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Urinary and Plasma Cell-Free DNA Integrity as Potential Biomarkers for Prostate Cancer

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Abstract

Prostate cancer is the second most common and the fifth leading cause of death from cancer in men worldwide. The origin of Cell-Free DNA (cfDNA) in healthy individuals is solely apoptosis which releases short DNA fragments. On the other hand, cancer cells release both short and long DNA fragments via apoptosis and necrosis respectively. This study aimed at assessing the efficacy of plasma and urine cfDNA integrity as non-invasive and practical screening and diagnostic biomarkers for PCa in comparison to the well-established PSA, elucidating the superiority of either plasma or urine samples regarding their diagnostic efficacy, in addition to investigating the use of these biomarkers in a sample of Egyptian patients, hence validating its genetic correlation to this ethnic group. A case-control study was designed recruiting one hundred and ten subjects; 46 prostate cancer patients, 44 benign prostatic hyperplasia patients and 20 apparently healthy individuals as a control group. Plasma and urine cfDNA integrity were measured using SYBR green-based quantitative Polymerase chain reaction for Arthrobacter luteus (ALU) repeats by measuring the ratio of longer fragments ALU 247 base pair to shorter fragments ALU 115 bp. Plasma and urine cfDNA integrity were significantly higher in the prostate cancer group compared to the other two groups ($p<0.001$). The area under receiver operating characteristic curve was 0.935 for plasma cfDNA integrity, 0.873 for urine cfDNA integrity and 0.792 for tPSA when comparing prostate cancer to benign prostate hyperplasia patients. Plasma and urine cell free DNA integrity could be used both as; screening and diagnostic biomarkers for prostate cancer. However, the plasma was superior to the urine among the studied Egyptian patients.

Keywords: Prostate cancer; Benign Prostatic Hyperplasia (BPH); cell-free DNA, DNA integrity; Screening.

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1. Introduction

Prostate cancer (PCa) is the second most common and the fifth leading cause of death from cancer in men worldwide. In 2018, the number of new cases diagnosed with PCa was estimated to be 1,276,106 and the number of deaths was 358,989 [1]. Although prostate specific antigen (PSA) screening is extremely sensitive, it lacks specificity and only 26% of patients within the grey zone PSA (4.1-9.9 ng/ml) are actual cases of PCa, this means that around three-quarters of the patients within this grey zone are subjected to needless biopsies [2]. Cell-free DNA (cfDNA) is composed of both genomic DNA (gDNA) and mitochondrial DNA (mtDNA) [3]**.** In healthy individuals, the main source of circulating cfDNA is apoptotic cells which release uniformly truncated DNA fragments ~200 base pair in length, in contrast to DNA released from malignant cells via necrosis or mitotic catastrophe, which varies in size because of random and incomplete digestion of gDNA [4]. In addition to circulating plasma cfDNA which is considered as a breakthrough in personalized medicine, urine cfDNA has the same merits of plasma cfDNA and even better in terms of patients' needs and compliance as it is non-invasive, non-hazardous, and large sample volume can be obtained easily [5].

Many published original articles have found that cfDNA is larger in cancer patients (> 200 bp) compared to normal individuals (180-200 bp). This was observed in different cancer types like prostate cancer, renal cell carcinoma, bladder cancer, oesophageal cancer, and others [4,6–12]. Moreover, Chen et al. (2017) published a review article on cell free DNA integrity among different cancer types and found that in the vast majority of the studies, cell free DNA integrity was higher in cancer patients than control groups [13]. In view of the aforementioned data the need for a diagnostic tool that enables early detection in a less invasive matter; and hence a better management protocol, is mandatory for clinical application in diagnosis and early detection PCa.

In this study, quantitative Polymerase chain reaction (qPCR) dependent on DNA fragment size was used to quantify and assess cfDNA integrity using the ALU repeated DNA sequence. ALU repeats are the most

abundant sequences in the human genome, with a copy number of about 1.4 million per genome [14]. ALU sequences are short interspersed elements (SINEs), typically 300 nucleotides, which account for more than 10% of the genome [15]. DNA integrity index is represented by the ratio of longer DNA fragments to shorter ones [4]**.** Considering all of the previously mentioned facts; the current study aimed to assess the efficacy of plasma and urine cfDNA integrity as non-invasive and practical screening and diagnostic biomarkers for PCa in comparison to the well-established PSA and to elucidate the superiority of either plasma or urine sample regarding their diagnostic efficacy.

2. Materials and Methods:

2.1 Participants

Compliance with Ethical Standards: The study protocol number (5/2015) was approved by the local ethics committee of the Clinical and Chemical Pathology Department, Faculty of Medicine, Cairo University, Egypt as to be in accordance with Helsinki Declaration II, Finland. Each participant provided written informed consent to take part in the study.
Patients were enrolled from Urology and Surgical Oncology outpatient clinics of the Faculty of Medicine, Cairo University, Egypt during the period from September 2016 to April 2017. Patients were selected according to the following criteria: Patients presenting with lower urinary tract symptoms, whose serum total PSA levels was greater than 4 ng/mL and did TRU/S and biopsy (laterally directed, 12 core biopsy). A transrectal approach was used, a biplane probe (5-8 MHz range) with a combination of end viewing and side viewing transducers was used. Ultrasound gel was applied over a latex condom applied onto the probe. All patients were examined in the left lateral decubitus position. A full urinary bladder helped in better visualization of the gland. Sampling of the prostate was performed either in the Sagittarius or in the axial plane. Biopsies were obtained using a needle gun (18 G x 20 cm). Confirmation of cancer diagnosis was achieved by TRU/S and biopsy for patients with PSA > 4, abnormal DRE, or both. In BPH group prostatitis was excluded by routine urine analysis and culture and sensitivity free samples. Risk stratification was calculated according to European Association of Urology Guidelines (European Association of Urology website, prostate cancer guidelines, accessed 22/01/2020, 13:36) [16] BPH was diagnosed by: a) Clinical symptoms and signs. 2) Digital rectal examination. 3) Laboratory: PSA, Urinalysis, and Urine culture and sensitivity. This allowed us to exclude other causes such as malignancy and prostatitis, highly suspected cases underwent biopsy and were included in the relevant group.
Control group: Healthy individuals at their twenties without any signs or symptoms. The control group was selected to be this age and not matching the age of the patients, as the prostate undergoes inevitable hyperplasia with age. They were healthy individuals without any lower urinary tract symptoms, enrolled from the outpatient clinics attendee and volunteers. They were consecutive.

