International Journal of Medicine Sciences
www.medicinejournal.in
Online ISSN: 2664-889X, Print ISSN: 2664-8881
Received Date: 05-11-2019 Accepted Date: 06-12-2019; Published: 13-01-2020
Volume 2; Issue 1; 2020; Page No. 17-24

Population-Based screening for prostate cancer by measuring zinc levels in prostatic fluid

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Abstract
Background: Prostate cancer (PCa) is an important and universal health problem of the man, particularly in developed countries. Prostate specific antigen (PSA) levels in blood do not provide the high reliability and precision required for an accurate screening for PCa. There is a need in a simple, rapid, direct, preferably non-invasive, and highly accurate biomarker and procedure for the screening for PCa.
Objective: To report the results from a Russian population-based randomized study of a screening using the zinc (Zn) level in expressed prostatic fluid (EPF) to detect PCa.
Methods: A total of 578 Russian ambulatory males aged over 50 years had their Zn levels in EPF determined. Men with their EPF Zn levels < 100 mg/L were subjected to further clinical examination including digital rectal examination (DRE) and transrectal prostate biopsy.
Results: The Zn levels in EPF (mean ± standard deviation, SD) in all the men (n=578) was 618±377 mg/L and, for those of them with PCa (n=3), the mean Zn level was 15±20 mg/L ($p \leq 0.001$). The Zn level in EPF increased with age up to 70 years but over 71 years old this metal's concentration began to decrease. In persons aged 51-60, 61-70, 71-80, and above 81 years, the mean ± SD values of the Zn levels in EPF were 602±329 (n=135), 655±395 (n=324), 539±354 (n=103), and 455±432 (n=16) mg/L, respectively. Among all the screened men, 33 (5.7%) had a Zn concentration in EPF < 100 mg/L. Of the patients who underwent prostate biopsy (n=11), 3 had PCa (positive predictive value, 27.3%).
Conclusion: The usefulness of Zn levels in EPF as a reliable biomarker for the screening for PCa needs to be confirmed in other randomized trials.

Keywords: Prostate, Screening for prostate cancer, Prostatic fluid, Biomarkers, Zinc

Introduction

Prostate cancer (PCa) is a most important medical, scientific and public health problem. Worldwide, PCa is the fifth leading cause of cancer deaths and the second most commonly diagnosed cancer in men [1]. PCa is especially prevalent in North America, Northern and Western Europe and Australia [2]. For example, the American Cancer Society declares PCa, with a lifetime prevalence of one in six men, is the most common cancer in American males and the second leading cause of their cancer death [3]. In terms of incidence and mortality PCa is also the leading cancer in men from Africa, Oceania, and the Caribbean [1, 2]. PCa has also become a major public health concern in China [4].

The survival rate is inversely depends on the stage reached at diagnosis, hence early-stage diagnosis using effective diagnostic tools is a key to reducing mortality due to PCa [5]. It is widely acknowledged that screening and early diagnosis of PCa are of vital importance for improving the likelihood of recovery. Historically used biomarkers such as prostatic acidphosphatase, serum prostate-specific antigen (PSA), and its precursor have not withstood the challenges of providing sensitivity and specificity. At present, current screening relies principally on PSA testing in blood serum and a PSA level of 4 ng/mL is used as the highest level compatible with non-malignant conditions. However, the PSA screening of PCa has some significant disadvantages.

Firstly, PSA is not a cancer-specific biomarker. So there can be an elevated serum concentration (≥4 ng/mL) among patients with benign prostate hyperplasia (BPH) and urogenital infections, including chronic prostatitis (CP). Reliance on PSA testing can result in significant over-detection of alleged PCa and hence inappropriate treatment of non-malignant disease [6]. Nearly 70-75% of prostate biopsies fail to detect PCa in men who undergo prostate biopsy procedures due to elevated PSA levels discovered after blood serum-test screening [5, 7]. In other words, it has been confirmed that only 25-30% of patients with a PSA value ≥ 4 ng/mL were finally diagnosed with PCa, leading to the over-treatment of low-risk patients, unnecessary biopsies and nonessential radical prostatectomies [8]. Thus, the level of PSA test specificity (selectivity) can be estimated as about 25-30%.

Secondly, the PSA test misses some aggressive tumors. For example, as was found by Thompson *et al*. (2004) that 20–25% men diagnosed with PCa including those with a poorly differentiated form (Gleason Score ≥ 8) have PSA levels below 4 ng/mL [6, 9] Data from other research shows that only 40% of patients with PCa have an abnormal PSA level [10]. Thus, the PSA test's sensitivity can be estimated as somewhere between 40-75%.

