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ORIGINAL PAPER

Are elevated mitochondrial DNA fragments in prostatic inflammation a potential biomarker for prostate cancer?

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Summary ***Background: We sought to determine whether two soluble forms with different size of mtDNA are linked to prostatic inflammation, and whether they discriminate prostate cancer (PCa) from inflammatory prostatic conditions.***
Methods: Histopathologically diagnosed prostatitis, PCa and benign prostatic hyperplasia patients (n = 93) were enrolled in this study and they were categorized as with and without prostate inflammation. Quantitative RT-PCR was used to analyze the levels of 79-bp and 230-bp fragments in urine and blood samples collected following prostate massage.
Results: The urine mtDNA-79 and mtDNA-230 were significantly increased in patients with prostate inflammation compared with those in without inflammation. Here, 79-bp fragment of apoptotic origin was significantly higher level than 230-bp fragment of necrotic origin. Although mtDNA-79 copy number in serum samples was also increased in patients with prostate inflammation, mtDNA-230 was similar in the two groups. Furthermore, mtDNA-79 and mtDNA-230 copy numbers in post-prostate massage urine were higher (about 16-fold and 22-fold, respectively) than those from serum samples. ROC analysis showed that, although post-prostate massage urine have relatively higher performance than blood, ability to discriminate cases of both fragments was not better than that of serum total PSA.
Conclusions: Our results demonstrate that shorter cf-mtDNA fragment size in particular, increase in the presence of prostate inflammation in post-prostatic massage urine but both fragments could never improve serum total PSA performance.

KEY WORDS: Prostate; Inflammation; Cancer; Mitochondrial DNA; Post-prostatic massage.

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INTRODUCTION

A possible link between inflammation and *prostate cancer* (PCa) has been suggested, but not yet confirmed. Several cross-sectional studies indicate that prostatic inflammation is more common in patients without cancer (1, 2). The factors contributing to prostate inflammation are largely unknown, and inflammation could play a role in false positive *prostate-specific antigen* (PSA) results in cancer screening (1, 3).
Inflammatory lesions contain dead cells, including apoptosis and necrosis, and evidence shows a bidirectional relationship between cell death forms and inflammation. Both *genomic DNA* (gDNA) and mitochondrial DNA (mtDNA) are released from dying cells, and circulating mtDNA fragments may trigger pro-inflammatory responses (4-12). Elevated levels of circulating cell-free mtDNA (cf-mtDNA) have been found in patients with various diseases, including urological malignancies, but the association with cancer is still debated (13-16).
Different diseases have been associated with specific mtDNA fragmentation patterns related to cell death type (17-19). There may be a link between mtDNA fragments and prostate inflammation, but it remains unclear, and the studies have mainly focused on blood samples. Evaluating the fragmentation pattern of cf-mtDNA in urine might provide insights into mtDNA-mediated inflammation and improve PCa biomarkers. Therefore, this study aimed to determine if the apoptosis- and necrosis-derived mtDNA fragments (79 bp and 230 bp, respectively) in post-prostate massage urine are related to prostate inflammation and can discriminate between PCa and inflammatory conditions, using serum PSA levels for comparison.

MATERIALS AND METHODS

Case selection

This study was conducted with 93 male patients, who applied the *Istanbul University-Cerrahpasa, Department of Urology*. Patients aged 40 and older, PSA value > 2.5 ng/mL, and suspicious findings on rectal examination were included in the study (Table 1). *Transrectal ultrasound* (TRUS) guided biopsies (12-28 cores) were performed for evaluation. The patients were divided into 3 groups according to the histopathological evaluations of TRUS biopsies: *prostate cancer* (PCa), prostatitis (Inflammation), and *benign prostatic hyperplasia* (BPH) groups. The results of biopsy showing adenocarcinoma and BPH were included in the PCa group. Similarly, the results showing prostatitis and BPH were included in the Inflammation group, whereas the results containing alone BPH were included in the BPH group. Additionally, according to the inflammation status in the results of TRUS biopsies, cases without inflammation (including cases of PCa and BPH groups) were defined as the Non-inflammation group. Patients with other malignant disease, distant metastases, infectious or inflammatory dis-

Table 1.
Demographic data of all groups.