Classification and staging of diagnosed PCa patients were applied using WHO histological classification of prostate tumors (2016), American Joint Committee on Cancer (AJCC) clinical, TNM and pathological classification of prostatic tumors (2012) and Histopathological grading[17,18]. While the state of metastasis was assessed using pan CT and bone scan, which was done only for patients with diagnostic confirmation for PCa. Diagnosis of BPH was based on PSA in the grey zone and negative prostate biopsy. Treatment strategies were as follows, patients were treated with open retro-pubic radical prostatectomy, or Radiation therapy combined with complete androgen deprivation therapy, according to their stage. Urine and plasma samples were collected accordingly, and then the entire biochemical and genetic workout was performed in the Molecular and Genetic Studies Unit.

2.2 Sample collection and qPCR for cfDNA

The urine and plasma samples were collected before TRU/S and biopsy. Additionally, sampling was done for de novo cases or cases on treatment and then comparison between these subgroups was analyzed. For patients on treatment, samples were collected before surgery, and two weeks after hormonal or radiotherapy.

2.2.1 Urine Sample Collection as follows:

Spontaneous micturition was encouraged, and the patients were asked not to do prostate massage prior to sampling as this induce the release of prostate fluid via the urethra into the urine. This will falsely increase the amount of the measured cfDNA and the sample in this case will be not valid due to the additional source of DNA which is not cfDNA [19]. Subsequently, ten milliliters of urine were collected in sterile leak-proof urine cups and centrifuged at 3000 rpm for 20 minutes within 1 hour of collection. The supernatant was aliquoted and frozen at -80 °C until analysis time.

2.2.2 Plasma Samples Collection and Processing

Blood samples were collected as follows; Six milliliters of blood were drawn and dispensed equally between a sterile Ethylenediamine tetra acetic acid (EDTA) vacutainer and sterile plain vacutainer. The EDTA vacutainer was centrifuged in two steps (3,000 rpm for 10 min and then 12,000 rpm for 10 min) to ensure collection of cell-free plasma, plasma was separated at room temperature and then the supernatant was aliquoted and frozen at -20°C till analysis time. The sterile plain vacutainer was used for serum separation for total and free PSA measurement by electro-chemiluminescence immunoassay on Cobas E601 analyzer (Roche Diagnostics, F. Hoffmann-La Roche Ltd).

2.2.3 Sample preparation for qPCR or cfDNA isolation and analysis

cfDNA was extracted from 400 µl urine and 200 µl plasma samples using ZYMO Quick-gDNA™ MiniPrep **(**Catalog number: D3024). The extracted DNA was eluted in 50 µl of elution buffer. This was followed by measuring the absorbance at 260 nm (A260) and A260/A280 ratio to determine cfDNA concentration (ranged from 40-60 ng/ul) and purity respectively, using the NanoDrop spectrophotometer. DNA purity ranged from 1.8 to 2.1.

2.2.4 qPCR conditions and quantification of ALU fragments

2.2.4.1 Primer Selection

cfDNA Quantification and integrity assays were performed by quantitative real-time PCR analysis. Two primer sets were used[4]; the first set for the 115-bp amplicons to amplify both shorter and long DNA fragments representing the total amount of cfDNA. The second set of primers for the 247-bp amplicons to amplify only long DNA fragments representing the DNA released from non-apoptotic cells. The ratio of ALU247 to ALU115 reflected the integrity of cfDNA. Quantitative real-time PCR was performed on Applied Biosystems Step One Real-Time PCR. For each sample, two tubes were used one for ALU115 and the other for ALU247. Each 20 µL reaction consisted of 10 µL Master Mix (*SuperReal PreMix Plus(SYBR Green)* kit, cat.no, FP205), 5 µL DNA sample, 3.8 µL nuclease-free water and 0.6 µL (25nmol); supplied by Invitrogen™ by life technologies™; Thermo Fisher Scientific , MA USA 02451**.** from each of the forward and reverse primers as indicated in (table 1).

2.2.4.2 PCR amplification conditions

Pre-cycling heat activation of DNA polymerase at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 30s, annealing at 64°C for 30s and extension at 72 °C for 30s. Following amplification, melt curve analysis was performed to assess the amplicon specificity and primer dimer formation; the thermal profile was as follows: 95 ˚C for 15s, 60 ˚C for 60s, this is followed by a ramp of 0.3 ˚C /s up to 95 ˚C for 15s. Amplification and melting curves are shown in Fig.(1)

2.2.4.3 Interpretation of the results

Done by absolute quantification of cfDNA using an external calibration curve method [6]. The five-point calibration curve was performed by serial dilutions (10, 1, 0.1, 0.01 and 0.001 ng/µL) of gDNA extracted from peripheral blood of a healthy volunteer and adjusted to the above mentioned concentration [4] A negative control (without template) was performed on each plate. cfDNA was calculated in ng/ml using the regression equation from the standard curve for both ALU 115 and ALU 247. This was done through the following equations.

Log concentration = CT– (intercept * R^2) / slope.
Concentration = log concentration to the power 10
Where, CT: is obtained from the PCR; Slope, Intercept, and R^2 are obtained from the standard curve, data were as follows; ALU 115, -3.7, 9.46, 1 and ALU 247, -3.6, 10.56, 1 respectively.

