1,1-diphenyl-2-picrylhydrazyl radical according to the method of Hatano et al. [27] using Hitachi U-2000 spectrophotometer. The results of the H-donating ability was calculated in inhibition percentage (Inhib%). Induced chemiluminescence of the plasma and erythrocytes was measured by a luminol-dependent chemiluminescence assay (H_2O_2/OH -microperoxidase-luminol-based system) according to the method of Blázovics et al. [28]. This method was adapted to a Berthold Lumat 9501 manual instrument (Berthold GmbH, Germany) [29]. The results had been expressed as a relative light unit percentage (RLU%) that is inversely proportional to the degree of scavenging capacity.

2.7. Measurements of micro and macro element concentration

The separated erythrocyte samples were measured on analytic balance then they were digested in the mixture of 10 ml of HNO_3 (65%), 2 ml HCl (37%) and 4 ml H_2O_2 (30%). The procedure was performed on 200 °C in block digester. The blind was prepared under the same condition. After digestion of the samples, they were diluted with ion exchange water (17 MΩ, 28 °C; Purelab Ultra MK2. ULXXIOM2 Model) to 25 ml. Measurements were performed with a Spectro Genesis simultaneous ICP-OES spectrometer equipped with CCD detector (Kleve, Germany) and axial plasma viewing [30].

The metal element concentrations in the bovine liver as Certified Reference Material (Institute for Reference Materials and Measurements, BCR[®]-185R) were determined for the demonstration of reliability and precision of the measurement (Table 3). The recovery (R) was calculated from the declared (DV) and measured values (MV) as follows: $R = MV/DV \cdot 100$. In the case of most elements, which had no certified values, the repeatability measurements were performed (five times) with standard solutions of 0.5 μg/mL, 5 μg/mL, and 10 μg/mL concentrations, and the recovery was calculated from these results (Table 3).

2.8. Statistical analysis

The basic statistical analysis was prepared using Microsoft Office Excel 2016 and Statistica 12 (StatSoft Inc., Tulsa. USA) software. To

Table 3
Element concentration in reference material (Bovine liver Certified Reference Material, BCR[®]-185R) and standard solution, as well as the measured values with their recovery data.

Elements	Certified value (μg/g)	Concentration of standard solution (μg/mL)	Measured value (μg/g and μg/mL) (N = 5)	Recovery (%)
Al		0.5	0.502 ± 0.002	100
B		0.5	0.503 ± 0.006	101
Ba		0.5	0.501 ± 0.002	100
Ca		10	9.99 ± 0.04	99.9
Cd	0.544		0.534 ± 0.027	98.2
Co		0.5	0.500 ± 0.002	100
Cr		0.5	0.497 ± 0.001	97.5
Cu	277		279 ± 6	99.4
Fe		10	9.99 ± 0.04	99.9
Li		0.5	0.501 ± 0.005	100
Mg		0.5	0.510 ± 0.002	102
Mn	11.07		10.8 ± 0.6	97.6
Ni		0.5	0.498 ± 0.006	99.6
P		50	49.9 ± 0.5	99.8
Pb	0.172		0.171 ± 0.006	99.4
S		50	50.5 ± 0.6	101
Sr		0.5	0.502 ± 0.003	100
Ti		10	9.99 ± 0.052	99.9
V		0.5	0.498 ± 0.007	99.6
Zn	138.6		140 ± 6	101

verify distribution, Shapiro–Wilks test was used. The data had non-parametric distribution. Therefore Mann–Whitney *U* test was used for the comparison of groups. The significance level was determined at $p < 0.01$. To calculate the correlation between data Spearman's rank correlations were used as they are more appropriate for not normal distribution data than a linear correlation (e.g Pearson). Significant correlations were determined at $p < 0.05$.

3. Results

There are significant differences between the mean values of essential and non-essential element concentrations measured in the treated and non-treated groups as it is shown in Table 4. Interestingly, the concentration of Pb was higher in the vitamin D₃ treated patient control and diseased groups than that of in the non-treated groups. The difference was significant ($p < 0.01$) between the vitamin D₃ treated cancerous group and the non-treated high-risk cancerous patients. Similar tendency could be observed in the Ca concentration, which was also elevated in the vitamin D₃ treated groups compared to the groups without vitamin D₃ administration. In addition, the Ca concentration level in the vitamin D₃ treated high-risk cancerous and cancerous groups approached the control value. A significant difference ($p < 0.01$) could be observed between the vitamin D₃ supplemented patient control individuals compared to the control group.

The study also demonstrates that the Cr ($p < 0.01$) level was significantly higher in the vitamin D₃ treated patient control and diseased groups than that of in the groups without vitamin D₃ administration.