	Non-inflammation		Inflammation
Variable	**BPH (n: 29)**	**PCa (n: 34)**	**Prostatitis (n: 30)**
Age (years)	65.51 ± 6.85	65.09 ± 9.18	63.32 ± 6.71
PSA (ng/ml)	5.72 ± 2.80	18.24 ± 21.04 [a***]	11.36 ± 9.81 [b ***]
Histology Adenocarcinoma, n (%)	NA	34 (100%)	NA
Pathological tumour stage pT1, n (%) pT2, n (%)	NA NA	15 (44.12%) 19 (55.88%)	NA NA
Gleason Grading Gleason Score < 7, n (%) Gleason Score = 7, n (%) Gleason Score > 7, n (%)	NA NA NA	12 (35.29%) 15 (44.12%) 7 (20.59%)	NA NA NA
Histology Biopsy core with cancer Median, n Range, n	NA NA	4.5 1-12	NA NA
Histology Biopsy core with inflammation Median, n Range, n	NA NA	NA NA	6 3-15

*Values were presented as Mean ± standard deviation; BPH: benign prostatic hyperplasia; PCa: prostate cancer; NA: not applicable; [a] PCa vs BPH; [b] Inflammation vs. BPH; *** $p < 0.001$.*

eases, histologically defined with both inflammation and cancer findings were excluded from the study. All patients provided written informed consent. This study was approved by the Istanbul *University-Cerrahpasa Ethics Committee* (*approval no. 83045809; Istanbul, Turkey*), and was performed according to the criteria set out by the Declaration of Helsinki.

Sample collection

Before TRUS biopsy, following a digital rectal examination of 3 strokes per prostate lobe, each subject provided 20-30 ml urine in a first catch specimen. All urine specimens were centrifuged at 2500 x g for 10 min at 4°C, followed by storage of the urine and pellet at -80°C. Five milliliter venous blood samples were collected into anticoagulant-free tubes after prostatic massage and centrifugation was performed at 2500×g for 10 min at 4°C. Then obtained supernatants were stored at -80°C until analyses.

DNA isolation, and Determination of mtDNA-79 and mtDNA-230 Copies

The *Plasma/Serum Cell-Free Circulating DNA Purification Kit* (*Norgen Biotek, Canada*) and *Urine Cell-Free Circulating DNA Purification Kit* (*Norgen Biotek, Canada*) were used to isolate circulating DNA from serum and urine. Cell-free DNA was isolated according to the kit manufacturers' protocols. Quantitative analysis of mtDNA fragments in serum and urine was performed by *quantitative real-time PCR* (qPCR). In this study, two primer sets specific for the mitochondrial ribosomal 16S RNA were used. The first primer pair amplified a 79-bp fragment (mtDNA-79), and the second primer pair amplified a 230-bp fragment (mtDNA-230) The sequence of the forward primer specific for both mtDNA fragments was 5'-CAGCCGCTATTAAAGGTTCG-3'. The sequence of the reverse primer specific for mtDNA-79 was 5'-CCTGGATTACTCCGGTCTGA-3', of the reverse primer specific for mtDNA-230 was 5'-GGGCTCTGCCATCTTAACAA-3'.

The qPCR was performed in duplicate on a *Real Time PCR Detection system* (*Bio Rad Laboratories, Inc., Hercules, CA, USA*). Each 20 µL reaction consisted of 2 mL DNA, 10 µl *EvaGreen 2X qPCR MasterMix* (*abm, Canada*) and 0.40 µL (10mM) forward/reverse primer. PCR conditions were 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 60 s, and 72 °C for 30 s. Each run included water blanks as a negative control. The specificity of the PCR products was confirmed by melting curve analysis. The quantity of each target gene in the samples was subsequently calculated according to the corresponding standard curve. The formula published by the Genomics and Sequencing Center of the University of Rhode Island (*Kingston, RI, USA; cels.uri.edu/gsc/cndna.html*) was used to calculate the mtDNA copy number (20).

Statistical analysis

Continuous variables were presented as means ± SD or medians, and categorical variables were presented as frequencies with percentages. Differences between groups were compared with Mann Whitney-U, and *Kruskal-Wallis* tests. *Receiver operating characteristic* (ROC) analysis, and area under the ROC curve (AUC) were used for determination of diagnostic performance of mtDNA levels. Point-biserial correlation was used for correlation between categorical variables and mtDNA levels. All statistical analyses were performed using *Prism 5.0* (*GraphPad Software, San Diego, CA*). A value of $p < 0.05$ was considered statistically significant.