The limitations and potential harm associated with PSA screening stimulate investigation of novel biomarkers with superior ability to detect PCa, compared with traditional PSA tests, so decreasing unnecessary biopsies. Much attention is now turning to fluid-based biomarkers, because obtaining fluid samples is in effect a minimally invasive procedure. Other relevant factors of great significance for any novel method of PCa detection include cost-effectiveness, capacity to generate real-time results, "simplicity-of-use", robustness, and functionality without excessive prior-processing of samples [11].

In our previous studies the significant role of Zn and some other trace elements (TEs) in prostatic function was studied in detail for both normal and pathophysiological glands [12-52]. One of the main functions of the this gland is the production of prostatic fluid [53]. It contains a high level of Zn and some other TEs, in comparison with their concentrations in prostate tissue, blood serum and other human body fluids.

The first finding of remarkably high levels of Zn in human expressed prostatic fluid (EPF) was reported in the early 1960s [54]. After analyzing EPF expressed from the prostates of 8 apparently healthy men, aged 25-55 years, it was found that Zn concentrations varied from 300 to 730 mg/L. After this finding several investigators suggested that the measurement of Zn levels in EPF may be useful as a marker of abnormal prostate secretory function [55, 56]. This suggestion promoted more detailed studies of the Zn concentrations in the EPF of healthy subjects and in those with different prostatic diseases, including PCa [56-58]. A detailed review of these studies was given in our earlier publication [58]. Moreover, the method and apparatus for micro analysis of Zn and some other TEs in the EPF samples using energy dispersive X-ray fluorescence (EDXRF) activated by radiation from the radionuclide source ^{109}Cd (^{109}Cd EDXRF) was developed by us [59]. We reasoned that apart from total amounts of TEs the ratios of Zn to some other TE content in EPF are likely to reflect a disturbance of prostate function. It was found that data on changes of TE content and Zn/TE concentration ratios in EPF of patients with PCa are very important, because these significant changes increase our knowledge and recognition of PCa pathogenesis and may prove useful as PCa diagnostic markers [60-78]. It was concluded that the Zn level in EPF, obtained by EDXRF, is a first candidate with the role of offering a new, simple, fast, reliable, and non-invasive diagnostic tool for PCa screening [79].

To study this issue, we performed a screening for PCa using Zn level determination in EPF as a first- line screening method in Russian men. All studies were approved by the Ethical Committees of the Medical Radiological Research Centre (MRRC), Obninsk. All the procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments, or with comparable ethical standards.

2. Materials and methods

2.1 Subjects and specimens

Men older than 50 years were invited by means of media communications (local newspaper and radio) to attend the Urological Department of the Medical Radiological Research Centre for a free determination of Zn level in a prostatic fluid assay. A total of 578 volunteers (all Caucasian), who agreed by informed consent and were ambulatory, participated. Their EPF's Zn levels were measured to provide an initial test for basic screening. This study population included residents of Obninsk, a non-industrial city in the Kaluga region of Russia. None of these males had a history of PCa. All individuals with a history of prostatectomy were excluded from the study. Use of any medication or food supplement that could influence the Zn value, such as any androgen or estrogen, polyvitamins with minerals and Zn supplements, was recorded.

EPF specimens were obtained using a standard rectal massage procedure in sterile containers, which were appropriately labeled. Twenty μL (microliters) of fluid were taken in duplicate by micropipette from every specimen for Zn analysis. Each 20 μL sample of the EPF was dropped on a 11.3 mm diameter disk made of thin, ash-free filter paper fixed on pieces of adhesive tape and dried in a desiccator at room temperature. Then the dried sample was covered with a 4 μm gage Dacron film and centrally pulled onto a Plexiglas cylindrical frame [59].