Other metal elements such as B, Co, Fe, Mg, Mn, Ti, V, and Zn represented a similar tendency to the previous results, with higher concentration in the vitamin D₃ treated patient control and cancerous groups. The Fe, Mg, Mn and Zn concentrations were remarkably higher in the vitamin D₃ treated groups compared to the non-treated ones. In the Fe level significant difference ($p < 0.01$) was found between the cancerous group with vitamin D₃ treatment and the non-treated high-risk cancerous group (Table 4). The concentration of Ni, Ca, Cr seemed to be significantly higher ($p < 0.01$) in the patient control group with vitamin D₃ administration than that of in the patient control without treatment.

The concentration of Cu and Li showed the opposite tendency to the previously mentioned metals because in the vitamin D₃ treated patient control and diseased groups the concentration of Cu and Li was lower than that of in the non-treated ones. A significant difference was found in the Cu concentration ($p < 0.01$) between the high-risk cancerous group and the vitamin D₃ treated cancerous group who received androgen deprivation therapy. The results also present that, despite the lower Cu concentration in the vitamin D₃ treated groups; its level is close to the control value in the vitamin D₃ treated high-risk prostate cancer group (Table 4), as it was also observed by the Ca concentration.

In the results of the redox parameters measured in plasma, a lower value (RLU%) could be observed in the vitamin D₃ treated patient control group compared to the non-treated patient control, which reflects higher scavenging capacity in the supplemented group (Table 5). That difference was more pronounced in the cancerous and high-risk cancerous groups while the RLU% results were lower and the RLU% in the cancerous group was significantly lower, compared to the non-treated high-risk cancer group, which indicates higher scavenging capacity in the treated groups. In erythrocyte, the induced chemiluminescence was found to have an opposite tendency to the plasma results. In the vitamin D₃ treated groups the RLU% results were higher than that of in the control group, which implies low free radical scavenging capacity in patient control and diseased groups too. The difference was significant ($p < 0.01$) between the vitamin D₃ treated diseased groups and the non-treated high-risk cancerous group (Table 5).

The outcome of the results of the H-donating ability in plasma was higher in the vitamin D₃ treated groups both in patient control and prostate cancer patients (Table 5), as it was expected from the

Table 4
Metal element concentration \pm standard deviation ($\mu\text{g/g}$) in erythrocyte after three-year treatment.

Elements	Patient control group (N = 8)	Patient control group with vitamin D ₃ treatment (N = 9)	High-risk prostate cancer group (N = 6)	High-risk prostate cancer group with vitamin D ₃ (N = 8)	Vitamin D ₃ treated cancerous group with androgen deprivation therapy (N = 11)
Al	< 0.058	< 0.058	< 0.058	< 0.058	< 0.058
B	4.57 \pm 5.89	6.18 \pm 3.106	2.39 \pm 2.42	7.04 \pm 1.97	9.39 \pm 5.05
Ba	0.447 \pm 0.273	0.245 \pm 0.172	0.316 \pm 0.311	0.115 \pm 0.149	0.113 \pm 0.136
Ca	2.84 \pm 2.36	4.32 \pm 0.99 [†]	2.44 \pm 1.91	2.94 \pm 1.79	3.32 \pm 1.46
Co	< 0.004	0.057 \pm 0.024	< 0.004	0.012 \pm 0.01	0.005 \pm 0.002
Cr	0.044 \pm 0.029	0.101 \pm 0.056*	0.026 \pm 0.015	0.097 \pm 0.025**	0.097 \pm 0.038**
Cu	0.054 \pm 0.048	0.021 \pm 0.014	0.080 \pm 0.042	0.043 \pm 0.060	0.027 \pm 0.025**
Fe	29.0 \pm 4.9	35.0 \pm 5.1	24.8 \pm 5.9	37.2 \pm 3.9	34.9 \pm 3.4**
Li	0.106 \pm 0.087	0.006 \pm 0.004	0.113 \pm 0.061	0.007 \pm 0.003	0.007 \pm 0.004
Mg	1.67 \pm 0.30	2.03 \pm 1.04	1.29 \pm 0.49	1.74 \pm 0.28	1.74 \pm 0.52
Mn	0.011 \pm 0.007	0.033 \pm 0.065	0.010 \pm 0.010	0.013 \pm 0.012	0.015 \pm 0.015
Ni	0.051 \pm 0.026	0.150 \pm 0.050*	0.461 \pm 0.500	0.170 \pm 0.109	0.248 \pm 0.304
P	14.1 \pm 3.6	10.7 \pm 2.7	12.1 \pm 3.3	12.5 \pm 3.3	10.5 \pm 2.9
Pb	0.132 \pm 0.046	0.197 \pm 0.129	0.131 \pm 0.053	0.723 \pm 0.919	1.36 \pm 0.78**
S	54.9 \pm 1.7	51.3 \pm 5.3	54.3 \pm 2.8	55.2 \pm 6.0	50.8 \pm 4.2
Sr	0.027 \pm 0.013	0.021 \pm 0.033	0.023 \pm 0.012	0.010 \pm 0.014	0.012 \pm 0.011
Ti	0.072 \pm 0.069	0.776 \pm 1.083	< 0.004	0.216 \pm 0.174	0.236 \pm 0.294
V	< 0.031	0.047 \pm 0.032	< 0.031	0.042 \pm 0.011	0.046 \pm 0.011
Zn	0.372 \pm 0.082	0.518 \pm 0.259	0.307 \pm 0.132	0.521 \pm 0.173	0.436 \pm 0.215