Results

Assessment of mtDNA levels in urine and serum according to inflammation status

Urine mtDNA-79 (median: 1.52×10^9 *vs.* 0.66×10^9 copies/ml) and mtDNA-230 (1.59×10^8 *vs.* 1.71×10^7 copies/ml) levels were increased in the Inflammation group compared to the Non-inflammation group ($p < 0.001$ and $p < 0.01$, respectively) (Figure 1 A-B).

Serum mtDNA-79 (median: 2.45×10^7 *vs.* 1.05×10^7 copies/ml) levels were higher in the Inflammation group compared with the Non-inflammation group ($p < 0.05$), but the differences were not significant in serum mtDNA-230 levels (median: 0.87×10^6 *vs.* 0.51×10^6 copies/ml) between these groups ($p > 0.05$) (Figure 1 C-D).

Assessment of urine and serum mtDNA levels in Inflammation, PCa, and BPH groups

Urine mtDNA-79 (median: 1.52×10^9 *vs.* 0.58×10^9 copies/ml) and mtDNA-230 (1.59×10^8 *vs.* 0.81×10^7 copies/ml) levels were increased in the Inflammation group compared to the BPH group ($p < 0.01$, for both) (Figure 2 A-B) whereas the differences were not significant in serum mtDNA-79 (median: 2.69×10^7 vs. 1.01×10^7 copies/ml) and mtDNA-230 levels (median: 0.51×10^6 *vs.* 0.88×10^6 copies/ml) between these groups ($p > 0.05$) (Figure 2 C-D).

There were statistically significant differences in urine

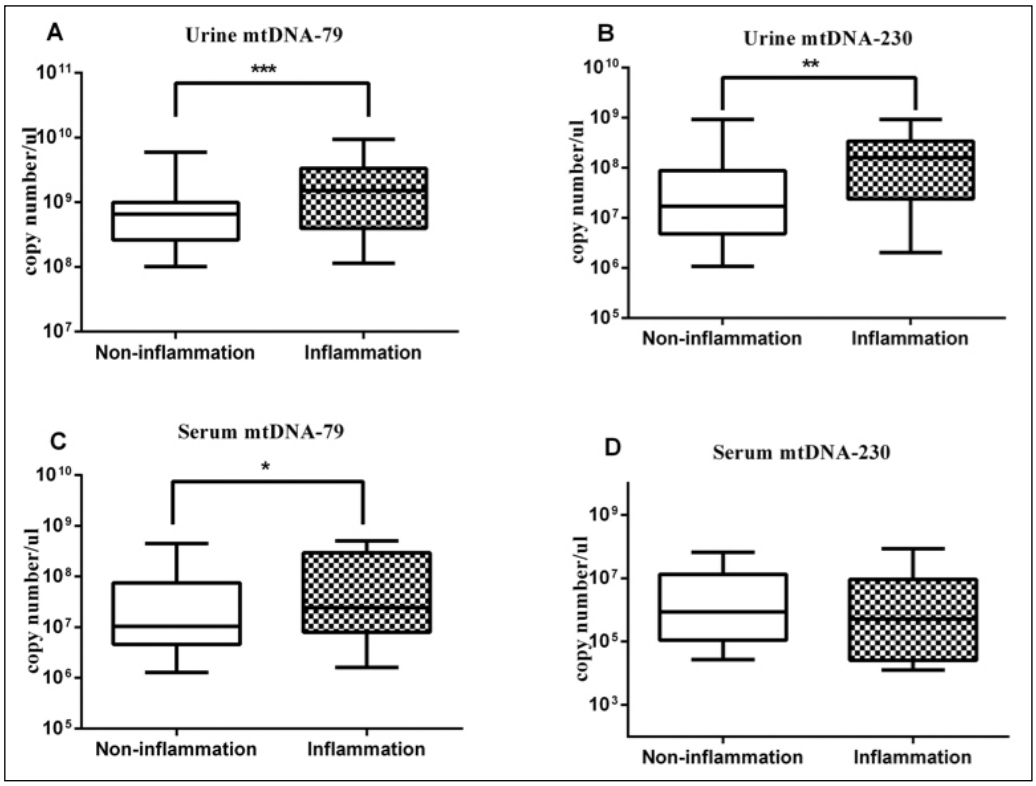

Figure 1.
*Comparison of the A. urine mtDNA-79, B. urine mtDNA-230, C. serum mtDNA-79, and D. serum mtDNA-230 between the Inflammation and Non-inflammation groups, *p < 0.05, **p < 0.01, ***p < 0.001. The bold line in the box plots represents the median value.*