2.3 Statistical methods

Data were statistically calculated using SPSS-18. Qualitative data were presented as frequencies and percentages. Abnormally distributed quantitative data were represented as median and interquartile range and were compared between two groups using Mann Whitney test and between more than two groups using Kruskal-Wallis test. Receiver operating characteristic curve (ROC) analysis was plotted to determine the Area under Curve (AUC), sensitivity and specificity of plasma and urine cfDNA levels and their integrity in PCa diagnosis. Forward stepwise logistic regression was run to select the minimum combination of variables that maximally discriminates between patients and control subjects. P <0.05 is considered statistically significant**.**

3. Results and discussion

This study recruited a total of 110 Egyptian participants, they were divided into three groups: 46 PCa patients, 44 benign prostate hyperplasia (BPH) patients and 20 apparently healthy individuals.

3.1 The clinical and demographic characteristics of study participants

The mean age was 69 ± 7 years for PCa patients, 59 ± 6 years for BPH and 21 ± 0.5 years for the control group. (Table 2) shows the characteristics of PCa patients regarding family history, disease severity, treatment status, the state of metastasis and risk stratification. Forty-five (45) % of cases were scheduled for treatment.

3.2 cfDNA concentration and integrity among the study participants

The median level of urine and plasma cfDNA integrity index were significantly higher in PCa patients than in BPH and control groups ($p < 0.05$), as shown in (Table 3). Furthermore, PCa patients were subdivided into non- metastatic and metastatic groups; plasma and urine cfDNA integrity were not statistically significantly different between metastatic and non-metastatic patients p=285 and p=784 for plasma and urine respectively. (table 4_a). No statistically significant difference was found in plasma integrity or urine integrity in patients who have not started treatment when compared to those on hormonal therapy, radiotherapy or underwent surgery p= 0.15 and p= 0.16 for plasma and urine integrity respectively (Table 4 $_b$) or between low risk, moderate and high risk patients p=0.39 for plasma integrity and p= 0.8 for urine integrity (table 4 $_c$). Finally, PCa patients were also compared regarding their family history, again there was no statistically significant difference among patients with positive and negative family history, p= 0.23 for plasma integrity and p= 0.42 for urine integrity (table 4 d).

3.3 Sensitivity, specificity and cut off values

As a tool in discriminating patients with PCa from BPH, ROC curve was plotted and yielded an AUC plasma and urine cfDNA integrity as shown in (Fig. 2&3). Additionally, plasma and Urine cfDNA integrity as well as tPSA sensitivity, specificity, positive predictive value (PPV), Negative predictive value (NPV) and overall diagnostic accuracy are presented in (Table 5).

3.4 Regression analysis and patient identification

Forward stepwise logistic regression was run to select the minimum combination of variables that maximally discriminates between patients with PCa and those with BPH. Disease status (PCa or BPH) was used as the (binary)

dependent variable; while plasma cfDNA integrity, urine cfDNA integrity and tPSA were entered as independent (predictor) variables. These variables were entered in the analysis in a stepwise fashion starting with the variable with the highest significant score. Plasma cfDNA integrity was associated was the highest score, 24.2 (P<0.001) and was the first predictor included in step one. At step two, tPSA was chosen to be included in the model because its score, 17.5, was significant (P<0.001). Urine cfDNA integrity was at step three with a score of 15.9 (p<0.001). The predicted probability of cancer prostate was calculated for the 90 studied patients and was compared against their actual cancer prostate found. The overall predictive accuracy was 91.1% (82 out of 90), better for predicting cancer prostate (42 out of 46; 91.3%) than BPH (40 out of 44; 90.9%). When comparing PCa patients and healthy controls, Plasma cfDNA integrity was associated with the highest score, 25 (P<0.001) and was the first predictor included in step one. At step two was urine cfDNA integrity with score 6.9 (P = 0.01). The predicted probability of cancer prostate was calculated for the 66 studied subjects and was compared against their actual cancer prostate found. The overall predictive accuracy is 95.4% (63 out of 66), better for predicting PCa (44 out of 46; 95.4%) than control subjects (19 out of 20; 95%) (Table 6)

This study was designed to evaluate the efficacy of plasma and urine cfDNA integrity as non-invasive screening and diagnostic biomarkers for PCa in comparison to the well-established tumour marker tPSA, in addition to elucidating the superiority of plasma or urine cfDNA integrity as regarding their diagnostic efficacy in PCa. To the best of our knowledge in prostatic diseases, several studies investigated the role of either plasma or urine cfDNA integrity alone to differentiate PCa from benign prostate hyperplasia (BPH) [7,9,12,20] However, none of them studied their combined role in PCa diagnosis. In this study, plasma was used rather than serum to generate more reliable results. cfDNA is remarkably more abundant in serum samples compared to plasma samples. Nevertheless, when this difference in yield was further analysed, it was found that serum samples show notably more variation between patients and the elevated levels of cfDNA concentration in serum samples may be due to gDNA contamination during the clotting process rather than being actual cfDNA. Therefore, it is advisable rather to use plasma than serum in evaluating circulating cfDNA [5,21,22].

In the current study, it was found that the median level of plasma cfDNA integrity was significantly higher in PCa group when compared to both BPH and healthy control groups. These results were in agreement with the results of Feng et al. and Khani et al. [8,12]in an Iranian population who found a significant difference in plasma cfDNA integrity between PCa group and BPH (p<0.001). Moreover, this finding agrees with the results of a study done on an Egyptian population which also found that plasma cfDNA integrity was higher in PCa compared to BPH patients (p >0.001)[6]. Similarly, a previous work included (123) PCa patients and (20) healthy controls, there was a statistically significant difference in plasma cfDNA integrity between the 2 groups (p<0.001) [10].