* significant difference to the patient control $p < 0.01$.

** significant difference to the high risk prostate cancer without vitamin D₃ treatment $p < 0.01$.

scavenging capacity results.

4. Discussion

Vitamin D₃ is an important vitamin for treatment against prostate cancer due to its probable protective effect against tumorigenesis [9,10,31,32]. In this study, the effect of vitamin D₃ on prostate cancer and its effect on the metal element- and redox homeostasis in patient control and diseased individuals were studied.

The PSA results (Table 1) show low values in the vitamin D₃ treated patient control and diseased groups. The vitamin D₃ level (Table 2) and the induced chemiluminescence in plasma (Table 5) show an adverse tendency that might refer to free radical scavenging contribution of vitamin D₃.

Significantly higher Pb concentration was found in the vitamin D₃ treated cancerous groups compared to the non-treated groups. In early and advanced stage prostate cancer other heavy metals tend to accumulate which was established by other investigations as well [33]. So far there are only a few studies that deal with the interaction between vitamin D₃ and Pb. Although, Ca absorption and metabolism are closely associated with vitamin D₃ [34] since Ca and vitamin D₃ interacts in many enzymatic pathways as it has a regulatory role in the Ca metabolism. Ca uptake is stimulated by vitamin D₃ [35] and Ca is built into the bone's structure with the intervention of vitamin D₃ [36]. One would expect that in all treated groups the level of Ca should be increased in red blood cells, but in this experiment Ca level was significantly elevated only in the patient control group. The large standard deviation can be explained by the patients' diverse eating habits. This

result needs to awake more attention while the high Ca concentration in the healthy body might lead to hypercalcemia which is among the adverse consequences of vitamin D₃ overdose.

The metabolism of Ca and Pb relates at many points in their homeostasis, and they compete for the same calcium binding sites [37–39]. It is known that Pb has a greater affinity to calcium binding proteins. Therefore small quantities of Pb can replace Ca [40]. Although, the interaction between Pb and vitamin D₃ biosynthesis is underway to be clarified [38], Pb accumulation in the erythrocyte is suspected to effect on vitamin D₃ metabolism. It has been scientifically proven that the controlled concentration of metals is essential for the normal function of the enzymes and transcription factors.

Higher Cu level, as well as lower Fe and Zn concentration, were detected in early-stage prostate cancer patients who didn't receive vitamin D₃ or androgen deprivation treatment compared to the control group that is in agreement with some studies in the literature of prostate cancer patients [41,42]. The metal element homeostasis change in cancer refers to different essential microelements and heavy metal status in the body. The abnormally elevated level of heavy metals and the accumulation or deficiency of some essential transition metals such as Co, Cu, and Fe, could also be a responsible factor for the impairment of receptor functions and enzymes activity such as tyrosine kinases [43]. It adversely affects signal molecules such as EGF, PDGF, VEGF, NF- κ B, AP-1, p53, NFAT and HIF-1 [44]. Fe deficiency can be a reason for anemia in cancer [45]. These metal elements showed higher concentration in the vitamin D₃ treated groups in the recent study. However, the level of Fe was elevated in the vitamin D₃ treatment both in patient control and diseased individuals, this result strengthens an

Table 5
Redox parameters in erythrocyte after three-year treatment.