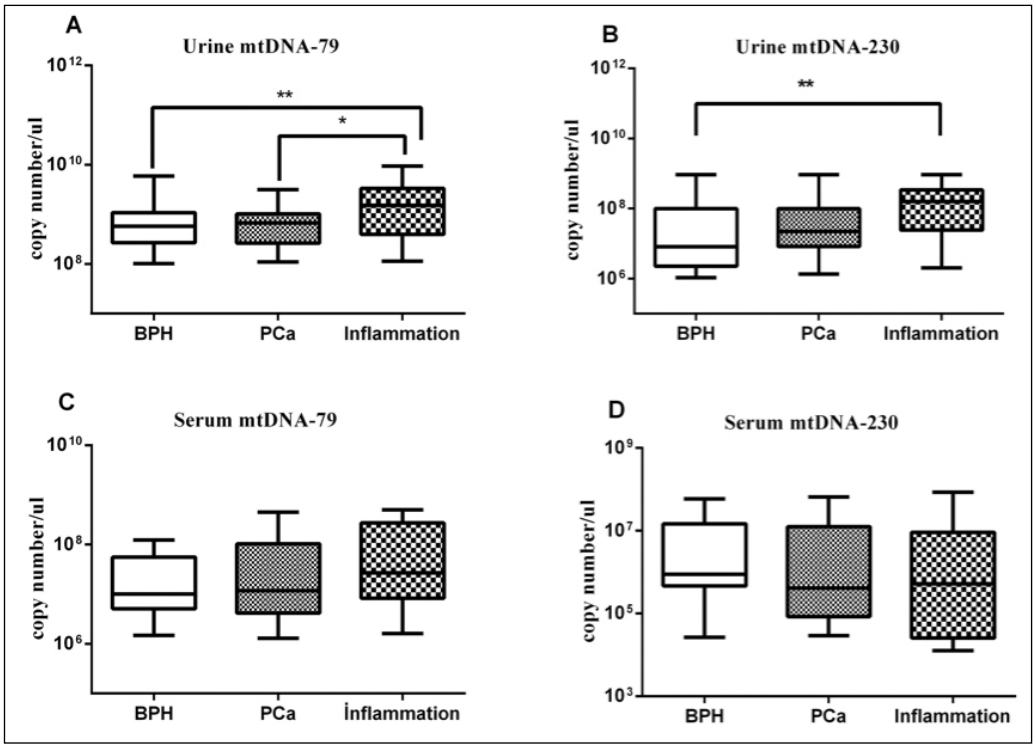

Figure 2.
*Pairwise comparisons of the A. urine mtDNA-79, B. urine mtDNA-230, C. serum mtDNA-79, and D. serum mtDNA-230 among the Inflammation, PCa, and BPH groups; *p < 0.05, **p < 0.01, The bold line in the box plots represents the median value.*

mtDNA-79 (median: 1.52 x 10^9 *vs.* 0.67 x 10^9 copies/ml) ($p < 0.05$), but not in urine mtDNA-230 (median: 1.59 x 10^8 *vs.* 2.23 x 10^7 copies/ml), serum mtDNA-79 (median: 2.69 x 10^7 *vs.* 1.18 x 10^7 copies/ml), and mtDNA-230 (median: 0.51 x 10^6 *vs.* 0.41 x 10^6 copies/ml) levels between the Inflammation and the PCa groups ($p > 0.05$, for all).

Additionally, there were no significant changes in urine mtDNA-79 (median: 0.67 x 10^9 *vs.* 0.57 x 10^9 copies/ml), urine mtDNA-230 (median: 2.23 x 10^7 *vs.* 0.81 x 10^7 copies/ml), serum mtDNA-79 (median: 1.18 x 10^7 *vs.* 1.01 x 10^7 copies/ml) and serum mtDNA-230 (median: 0.41 x 10^6 *vs.* 0.88 x 10^6 copies/ml) levels between the comparison of the PCa and BPH groups (Figure 2 A-D).

Table 2.
Diagnostic performance of serum PSA, serum and post-prostatic massage urine specimens mtDNA.