Concerning the median level of urine cfDNA integrity, the PCa group had significantly higher values when compared to BPH and healthy control groups (p<0.001). These results support those of Casadio et al. [23], who compared urine cfDNA integrity between PCa group and healthy control group and found a statistically significant difference between the two groups (p<0.01). However, this outcome was contradictory to the results of Salvi and colleagues who found no significant difference in median levels of urine cfDNA integrity between the two groups (p>0.05) [9]. The reason for the deviant results is that the Salvi et al. study included patients with benign diseases that also lead (like PCa) to an elevated release of cfDNA and longer fragments. So that cfDNA integrity index might be feasible in distinguishing PCa from BPH, but seems not well suited to distinguish PCa from all benign diseases that lead to an increase cell death (like inflammations). Further studies may be required to address this issue. So far and to the best of the researchers' knowledge it can only be said that the cfDNA integrity index has some value in distinguishing BPH from PCa.

The present study found no statistically significant difference in the median level of plasma DNA integrity between low risk (5.9), moderate risk (2.6) and high risk PCa patients (3.1) (p=0.39). These results are consistent with the results of four studies done on different populations including Egyptian, Iranian and Chinese, and found no statistically significant difference in plasma cfDNA integrity in PCa patients of different Gleason scores ($p \geq 0.05$) [6,8,10,12]. On the other hand, Arko-Boham and co-workers [20] have found that cfDNA integrity increases with disease progression Arko- Boham recruited 31 prostate cancer patients and 30 healthy controls. However,, their results could be limited by the small sample size.

The concentration of ALU 247 was statistically significantly higher in PCa group compared to the other two groups, while only urine ALU 115 concentration was significant when comparing PCa to BPH or the healthy control group. According to Umetani et al. [15] the concentration of ALU 115 should always be higher than the concentration of ALU 247 based on the assumption that ALU 115 primers amplify both ALU 115 and ALU 247 fragments, which was not the case in this study. This can be attributed to the type of cancer, the nature of sample, primer design, quality of sample, PCR reagents, PCR instrument or other confounders that need further research. The integrity index was calculated as ratio between ALU 247/ALU115 concentrations. The integrity index was sometimes greater than one. According to Umetani et al.[15], ALU115 primers amplify both ALU115 and ALU247. Therefore, theoretically DNA integrity index should be less than one. However, practically we found that DNA integrity can be more than one. Moreover, several studies have found the same observation [7,24–27]. Plasma cfDNA profile can be used to study cancer-related genetic and epigenetic changes and may cost-efficiently provide somatic information in clinical trials designed to identify predictive biomarkers [6]. Additionally, urine cfDNA integrity test has the advantage of being non-invasive, rapid, and easy to perform, with only a few millilitres of urine needed to carry out the analysis [5].

To sum it up, the quantification of circulating cfDNA still has some limitations such as the differences in methodology and the lack of standardization in these methodologies, which have hampered the implementation of these tumor markers in clinical practice.

Table 1 :The sequence of ALU115 and ALU247 primers

	ALU115	ALU247
Forward	5′-CCTGAGGTCAGGAGTTCGAG-3′	5′-GTGGCTCACGCCTGTAATC-3′
Reverse	5′-CCCGAGTAGCTGGGATTACA-3′	5′-CAGGCTGGAGTGCAGTGG-3′.

Table 2: Clinical and demographic characteristics of PCa patients

PCa n=46	
Family history	
Negative	24 (52.1%)
Positive	22 (47.9%)
Gleason score	
6	6 (13%)
7	25 (54.3%)
8	8 (17.3%)
9	3 (6.5%)
10	4 (8.9%)
Treatment	
No treatment	21 (45.7%)
Hormonal	11 (23.9%)
Radiotherapy	7 (15.2%)
Surgery	7 (15.2%)
Distant metastasis	
M0	22 (47.8%)
M1	24 (52.2%)
Risk stratification	
Low	6 (13%)
Moderate	25 (54.3%)
High	15 (32.7%)
PSA levels	
>10 ng/ml	25 (54.5%)
4 – 10 ng/ml	21 (45.5%)
Risk stratification was calculated according to European Association of Urology Guidelines 2019. Low risk was defined as PSA <10 ng/ml and GS < 7 (ISUP grade 1) and cT1-2a; Moderate risk: PSA 10 – 20 ng/ml or GS 7 (ISUP grade 2/3) or cT2b; High risk: PSA > 20 ng/ml or Gleason score > 7 (ISUP 4/5) or cT2c GS: Gleason score; ISUP: International Society for Urological Pathology; PSA: Prostate specific antigen.	

Table 3: Biochemical and Genetic markers among the studied groups

Variable	Control Group N=20	BPH Group N=44	PCa Group N=46	P-value
tPSA ng/ml	0.6[c] (0.51-0.7)	2.1 [b] (0.87-3.72)	15.3 [a] (3.6-35.1)	<0.001
fPSA ng/ml	N/A	0.95 (0.31-2.0)	0.78 (0.5-0.91)	0.27
f/t PSA ratio	N/A	0.27 (0.22-0.31)	0.18 (0.11-0.32)	0.1
Plasma cfDNA integrity	0.36 [b] (0.22-0.54)	0.32 [b] (0.21-0.64)	3.15 [a] (1.69-5.48)	<0.001
Plasma ALU 115 conc. ng/ml	24.7 (7.6-46.8)	33.1 (13.5-64.9)	15.2 (3.5-41.4)	0.149
Plasma ALU 247 conc. ng/ml	5.9 [b] (1.8-12.5)	8.5 [b] (2.65-32.2)	32.3 [a] (15.6-94.9)	<0.001
Urine cfDNA integrity	0.63[b] (0.28-1.0)	0.42 [b] (0.13-1.0)	1.64 [a] (1.0- 3.7)	<0.001
Urine ALU 115 conc. ng/ml	3.7 [b] (2.69-6.17)	7.2 [b] (1-35.3)	1.1 [a] (1-16.3)	0.028
Urine ALU 247 conc. ng/ml	2.4 [b] (1.0-3.5)	1.5 [b] (1-4.2)	4.9 [a] (1.2 - 12.5)	0.003

Data are expressed as median (25th-75th percentile).
P value < 0.05 was considered significant.
Groups carrying different initials are statistically different (P <0.05).
N/A, Not available; PCa, prostate cancer; BPH, benign prostate hyperplasia.