2.2 Standards and Certified Reference Material

To determine concentration of the Zn by comparison with known standards, aliquots of solutions of commercial, chemically pure compounds were used for calibration [80]. The standard samples for calibration were prepared in the same way as the samples of prostate fluid. Because there were no available liquid Certified Reference Materials (CRMs), ten sub-samples of the powdered CRM IAEA H-4 (animal muscle) were analyzed to estimate the precision and accuracy of results. Every CRM sub-sample weighing about 3 mg was applied to the piece of adhesive tape serving as an adhesive fixing backing. An acrylic stencil made in the form of a thin-walled cylinder with 11.3 mm inner diameter was used to apply the sub-sample to the adhesive tape. The polished-end acrylic pestle, which is a constituent of the stencil set, was used for uniform distribution of the sub-sample upon the adhesive tape surface restricted by the stencil's inner cylindrical surface. After the sub-sample was lightly pressed onto the adhesive tape carrier, the stencil was removed. Then the sub-sample was covered with 4 μm gage Dacron film. Before the sample was applied, pieces of adhesive tape and Dacron film were weighed using an analytical balance. They were reweighed after the sample had been placed inside to determine precisely the sub-sample mass.

2.3 Facility for Zn level analysis

The facility for the radionuclide-induced EDXRF included an annular ^{109}Cd source with an activity of 2.56 GBq, A Si(Li) detector with an electric cooling system and a portable multi-channel analyzer based on a personal computer, comprised the detection system. Its resolution was 270 eV at the 6.4 keV line.

The facility functioned as follows. Photons with energy 22.1 keV from the ^{109}Cd source arrived at the surface of the specimen and penetrated it, inducing fluorescent K_α X-rays from the Zn. The fluorescence reached the detector after passing through a 10 mm diameter collimator. Then the X-ray's arrival was recorded. The duration of the measurements of Zn concentration was 10 min for each sample. If the Zn concentration in a sample was lower than 100 mg/L, the analysis of each sample was repeated and the duration of the measurement was 60 min/ The intensity of the K_α-

line of Zn for EPF samples and standards was estimated from a calculation of the total area under the corresponding photopeak in the spectra.

2.4. Computer programs and statistic

All EPF samples for EDXRF were prepared in duplicate and mean value of Zn content was used in the final calculation. Using the Microsoft Office Excel programs, some statistical characteristics, such as arithmetic mean (M), standard deviation (SD), standard error of the mean (SEM), minimum and maximum values (Range), and median were calculated for Zn concentrations in the EPF of four age groups: group 1 (51-60 years), group 2 (61-70 years), group 3 (71-80 years), and group 4 (over 81 years). The difference in the results between the six pairs of samples in groups 1 and 2, groups 1 and 3, groups 1 and 4, groups 2 and 3, groups 2 and 4, and groups 3 and 4 was evaluated by the parametric Student's t-test and non-parametric Wilcoxon-Mann-Whitney U-test. Values of $p<0.05$ were considered to be statistically significant. For the construction of the diagram illustrating individual data set for Zn concentrations in the EPF the Microsoft Office Excel software was also used.

2.5 Clinical study

If the value of Zn concentration in EPF was < 100 mg/L, the subject was referred for DRE and transrectal prostate biopsy in clinical conditions. Palpable abnormalities were characterized according to the International Union Against Cancer (UICC) 1992 [81]. The histological criteria for prostate cancer used were those of the World Health Organization (WHO) [82]. The clinical stage was evaluated according to the tumor node metastasis (TNM) classification UICC and the American Joint Committee on Cancer (AJCC) [83]. One urological pathologist did all pathological evaluations. Information on the patient's age, race, DRE result, Zn level in EPF, and biopsy result (cancer or no cancer) was entered into a computerized database for analysis.

3. Results

A total of 578 subjects were recruited in this study. The mean age of these males was 66 years ranging from 53 to 93 years. Of these, 135 (23.3%) were between 51 and 60 years (age group 1), 324 (56.1%) were between 61 and 70 years (age group 2), 103 (17.8%) were between 71 and 80 years (age group 3), and 16 (2.8%) were ≥ 81 years of age (age group 4). The concentrations of Zn were measured in all prostatic fluid samples of the study (Figure 1). Table 1 presents certain statistical parameters (arithmetic mean, standard deviation, standard error of mean, minimal and maximal values, median, percentiles with 0.025 and 0.975 levels) of the Zn concentrations in EPF of males investigated in this study.