	Sensitivity	Specificity	AUC (95%CI)	P
Urine mtDNA-79				
Inflammation *vs.* Non-Inflammation	%73.3	%71.4	0.712 (0.588-0.836)	0.001
Inflammation *vs.* BPH	%73.4	%72.4	0.709 (0.570-0.847)	0.006
Inflammation *vs.* PCa	%73.2	%70.6	0.714 (0.580-0.849)	0.003
PCa *vs.* BPH	%41.2	%68.9	0.534 (0.388-0.679)	NS
Urine mtDNA-230				
Inflammation *vs.* Non-Inflammation	%70.1	%66.7	0.707 (0.591-0.823)	0.001
Inflammation *vs.* BPH	%63.3	%72.4	0.725 (0.594-0.857)	0.002
Inflammation *vs.* PCa	%66.7	%73.5	0.691 (0,557-0.825)	0.008
PCa *vs.* BPH	%58.8	%58.6	0.595 (0.449-0.741)	NS
Serum mtDNA-79				
Inflammation *vs.* Non-Inflammation	%54.2	%60.4	0.653 (0.511-0.794)	0.04
Inflammation *vs.* BPH	%56.1	%63.6	0.682 (0.528-0.836)	0.03
Inflammation *vs.* PCa	%48.1	%65.4	0.651 (0.498-0.803)	NS
PCa *vs.* BPH	%42.3	%63.6	0.542 (0.377-0.707)	NS
Serum mtDNA-230				
Inflammation *vs.* Non-Inflammation	%47.8	%75.6	0.585 (0.427-0.744)	NS
Inflammation *vs.* BPH	%52.2	%75.0	0.602 (0.429-0.776)	NS
Inflammation *vs.* PCa	%47.8	%68.1	0.572 (0.403-0.741)	NS
PCa *vs.* BPH	%56.1	%75.0	0.580 (0.408-0.752)	NS
Serum PSA				
Inflammation *vs.* Non-Inflammation	70.0%	60.3%	0.609 (0.495-0.725)	NS
Inflammation *vs.* BPH	70.1%	89.7%	0.823 (0.711-0.935)	< 0.001
Inflammation *vs.* PCa	56.7%	61.8%	0.572 (0.429-0.716)	NS
PCa *vs.* BPH	64.7%	89.6%	0.793 (0.679-0.907)	< 0.001

AUC: area under an ROC curve; BPH: benign prostatic hyperplasia; PCa: prostate cancer; NS: not significant.

Table 3.
Diagnostic performances of mtDNA combinations in serum and post-prostatic massage urine specimens.

	Sensitivity	Specificity	AUC (95%CI)	P
Combination of urine mtDNA-79 and mtDNA-230				
Inflammation *vs.* Non-Inflammation	66.67%	85.36%	0.715 (0.592-0.837)	0.001
Inflammation *vs.* BPH	80.02%	76.67%	0.810 (0.694-0.926)	< 0.001
Inflammation *vs.* PCa	72.72%	73.33%	0.756 (0.634-0.877)	< 0.001
PCa *vs.* BPH	51.51%	53.57%	0.488 (0.342-0.634)	NS
Combination of serum mtDNA-79 and mtDNA-230				
Inflammation *vs.* Non-Inflammation	47.6%	60.97%	0.647 (0.494-0.800)	NS
Inflammation *vs.* BPH	50.02%	63.16%	0.651 (0.481-0.821)	NS
Inflammation *vs.* PCa	54.17%	68.18%	0.671 (0.489-0.811)	NS
PCa *vs.* BPH	50.20%	55.56%	0.542 (0.364-0.719	NS

AUC: area under an ROC curve; BPH: benign prostatic hyperplasia; PCa: prostate cancer; NS: not significant.

ROC curve analysis of serum and urine mtDNA, and serum PSA according to inflammation status

Generally, AUC values demonstrated moderate discriminatory power, and urine AUC results (mtDNA-79, and mtDNA-230, 0.712, and 0.707, respectively) were higher than serum (mtDNA-79, and mtDNA-230, 0.653, and 0.585, respectively) in the Inflammation group compared to the Non-inflammation group. Similarly, urine mtDNA-79 and mtDNA-230 AUC values were higher than serum PSA AUC values (0.712, 0.707, and 0.585, respectively). Urine mtDNA-79 and mtDNA-230 levels showed similar diagnostic performance with urine combined mtDNA-79 and mtDNA-230 results (Figure 3). Sensitivity and specificity values for all testing were given in Tables 2, 3.