Table 4 [a]: The median and interquartile levels of the studied parameters in Prostate cancer group regarding the state of metastasis

Variables	Non-metastatic N= 22	Metastatic N=24	P value
Plasma cfDNA integrity	3.57 (2.14-5.79)	2.78 (1.59-4.77)	0.285
Urine cfDNA integrity	2 (1.2-3.1)	1.15 (1.0-4.67)	0.784

Table 4 b: Comparison of cfDNA integrity in PCa group regarding the treatment

	No treatment N= 20	Received treatment N= 26	P- value
Plasma integrity	2.51 (1.62 – 4.35)	4 (1.6 – 6)	0.15
Urine integrity	2.30 (1.0 - 5.85)	1.3 (1 – 3)	0.16

Table 4c: The median and interquartile levels of the studied parameters in Prostate cancer group regarding risk stratification

Variables	Low Risk N= 6	Moderate risk N= 25	High N=15	p value
Plasma integrity	5.9 (3 - 8.3)	2.6 (1.4 - 4.8)	3.1 (1.7 - 4.6)	0.39
Urine integrity	1.5 (1.3 - 4.9)	1.9 (1 - 2.7)	1.4 (1 - 4.7)	0.8

Table 4 d: The median and interquartile levels of the studied parameters in Prostate cancer group regarding family history

Variables	Positive N= 22	Negative N= 24	p value
Plasma cfDNA integrity	2.7 (1.2 - 4.6)	3.6 (1.9 - 5.6)	0.23
Urine cfDNA integrity	1.6 (1 - 4.3)	1.6 (1 - 2.7)	0.42

Table 5: The cut off values from ROC curve statistics for plasma and urine cfDNA integrity to differentiate PCa from BPH patients.

parameter	Cut off value	Sensitivity	Specificity	PPV	NPV	Diagnostic accuracy
Plasma integrity	0.989	93.47%	81.81%	84.31%	92.30%	87.77%
Urine integrity	0.900	84.78%	72.72%	76.47%	82.05%	78.88%
tPSA (ng/ml)	4.1	71.73%	77.27%	76.74%	72.34%	74.34%
PPV, Positive predictive value; NPV, Negative predictive value; PCa, prostate cancer						

Table 6: The predictive probability of the regression model to differentiate BPH from PCa patients

	True positive	False Negative	True Negative	False positive	Total	Overall predictive accuracy
BPH and PCa	42	4	40	4	90	91.1% (82/90)
Control and PCa	44	2	19	1	66	95.4% (63/66)