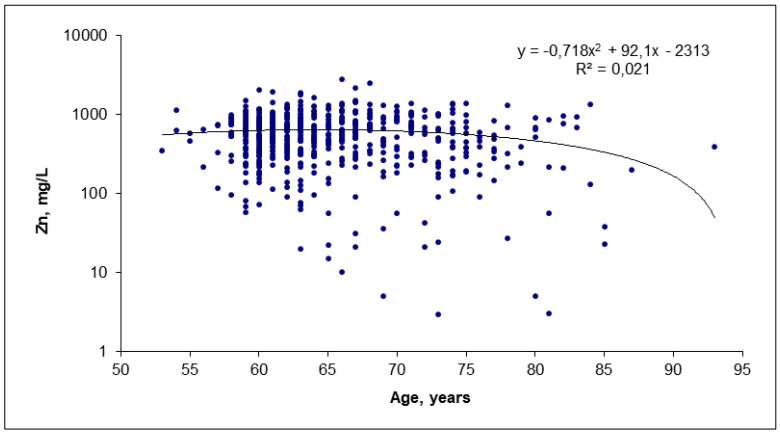

Fig 1: Data sets of individual concentrations of Zn in prostatic fluid of men aged 53-93 years and trend of concentrations with age.

Table 1: Some basic statistical parameters of Zn concentration (mg/L) in prostate fluid for four age groups of men recruited for prostate cancer screening

Age Group years	n (%)	Mean	SD	SEM	Min	Max	Median	Per. 0.025	Per. 0.975
51-60	135 (23.3%)	602	329	28	58	2048	585	87	1180
61-70	324 (56.1%)	655	395	22	5	2813	610	56	1481
71-80	103 (17.8%)	539	354	35	3	1411	507	22	1362
≥81	16 (2.8%)	455	432	111	3	1340	217	10	1212
Total53-93	578 (100%)	618	377	16	3	2813	589	37	1410

M - arithmetic mean, SD – standard deviation, SEM – standard error of mean, Min – inimum value, Max – maximum value, Per. 0.025 – percentile with 0.025 level, Per. 0.975 – percentile with 0.975 level.

Levels of Zn concentration in EPF depended on age (Figure 1 and Tables 1-3). In males aged 51–60, 61–70, 71–80 and ≥81 years, the Zn values (mean ± SD) in EPF were 602±329, 655±395, 539±354 and 455±432 mg/L, respectively (Table 1). Medians of the Zn values in EPF for age group 1, 2, 3, and 4 were 585, 610, 507, and 217 mg/L, respectively (Table 1).

The distribution of Zn levels in EPF of all subjects (n=578) was a little skewed to the left, unlike a normal distribution (Figure 2). Therefore, age group comparisons were performed for each pair of groups using parametric Student's *t*-test and non-parametric Wilcoxon-Mann-Whitney *U*-test (Tables 2 and 3).

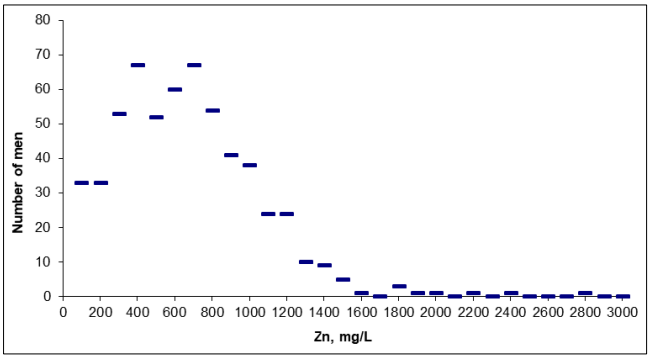

Fig 2: The histogram of Zn concentration (mg/L) in in prostatic fluid of men recruited for screening for prostate cancer (total n=578)

Table 2: Ratio of means and the difference (Student's *t*-test) between mean values of Zn concentration (mg/L) in prostate fluid of four age groups: 51-60 (1), 61-70 (2), 71-80 (3), and ≥81years (4)

1 and 2		1 and 3		1 and 4		2 and 3		2 and 4		3 and 4	
Ratio 2/1	***p*≤**	**Ratio 3/1**	***p*≤**	**Ratio 4/1**	***p*≤**	**Ratio 3/2**	***p*≤**	**Ratio 4/2**	***p*≤**	**Ratio 4/3**	***p*≤**
1.09	0.13	0.90	0.16	0.76	0.22	0.82	0.005	0.69	0.09	0.84	0.48

Table 3: Ratio of medians and the difference (Wilcoxon-Mann-Whitney *U*-test) between mean values of Zn concentration (mg/L) in prostate fluid of four age groups: 51-60 (1), 61-70 (2), 71-80 (3), and ≥81years (4)

1 and 2		1 and 3		1 and 4		2 and 3		2 and 4		3 and 4	
Ratio 2/1	***p*>**	**Ratio 3/1**	***p*>**	**Ratio 4/1**	***p*>**	**Ratio 3/2**	***p*≤**	**Ratio 4/2**	***p*≤**	**Ratio 4/3**	***p*>**
1.04	0.05	0.87	0.05	0.37	0.05	0.83	0.01	0.36	0.05	0.43	0.05