ROC curve analysis of serum and urine mtDNA and serum PSA in Inflammation, PCa, and BPH groups

AUC values of ROC curve analysis were determined for urine and serum mtDNA, and serum PSA in the Inflammation, PCa, and BPH groups. Mostly the results in Inflammation *vs.* BPH, and Inflammation *vs.* PCa groups had moderate diagnostic performance (Figure 4). Urine mtDNA-79 and mtDNA-230 had better diagnostic performance (AUC range 0.725-0.691) than serum mtDNA-79, and mtDNA-230 (AUC range 0.682-0.572). AUC values of serum PSA were higher than urine mtDNA-79, and mtDNA-230. The combination of urine mtDNA-79 and mtDNA-230 results had moderate AUC values similarly as urine mtDNA-79 and mtDNA-230.

ROC curve analysis between the PCa and the BPH groups indicated that except for serum PSA (AUC:0.793) , all other parameters had mild AUC values (range 0.534-0.594). Urine mtDNA-79 and mtDNA-230 values were higher than serum, but all of them were in mild AUC levels (Tables 2, 3).

Correlation analyzes of urine and serum mtDNA

The correlation analysis showed that the presence of prostatic inflammation was positively correlated with urine mtDNA-79, urine mtDNA-230, and serum mtDNA-79 levels. Also, urine mtDNA-79 levels were positive correlated with urine mtDNA-230 results. Similarly, serum mtDNA-79 levels were positively associated with serum mtDNA-230 (Table 4).

Discussion

To date, a few studies have quantified cf-mtDNA levels and mtDNA fragmentation pattern in urologic cancer patients. Some studies found elevated cf-mtDNA content in PCa (15, 21, 22), but another study on cf-mtDNA could-

Table 4.
Correlation analyzes of urine and serum mtDNA according to inflammation status.

Parameter	Correlation Coefficient Sig. (2-tailed)
Inflammation status *vs.* urine mtDNA-79	r = 0.343 p = 0.001
Inflammation status *vs.* urine mtDNA-230	r = 0.335 p = 0.001
Inflammation status *vs.* serum mtDNA-79	r = 0.250 p = 0.035
Urine mtDNA-79 *vs.* urine mtDNA-230	r = 0.601 p < 0.001
Serum mtDNA-79 *vs.* serum mtDNA-230	r = 0.262 p = 0.035