Patients	Induced chemiluminescence in plasma (RLU%)	Induced chemiluminescence in erythrocyte (RLU%)	H-donating ability in plasma (inhibition%)
Patient control group (N = 8)	144 \pm 157	144 \pm 46	63.6 \pm 12.5
Patient control group with vitamin D ₃ treatment (N = 9)	105 \pm 122	177 \pm 39	72.0 \pm 4.2
High-risk prostate cancer group (N = 6)	217 \pm 179	125 \pm 23	62.8 \pm 14.0
High-risk prostate cancer group with vitamin D ₃ (N = 8)	71 \pm 40	156 \pm 57**	75.5 \pm 12.5**
Vitamin D ₃ treated cancerous group with androgen deprivation therapy (N = 11)	71 \pm 49**	170 \pm 70**	69.2 \pm 9.9

** significant difference to the high-risk prostate cancer without vitamin D₃ treatment $p < 0.01$.

earlier experiment that vitamin D₃ is needed to administer Fe uptake into red blood cells [46]. Zn metabolism is specific in prostate cancer since in malign prostate cells Zn level was found to be significantly low [47–49] and it has been established that Zn depletion in the prostate’s peripheral zone correlates with the Gleason score [33]. The higher Zn level can be favorable since Zn is an important microelement in the immune processes by protecting the erythrocytes against oxidative stress [50]. However, the high ratio of Zn/Fe can assist the red blood cells against the free radical load in prostate cancers progression. In our results, the Zn/Fe ratio was reduced in the early stage prostate cancer group who did not receive any treatment, however in the vitamin D₃ treated patient control group and high-risk cancerous groups the Zn/Fe ratio was higher, that was caused by the higher Zn concentration in the erythrocyte. Significantly elevated Zn-protoporphyrin concentration can be observed in erythrocytes in cancerous processes which is very important in cancer-associated anemia.[51].

The patients who received vitamin D₃ treatment had significantly high Cr level both in patient control and diseased population. In the literature so far there were very few investigations about the role of Cr in prostate cancer or the importance of vitamin D₃ in the Cr metabolism, however, in our results, the significant differences are shown between the treated and non-treated groups. Cr is an important factor in the glucose metabolism through a low molecular weight chromium (LMWCr)-binding substance [52]. As it is already established that glucose induces cell proliferation in prostate cancer [53], our result should bring up more questions about the effect of Cr in prostate cancer and of vitamin D₃ on Cr metabolism.

There are some non-significant changes for elements nevertheless their tendency can show valuable information. For example, the level of Mg was elevated in the vitamin D₃ treated groups which are in accordance with previous studies, where increased Mg absorption was observed after a pharmacological dose of vitamin D₃ supplementation [54,55]. However, Mg also effects on vitamin D₃ metabolism that enables binding to the vitamin D₃ transporter protein and facilitates the hydroxylation of vitamin D₃ to be converted into its active form in the liver and kidney [56]. Nevertheless, the role of Mg in prostate cancer needs further investigations.

Ni concentration was high in the untreated high-risk prostate cancer group but in the vitamin D₃ treated and the control groups the Ni concentration was low. The role of Ni in prostate cancer and of vitamin D₃ in Ni metabolism hasn’t been clarified yet. According to the literature, the competition between Ni and Mg in the DNA repair mechanism has been already discovered [54,57], but there are still no results investigating the interaction of these two metals in prostate cancer and how vitamin D₃ has an influence on their metabolism in normal or even in diseased conditions. According to our results, there might be a

connection in the metabolism of the vitamin D₃ and Ni, that could be beneficial, as Ni concentration approaches the control values that could also be observed by Ca, Cu, Mn and Mg values too.

It seems from our results that in the groups with vitamin D₃ treatment the Li level was lower in erythrocyte. This could be a dangerous side effect as Li deficiency can lead to neurologic diseases [58] through the inhibition of the signal transduction of phosphoinositide cycle by reducing the cellular level of inositol which is linked to memory impairment and depression [59]. Li may protect against oxidative stress, which occurs during neurologic defects, by up-regulating the NADH dehydrogenase and the cytochrome b-c1 complexes of the mitochondrial electron transport chain [59]. There is little data available in the literature about the role of Li in prostate cancer. Its anti-cancer potential was investigated and it was established that Li inhibits the glycogen synthase kinase 3 (GSK-3) action, that’s activation is associated with the prostate cancer’s progression [60].

We found lower induced chemiluminescence in the plasma (Table 5) of vitamin D₃ treated patient control and cancerous individuals compared to the patient control and cancerous group without treatment that could refer to higher free radical scavenging capacity in those groups. The results of redox parameters allow us to suppose that the permanent vitamin D₃ intake affects the redox homeostasis through its metabolism in the diseased environment and normal conditions, too. The differences between patient control groups with and without vitamin D₃ administration proposes the question whether the persistent treatment of vitamin D₃ is necessary with the current instructions. Normally, in cancer, the free radical scavenging capacity is lower than that of in the control groups, and it is usually elevated in the final stage of metastasis [61] that may show a connection to the vitamin D₃ deficiency that could lead to the activation of glutathione-dependent enzymes [62].