Among the screened males, 33 (5.7%) had Zn concentration in EPF < 100 mg/L. They were selected as the group with high risk of PCa. Age distributions of these 33 males were 5 (15.2%), 16 (48.5%), 8 (24.2%) and 4 (12.1%) in ranges 51–60, 61–70, 71–80 and ≥ 81 years, respectively. The relative portions (%) of males with Zn concentrations in EPF under the cut- off value < 100 mg/L in the different age groups increased with age and were 3.7%, 4.9%, 7.8%, and 25% in groups 1, 2, 3, and 4, respectively. The Zn value (mean ± SD) in EPF in all subjects (n=578) was 618±377 mg/L and in those with Zn level under the cut-off value (n=33), 47±31 mg/L (p= 0.0001).

Of the 33 subjects with Zn levels in EPF under the cut-off value 100 mg/L, only 11 (33.3%) underwent prostate biopsy. In the other 22 cases biopsy was not done because of patient refusal. Of 11 patients who underwent prostate biopsy, 3 had PCa, 4 had benign prostatic hyperplasia (BPH), and 4 benign prostatic hyperplasia combined with chronic prostatitis (BPH+CP). Thus, the positive predictive value {PPV) was estimated to be 27.3% (3 from 11 persons) for Zn levels in EPF alone. Mean (mean ± SD) values of Zn concentration in EPF of patients with PCa, BPH, and BPH+CP in these selected groups were 15±20, 65±32, and 66±28 mg/L, respectively. In the 2 men with prostate cancer, the clinical tumor stage was T2N0M0 (66.6%) and in one person T3N0M0 (33.3%).

4. Discussion

As was shown by us in previous studies [56-79], results from the use of CRM IAEA H-4 as certified reference materials for the analysis of samples of EPF is acceptable. Good agreement of the Zn content, analyzed by the ^{109}Cd EDXRF method, with the certified data of reference materials indicates an acceptable accuracy for the results obtained in the current study and presented in Tables 1-3 and Figures 1 and 2.

In our previous study a strongly pronounced tendency for an age-related increase of Zn concentration was observed in EPF of healthy males for the third to seventh decades [60]. Results of the present work confirmed this conclusion (Tables 1-3 and Figure 1). Moreover, it was found that the Zn level in EPF began to decrease in age over 70 years. Our finding for the Zn age-dependence does not agree with data published by Kavanagh *et al*. (1982) [84] when 33 specimens obtained from normal male subjects in age from 15 to 85 years were measured by atomic absorption spectrometry and the Pearson correlation between age and Zn concentration was used.

The PCa screening era using PSA started in 1991, the year when Catalona *et al*. showed that PSA could be used as a first-line screening test for PCa in men without suspicious DRE findings [85]. In Catalona *et al*.'s study 1653 healthy men of the US population, 50 or more years old, were enrolled in the investigation. The initial blood serum PSA values were > 4.0 ng/ml in 137 persons. In other words, the group with high risk of PCa consisted of 137 from 1653 men (8%). Similar results were obtained in further studies in many European countries in which blood serum PSA concentrations of 4 ng/ml or higher were detected in a range from 5% [86] to 13% of screened men.[87] In our study Zn concentrations in EPF below the cut- off value, 100 mg/L, were detected in 33 out of 578 men (5.7%). Thus, the selection possibilities of serum PSA and Zn in EPF tests in recognizing the PCa high risk group during a population screening were similar.

In the primary healthcare setting in many countries there was a significant problem in getting men with an elevated serum PSA level to undergo a prostate biopsy. The biopsy rates reported in the published work ranged between 19% and 90%.[87,88[Of the 33 males in the group with high risk of PCa in our study only 11 agreed for further clinical examination with underwent prostate biopsy. So, the biopsy rate in our study equalled 33.3% and was inside the range of biopsy rates usual for population screening for PCa.

In large-scale randomized PSA screening trials in the USA and Europe [85-87,89,90], as well as in PSA population screening studies in China [91, 92] and some other countries [88, 93, 94] PPV values varied in range between 11% and 30% for PSA alone with median about 20-25%. In Catalona *et al*.'s study [85] PPV was estimated to be 27%, which was very similar to our finding in a screening study with Zn in EPF as PCa biomarker (PPV=27.3%). The PPV value in our Zn EPF screening study (27.3%) was somewhat higher than the PPV of PSA screening trials in Russia (24.1%) [95].