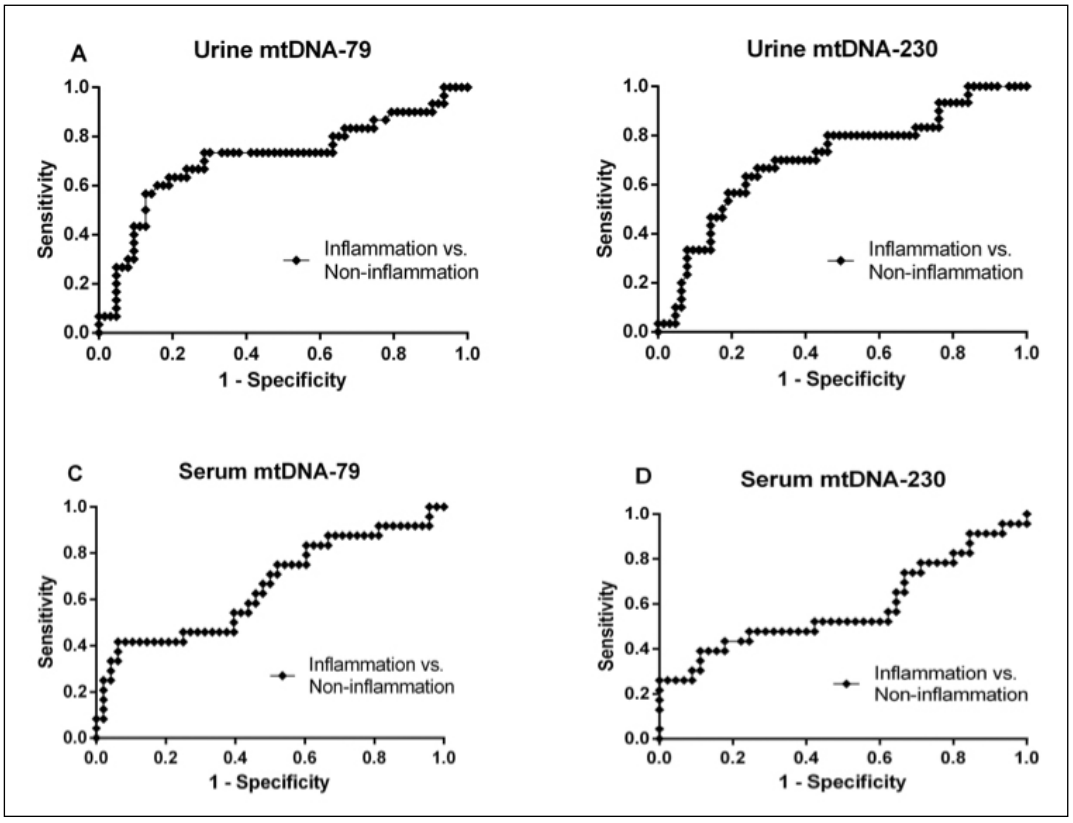

Figure 3.
Receiver operating characteristic (ROC) curves of the A. urine mtDNA-79, B. urine mtDNA-230, C. serum mtDNA-79, and D. serum mtDNA-230 between the Inflammation and Non-inflammation groups.

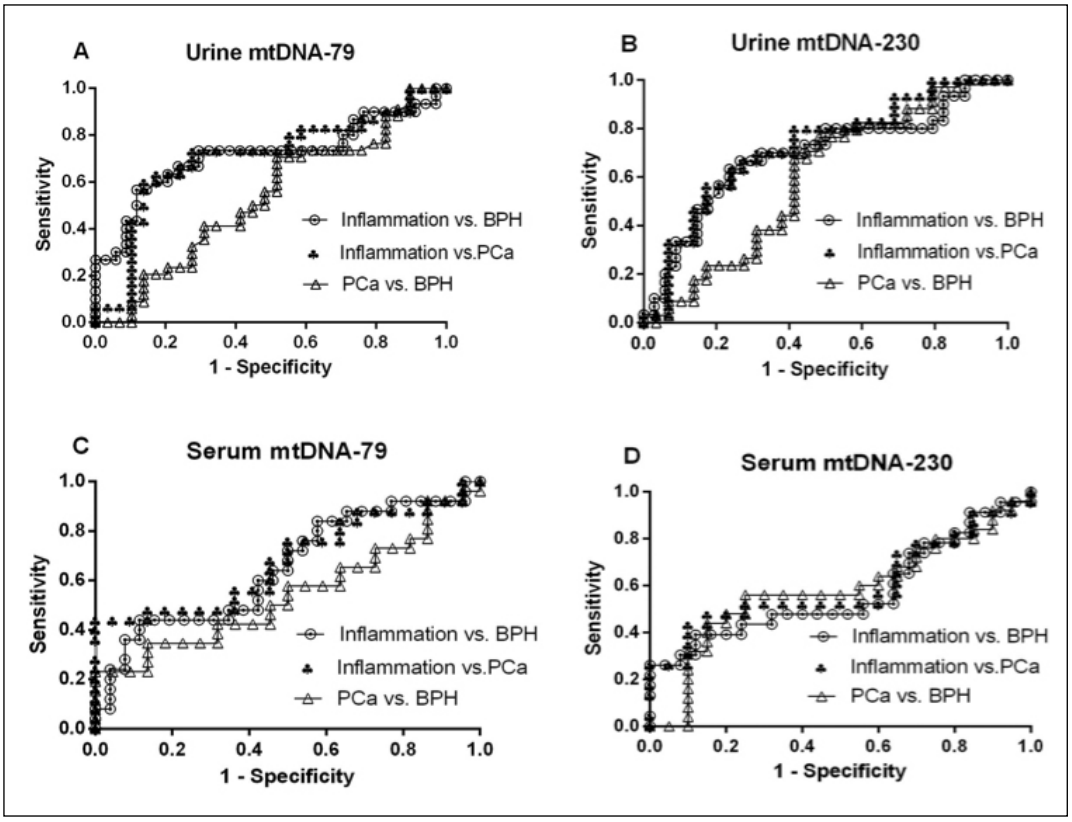

Figure 4.
Pairwise comparisons of the receiver operating characteristic (ROC) curves of the A. urine mtDNA-79, B. urine mtDNA-230, C. serum mtDNA-79, and D. serum mtDNA-230 among the Inflammation, PCa, and BPH groups.

n't distinguish between PCa and BPH (15). In addition to previous publications on blood samples (23), this study found higher levels of mtDNA fragments in post-prostate massage urine in cases with prostate inflammation than those without inflammation.