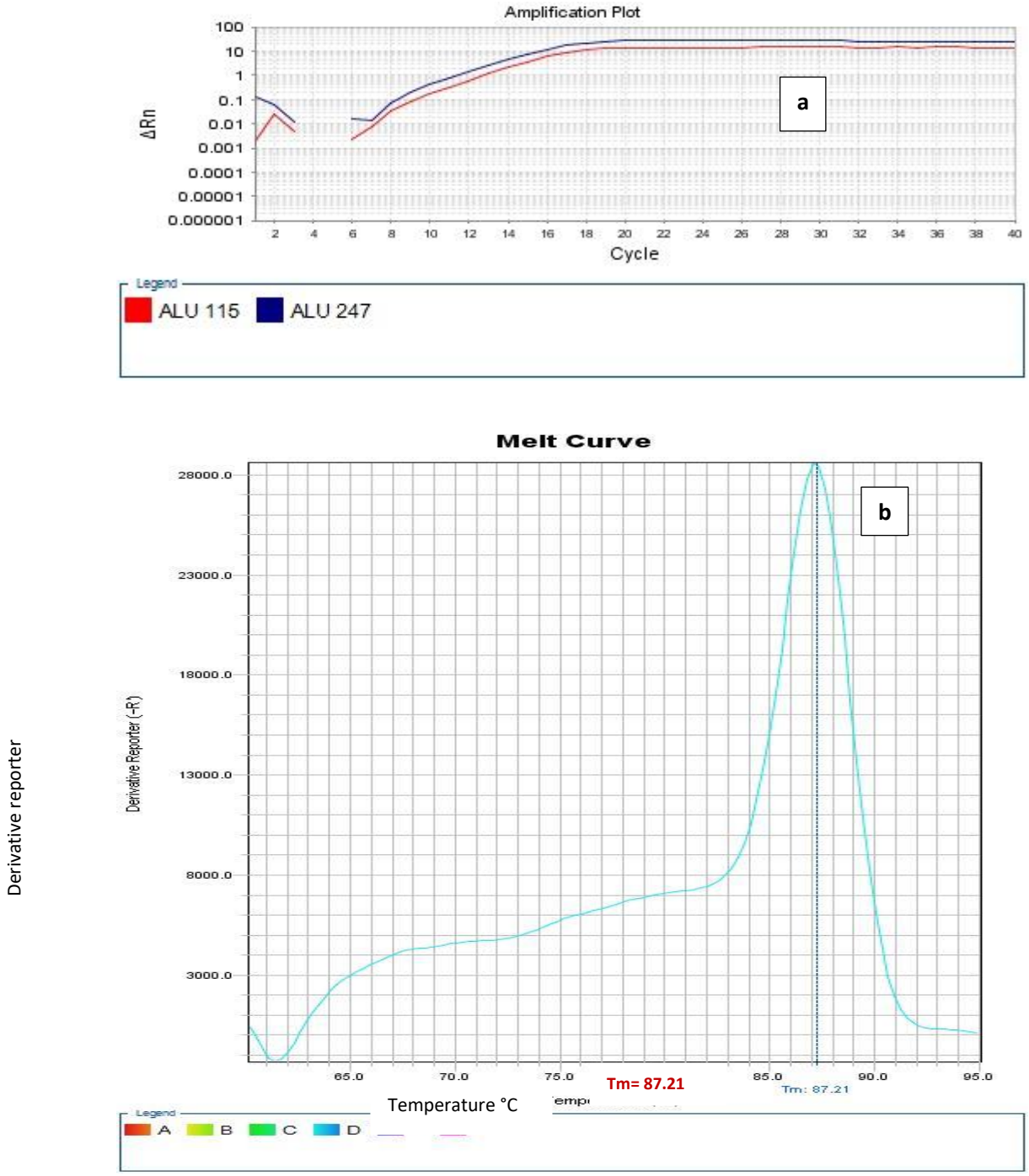

Figure 1: (a): Amplification plot of qPCR result obtained with both primer sets (ALU115: red line, ALU 247: blue line). (**b):** Melting curve analysis using Step One real time PCR. Tm, melting temperature.

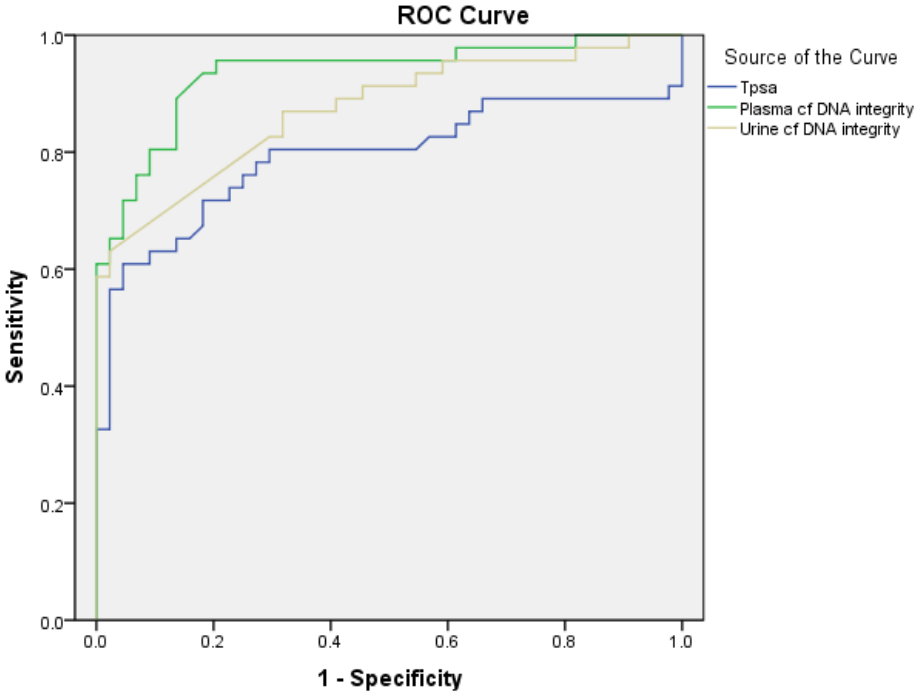

Figure 2: Multiple ROC curve for combined tPSA, plasma cfDNA integrity and urine cfDNA integrity to differentiate Prostate cancer from BPH patients. For plasma cfDNA integrity the AUC= 0.935; 95% CI= 0.883 – 0.987; $p < 0.001$; urine cfDNA integrity the AUC= 0.873; 95% CI= 0.800 – 0.946; $p < 0.001$and tPSA has the following values, AUC= 0.792; 95% CI= 0.691 – 0.892; $p < 0.001$.

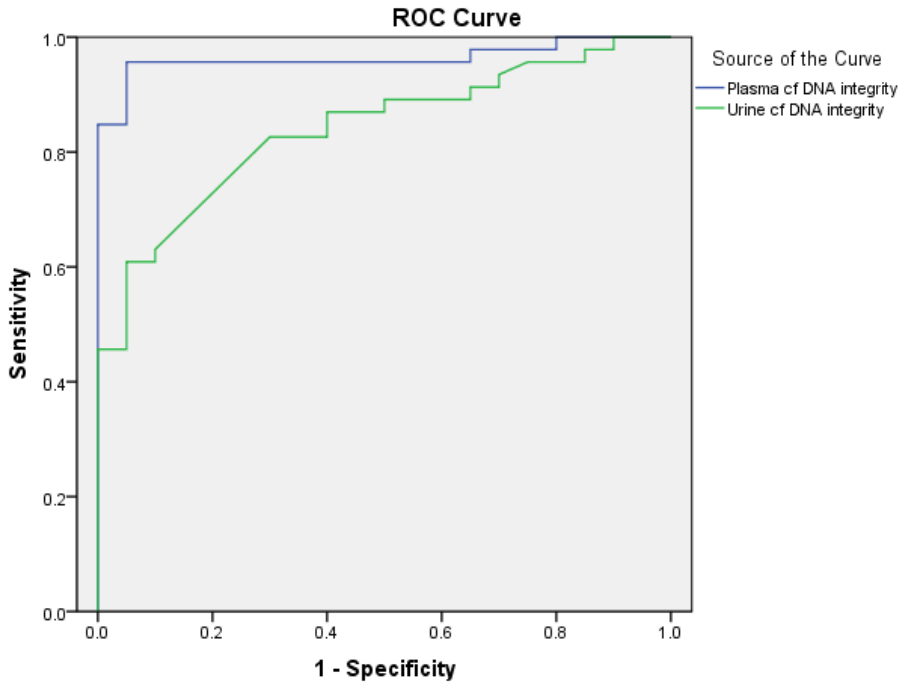

Figure 3: Multiple ROC curve for combined plasma cfDNA integrity and urine cfDNA integrity to differentiate Prostate cancer from healthy control group. The AUC was 0.963 for plasma cfDNA integrity and 0.840 for urine cfDNA integrity. The p value for both is < 0.001.