In our study the prostate biopsy was performed in only 33.3% of those who had an indication for biopsy (11 of 33 persons) and the

PPV value was 27.3%. If we assume that PPV was the same in group of participants who refused biopsy (22 persons), number of prostate cancers detected per 1,000 screened men would be 16. Thus, the PCa detection rate in Russian men, 50 years or older, estimated by our data of Zn EPF screening was 1.6%. This result was very close to data reported in larger PSA screening studies in European countries including Russia [86, 87, 90, 95].

Thus, all characteristics of Zn in EPF's usefulness for the screening for PCa estimated in present study are at least no worse than those of the PSA test. However, the new method developed by us has many advantages in comparison with PSA test. First of all, it is not an invasive method because samples of EPF can be obtained using a standard rectal massage procedure during DRE, while the serum PSA test damages the blood vessels' walls by venipuncture. Levels of Zn in EPF as a PCa biomarker depend less strongly than PSA on age, prostate volume and the presence of absence of prostatitis [60, 79, 89]. The new method is very fast because the EDXRF used for Zn determination is a fully instrumental and non-destructive method and a drop of EPF is investigated without requiring any sample pretreatment or its consumption. Modern devices for EDXRF analysis with X-ray tubes, including "the total reflection" version (TRXRF) of the method, allow reliable determinations of Zn concentration in a drop of EPF within a few minutes [96]. Therefore, in our opinion, our results suggest strongly that the Zn in EPF test without suspicious DRE findings can at least replace the PSA level determination in screening for PCa.

After large-scale use of PSA screening for PCa in the USA and Europe during the last 15 years, the PSA test's value has been challenged. This is because the application of such screening leads to unnecessary biopsies (up to 75-80% of resulting biopsies are in principle avoidable) and other treatment may lead to alteration of quality of life issues, such as incontinence or impotence.[89,90] At the end of these debates numbers of PSA tests declined in the USA and Europe after 2008. However, recent studies in the US shown that during the PSA screening era (1994-2008) there was a 53% decrease in the PCa mortality rate and therefore the recommendation against all PSA screening was patently wrong [89]. Because the usefulness of PSA screening for PCa detection was proven, it is obvious that novel early-diagnostic biomarkers are urgently needed to be discovered and evaluated to distinguish persons with prostatic cancers from healthy men. Numerous new PCa diagnostic tests are currently under development [90] but none show diagnostic characteristics much better than PSA test.

In our opinion, results of population screenings for PCa can be significantly improved by using a combination of PSA and Zn detection in EPF. In Whelan *et al.*'s (2013) study it was found that the level of PSA mRNA was significantly elevated in EPF specimens obtained from patients with a subsequent diagnosis of prostate cancer [97]. Thus, if in patients with PCa the levels of Zn and PSA in EPF decreased and increased, respectively, since the mechanisms of these parameters' formation is different, therefore the ratio of concentrations of Zn/PSA in EPF seems to be a promising biomarker for early prostatic cancer detection.

Our main study limitation was the number of subjects screened in the single research center (MRRC, Obninsk, Russia) where this work was done. It is the first pilot study so it needs to be repeated on a larger scale.

5. Conclusions

There is a critical need for a highly reliable, accurate, simplified biomarker and procedure for the screening for PCa, or as an adjunct for the PSA test during the urological examination of patients as candidates for prostate biopsy. In the present work, for the first time Zn concentration measurements were carried out in the EPF samples from 578 apparently healthy men as a screening test for PCa. Concentrations of Zn in EPF samples were measured by the non-destructive, instrumental EDXRF micro method developed by us. The present study reveals that Zn level in EPF alone is a fast, reliable, and non-invasive diagnostic tool that can be successfully used by physicians at the point-of-care as a first-line and as a repeat screening test for PCa. A larger study is necessary to confirm our observations.

Competing Interests

Authors have declared that no competing interests exist. The authors alone are answerable for the substance and composing of the paper. The authors did not receive any funds from any source.

Ethical Statement

All the procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments, or with comparable ethical standards.

Acknowledgment

The authors are grateful to Dr Tatyana Sviridova, Medical Radiological Research Center, Obninsk for supplying EPF samples. The authors are also extremely grateful to Dr. Sinclair Wynchank for a very valuable and detailed discussion of the results of this work and his help in English.

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