Different studies revealed a positive link between elevat-

ed systemic mtDNA levels and inflammation-associated diseases (9, 24, 25) and inflammatory cell death is often necrotic (7). We expected that a possible increase in the long mtDNA fragments (> 200 bp) derived from non-apoptotic types of cell death (i.e. necrosis) may be associated with prostate inflammation according to the histopathological examination. Surprisingly, we found that 230 bp fragment was significantly lower than 79 bp fragment (an implication of an active apoptotic phenomenon) in prostate inflammation. Additionally, our results show no correlation between urine and serum mtDNA-230 fragment in patients with inflammation, implying different factors contribute to elevated cf-mtDNA levels in each compartment.

Prostate massage can stimulate the release of tissue's viable cells as well dying/dead cells and increase the distribution concentration of circulating cf-mtDNA in urine. The influence of exfoliated inflammatory or endothelial cells on mtDNA fragments cannot be excluded, complicating our results. The cause of changes in cf-mtDNA fragment levels due to prostate inflammation remains unclear with our experimental design and existing literature. Although the origin of cf-mtDNA and cf-DNA lacks consensus (26), excessive release by apoptotic or necrotic cells and reduced clearance by inflammatory cells may alter mtDNA content. The mtDNA-79 fragment might be more resistant to DNAse activity than long fragments. Furthermore, whether the higher levels of cf-mtDNA fragments is a cause or a consequence for prostate inflammation in the context of mitochondrial damage, is the chicken or the egg causality dilemma.

Although cf-mtDNA is found in blood, urine, or saliva samples, data for prostate diseases associated with malignancy were mostly based on blood samples. Urine samples collected after prostate massage may provide more evidence for prostate-associated conditions due to fewer confounding factors. Therefore, we expected urine mtDNA fragments to reflect prostate inflammation magnitude better than blood ones. As anticipated, post-prostate massage urine from patients with prostate inflammation showed higher mean mtDNA-79 and mtDNA-230 copy numbers (approximately 16-fold and 22-fold, respectively) compared to serum samples. Serum mtDNA-79 was significantly increased in patients with inflammation ($p < 0.05$), while serum mtDNA-230 remained similar between inflamed and non-inflamed patients ($p > 0.05$). Post-prostate massage urine mtDNA-79 and mtDNA-230 copy numbers showed slightly higher AUC values for discriminating prostate inflammation than serum. The differences in fragment levels between urine and serum could be due to urine's enrichment in prostate-derived cf-mtDNA and the rapid clearance of nucleic acids from blood. Furthermore, urinary cf-mtDNA concentrations have lower *coefficient of variations* (CV) than serum, but further investigation is needed as their CVs still exceed 100%.

In this work, we also performed ROC curve analysis for urine cf-mtDNA fragments to differentiate PCa from inflammatory prostatic conditions, and compared it with the diagnostic performance of serum PSA levels. Considering the AUC, sensitivity and specificity, the levels of post-prostate massage urine mtDNA-79 and mtDNA-230 had slightly higher performance in distinguishing prostate inflammation than their serum levels. Here, the discrimination performance of single parameter for prostate inflammation is moderate with an AUC of about 0.7. Nevertheless, each fragment yielded performance similar to than serum PSA levels. Furthermore, when we tested the ability of these fragments in urine to discriminate PCa from BPH, PCa from prostate inflammation, and prostate inflammation from BPH, the AUC values of both fragment were within the range of 0.53-0.73, which were close to those (0.58-0.82) for the serum PSA. Moreover, combining of two fragment copy numbers did not significantly improve performance over using a single fragment alone. Therefore, mtDNA fragments alone does not seem to be a reliable predictor of differentiating PCa or BPH from inflammatory prostatic conditions.

This study had several limitations, including focusing only on histologically measurable inflammation, being a single-center trial, and lacking adjustment for prostate volume-related variables and other potential confounding factors due to small sample sizes. Additionally, although this study was conducted on the samples from PCa patients without prostatitis, it is essential to keep in mind that presence of prostatitis in PCa is not an infrequent event.

In conclusion, the present data demonstrate that cf-mtDNA fragments in post-prostatic massage urine increase in the presence of prostate inflammation; shorter cf-mtDNA fragment size in particular, may be a result of mostly an active apoptotic phenomenon associated with prostate inflammation. Even though both mtDNA fragments in the urine samples show relatively higher performance than blood, they do not seem to help identify patients with PCa from non-PCa patients more than the conventional PSA test. Further research is required to elucidate the sources of inter-individual variability observed in mtDNA copy numbers.

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