For instance, several studies used plasma to quantify the circulating cfDNA, while others used serum as a template. Moreover, some studies performed DNA extraction and measured the levels of circulating cfDNA by qPCR, while other studies used serum or plasma as a direct template to quantify cell-free DNA [28]. Nevertheless, most of the published reports considered the integrity of serum and/or plasma cfDNA as a promising molecular biomarker for detecting various types of malignancies.

4. Conclusions: This study revealed that plasma cfDNA integrity is superior to urine in discriminating between PCa and BPH. Although the results of urine cfDNA integrity in comparison to plasma were not very promising; urine cfDNA was quantifiable in almost all samples and can be used for the detection of genetic and epigenetic alterations to discriminate PCa from benign prostatic lesions.
PSA is prostate specific but cancer nonspecific as it increases in many prostate diseases such as PCa, BPH, prostatitis and prostate massage. Likewise, cfDNA integrity is not prostate cancer specific and could increase in many types of cancers. So, both PSA and cfDNA could complement each other to diagnose prostate cancer.
In conclusion, plasma cfDNA integrity is a promising noninvasive biomarker in the screening and diagnosis of PCa.

Abbreviations: ALU: Arthrobacter luteus; BPH: Benign prostate hyperplasia; cfDNA: cell free DNA; EDTA: Ethylenediamine tetra-acetic acid ; PCa: prostate cancer; SINEs: short interspersed elements.

Ethics approval and consent to participate: The study protocol was approved by the local ethics committee of the department of Clinical and Chemical pathology, Faculty of Medicine, Cairo University (#5/2015) as to be in accordance with Helsinki Declaration II, Finland. Written informed consent was obtained from all participants.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: NM collected the data and samples, performed laboratory work-up, statistically analysed and interpreted the data and wrote the manuscript; WR designed the work and revised the draft; DE designed the work, statistically analysed the data and revised the script; MA examined the patients and revised the manuscript. All authors read and approved the final manuscript.

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References

[1] F. Bray, J. Ferlay, I. Soerjomataram, R.L. Siegel, L.A. Torre, A. Jemal (2018). Global cancer statistics GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, CA. Cancer J. Clin. 68: 394–424. doi:10.3322/caac.21492.

[2] T. Ross, K. Ahmed, N. Raison, B. Challacombe, P. Dasgupta (2016). Clarifying the PSA grey zone: The management of patients with a borderline PSA, Int. J. Clin. Pract. 70: 950–959. doi:10.1111/ijcp.12883.

[3] J. Ellinger, P. Albers, S.C. Müller, A. Von Ruecker, P.J. Bastian (2009). Circulating mitochondrial DNA in the serum of patients with testicular germ cell cancer as a novel noninvasive diagnostic biomarker, BJU Int. 104:48–52. doi:10.1111/j.1464-410X.2008.08289.x.

[4] P.O. Delgado, B.C.A. Alves, F. De Sousa Gehrke, R.K. Kuniyoshi, M.L. Wroclavski, A. Del Giglio, F.L.A. Fonseca (2013). Characterization of cell-free circulating DNA in plasma in patients with prostate cancer, Tumor Biol. 34: 983–986. doi:10.1007/s13277-012-0634-6.

[5] V. Casadio, S. Salvi (2019). Cell-free DNA as Diagnostic Markers Methods and Protocols Methods in Molecular Biology.

[6] A. Fawzy, K.M. Sweify, H.M. El-Fayoumy, N. Nofal (2016). Quantitative analysis of plasma cell-free DNA and its DNA integrity in patients with metastatic prostate cancer using ALU sequence, J. Egypt. Natl. Canc. Inst. 2:8 235–242. doi:10.1016/j.jnci.2016.08.003.

[7] B. Arko-Boham, N.A. Aryee, R.M. Blay, E.D.A. Owusu, E.A. Tagoe, E.S. Doris Shackie, A.B. Debrah, N.A. Adu-Aryee (2019). Circulating cell-free DNA integrity as a diagnostic and prognostic marker for breast and prostate cancers, Cancer Genet. 235–236 :65–71. doi:10.1016/j.cancergen.2019.04.062.

[8] J. Feng, F. Gang, X. Li, T. Jin, H. Houbao, C. Yu, L. Guorong (2013). Plasma cell-free DNA and its DNA integrity as biomarker to distinguish prostate cancer from benign prostatic hyperplasia in patients with increased serum prostate-specific antigen, Int. Urol. Nephrol. 45:1023–1028. doi:10.1007/s11255-013-0491-2.

[9] S. Salvi, G. Gurioli, F. Martignano, F. Foca, R. Gunelli, G. Cicchetti, U. De Giorgi, W. Zoli, D. Calistri, V. Casadio (2015). Urine Cell-Free DNA Integrity Analysis for Early Detection of Prostate Cancer Patients, Dis. Markers. doi:10.1155/2015/574120.

[10] R. Hanley, K.M. Rieger-Christ, D. Canes, N.R. Emara, A.P. Shuber, K.A. Boynton, J.A. Libertino, I.C. Summerhayes (2006). DNA integrity assay: A plasma-based screening tool for the detection of prostate cancer, Clin. Cancer Res. 12:4569–4574. doi:10.1158/1078-0432.CCR-06-0130.

[11] S. Salvi, G. Gurioli, U. De Giorgi, V. Conteduca, G. Tedaldi, D. Calistri, V. Casadio (2016). Cell-free DNA as a diagnostic marker for cancer: Current insights, Onco. Targets. Ther. 9:6549–6559.

doi:10.2147/OTT.S100901.