The elevation of the inhibition% in plasma might be due to the indirect concentration growth of those molecules which have an H-donating capacity. It is assumed that vitamin D₃ treatment has a potential role in the inflammatory suppression processes.

The oxidative stress induction by the extended concentration of certain metal elements is known and it is agreed with our results. The free scavenging capacity in erythrocytes was lower in the groups where a high concentration of Fe, Pb and Cr could be observed. Decreased Zn/Fe ratio could be observed in the groups with high RLU%. The concentration of Zn was elevated in the groups where the antioxidant capacity was high. From the literature, it is already known that Zn assists the immune strengthening processes that is possibly related to the higher scavenging capacity and facilitates the absorption of vitamin D₃ [63].

Significantly positive ($p < 0.05$) correlations were found between

Table 6

Significant correlation values (Spearman's rank correlation coefficient) between the metal elements and redox parameters or PSA; and between the vitamin D₃ level and metal elements measured from erythrocyte ($p < 0.05$).

	Patient control group (N=8)	Patient control group with vitamin D ₃ treatment (N=9)	High-risk prostate cancer group (N=6)	High-risk prostate cancer group with vitamin D ₃ (N=8)	Vitamin D ₃ treated cancerous group with androgen deprivation therapy (N=11)
PSA-Fe		0.866			
PSA-Mg					0.904
PSA-Sr		0.866			0.813
(RLU%) erythrocyte-Ba	-0.809				
(RLU%) erythrocyte-Cu	-0.762				-0.673
(RLU%) erythrocyte-Li	-0.786		-0.667		
(RLU%) erythrocyte-Mg					
(RLU%) erythrocyte-Mn	0.711				-0.775
(RLU%) erythrocyte-P			-0.767		
(RLU%) erythrocyte-Sr	-0.755		-0.812		
vitamin D ₃ level-B		0.821			
vitamin D ₃ level-Mn					0.829

the vitamin D₃ level and metal concentrations in the vitamin D₃ treated patient control group and the vitamin D₃ treated cancerous group (Table 6). In the vitamin D₃ treated patient control group the vitamin D₃ level correlated in a significantly positive way with B level (+0.821) and in the vitamin D₃ treated cancerous group the vitamin D₃ level correlated in a significantly positive way with the Mn level (+0.829). In the other groups, there were no significant correlations between the metal elements and the vitamin D₃ level.

We looked at correlations of the metal elements, the redox parameters and the PSA (Table 6). The correlations in the vitamin D₃ treated groups are different to the correlations found in the control. We can come to the conclusion that the vitamin D₃ treatment obviously had an effect on the metal element- and the redox-homeostasis. In the high-risk prostate cancer group with vitamin D₃ treatment, there was no correlation found, that can be by reason of a total homeostasis disorder. It is not reflected on the metal elements but can be generated by a large-scale change of the redox parameters. The positive correlation between PSA and Fe suggests that the high Fe level could assist the increase of PSA level.

5. Conclusion

According to our experiment vitamin D₃ plays an essential role in the inorganic elements level that can indicate its influence on the element metabolism in prostate cancer. The metal element concentration difference can further influence the overcome the disease. In the erythrocyte of patients with prostate cancer, the vitamin D₃ treatment had a positive effect on the metabolism of the most important essential elements as Ca, Cu, Mn, Mg and Ni because their concentration converged to the control values. At the same time, the potential formation of Li deficiency by the treatment of vitamin D₃ in prostate cancer needs further investigation.

From the induced chemiluminescence and H-donating ability results, it appears that the vitamin D₃ treatment favorably affected the antioxidant system because we got consistently better results in the patient control and cancerous groups. It is worth considering from our results that, the difference was more expressed in the high-risk cancerous group with vitamin D₃.

There is still no existing optimal factor for the prognosis of prostate cancer. Therefore our results could be one further step to the feasible diagnosis of prostate cancer. Besides, metal element status could be another prognostic index in prostate adenocarcinoma to differentiate between low and high-risk prostate cancer's progression phases. The significant decrease of PSA with the increase of the scavenging capacity of the treatment might be attributable to the beneficial effect of vitamin D₃ on the antioxidant system. However, further comprehensive investigations are required to understand the role of vitamin D₃ on the element status in prostate adenocarcinoma.

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Conflict of interest

Authors declare that they have no conflict of interest.

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