[12] M. Khani, J. Hosseini, R. Mirfakhraie, M. Habibi, E. Azargashb, F. Pouresmaeili (2019). The value of the plasma circulating cell-free DNA concentration and integrity index as a clinical tool for prostate cancer diagnosis: A prospective case-control cohort study in an Iranian population, Cancer Manag. Res. 11: 4549–4556. doi:10.2147/CMAR.S192646.

[13] J. Cheng, Q. Tang, X. Cao, B. Burwinkel (2017). Cell-free circulating DNA integrity based on peripheral blood as a biomarker for diagnosis of cancer: A systematic review, Cancer Epidemiol. Biomarkers Prev. 26:1595–1602. doi:10.1158/1055-9965.EPI-17-0502.

[14] Z. Gu, H. Wang, A. Nekrutenko, W.H. Li (2000). Densities, length proportions, and other distributional features of repetitive sequences in the human genome estimated from 430 megabases of genomic sequence, Gene. 259: 81–88. doi:10.1016/S0378-1119(00)00434-0.

[15] N. Umetani, A.E. Giuliano, S.H. Hiramatsu, F. Amersi, T. Nakagawa, S. Martino, D.S.B. Hoon (2006). Prediction of breast tumor progression by integrity of free circulating DNA in serum, J. Clin. Oncol. 24 4270–4276. doi:10.1200/JCO.2006.05.9493.

[16] N. Mottet, R.C.N. van den Bergh, E. Briers, T. Van den Broeck, M.G. Cumberbatch, M. De Santis, S. Fanti, N. Fossati, G. Gandaglia, S. Gillessen, N. Grivas, J. Grummet, A.M. Henry, T.H. van der Kwast, T.B. Lam, M. Lardas, M. Liew, M.D. Mason, L. Moris, D.E. Oprea-Lager, H.G. van der Poel, O. Rouvière, I.G. Schoots, D. Tilki, T. Wiegel, P.P.M. Willemse, P. Cornford, EAU-EANM-ESTRO-ESUR-SIOG Guidelines on Prostate Cancer—2020 Update (2020). Part 1: Screening, Diagnosis, and Local Treatment with Curative Intent, Eur. Urol. 1–20. doi:10.1016/j.eururo.2020.09.042.

[17] P.A. Humphrey, H. Moch, A.L. Cubilla, T.M. Ulbright, V.E. Reuter (2016). The 2016 WHO Classification of Tumours of the Urinary System and Male Genital Organs—Part B: Prostate and Bladder Tumours, Eur. Urol. 70:106–119. doi:10.1016/j.eururo.2016.02.028.

[18] S.B. Edge, C.C. Compton, The american joint committee on cancer (2010). The 7th edition of the AJCC cancer staging manual and the future of TNM, Ann. Surg. Oncol. 17:1471–1474. doi:10.1245/s10434-010-0985-4.

[19] G.F. Giskeødegård, T. Andreassen, H. Bertilsson, M.B. Tessem, T.F. Bathen (2019). The effect of sampling procedures and day-to-day variations in metabolomics studies of biofluids, Anal. Chim. Acta. doi:10.1016/j.aca.2019.07.026.

[20] A. Condappa, D. McGrowder, W. Aiken, W. McLaughlin, M. Gossell-Williams (2020). Evaluation of Plasma Circulating Cell Free DNA Concentration and Integrity in Patients with Prostate Cancer in Jamaica: A Preliminary Study, Diseases. 8:34. doi:10.3390/diseases8030034.

[21] A.J. Bronkhorst, J. Aucamp, P.J. Pretorius, Cell-free DNA (2015). Preanalytical variables, Clin. Chim. Acta. 450: 243–253. doi:10.1016/j.cca.2015.08.028.

[22] T.H. Lee, L. Montalvo, V. Chrebtow, M.P. Busch (2001). Quantitation of genomic DNA in plasma and serum samples: Higher concentrations of genomic DNA found in serum than in plasma, Transfusion. 41:276–282. doi:10.1046/j.1537-2995.2001.41020276.x.

[23] V. Casadio, D. Calistri, M. Tebaldi, S. Bravaccini, R. Gunelli, G. Martorana, A. Bertaccini, L. Serra, E. Scarpi, D. Amadori, R. Silvestrini, W. Zoli (2013). Urine Cell-Free DNA integrity as a marker for early bladder cancer diagnosis: Preliminary data, Urol. Oncol. Semin. Orig. Investig. 31: 1744–1750. doi:10.1016/j.urolonc.2012.07.013.

[24] O.J. Stötzer, J. Lehner, D. Fersching-Gierlich, D. Nagel, S. Holdenrieder (2014). Diagnostic relevance of plasma DNA and DNA integrity for breast cancer, Tumor Biol. 35: 1183–1191. doi:10.1007/s13277-013-1158-4.

[25] M. Agostini, S. Pucciarelli, M.V. Enzo, P. Del Bianco, M. Briarava, C. Bedin, I. Maretto, M.L. Friso, S. Lonardi, C. Mescoli, P. Toppan, E. Urso, D. Nitti (2011). Circulating cell-free DNA: A promising marker of pathologic tumor response in rectal cancer patients receiving preoperative chemoradiotherapy, Ann. Surg. Oncol. 18: 2461–2468. doi:10.1245/s10434-011-1638-y.

[26] D. El-Gayar, N. El-Abd, N. Hassan, R. Ali (2016). Increased free circulating DNA integrity index as a serum biomarker in patients with colorectal carcinoma, Asian Pacific J. Cancer Prev. 17: 939–944. doi:10.7314/APJCP.2016.17.3.939.

[27] G. Leszinski, J. Lehner, U. Gezer, S. Holdenrieder (2014). Increased DNA integrity in colorectal cancer, In Vivo (Brooklyn). 28: 299–303.

[28] A.R. Thierry, S. El Messaoudi, P.B. Gahan, P. Anker, M. Stroun (2016). Origins, structures, and functions of circulating DNA in oncology, Cancer Metastasis Rev. 35: 347–376. doi:10.1007/s10555-016-9629-x.