



OPEN The role of urine derived exosome metalloproteinases as biomarkers for prostate cancer detection may be confounded by non-malignant prostatic inflammation

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Matrix metalloproteinases (MMPs) are emerging as promising diagnostic and prognostic biomarkers for prostate cancer (PCa). This study explored the association between urinary exosome MMP levels and the incidence of prostate cancer. Urine samples were collected from patients undergoing prostate biopsy or prostatectomy, and from age-matched healthy controls. A total of 147 patients participated, including 37 patients who provided samples before prostatectomy, 41 patients before a biopsy that turned out positive, 21 patients before a biopsy that turned out negative, and 48 healthy controls. The study found that MMP-2 expression was similar in patients with prostate cancer and healthy controls but significantly higher in those with a negative biopsy. MMP-9 expression was elevated in patients with a positive biopsy and even higher in those with a negative biopsy. Multivariate logistic regression, adjusted for age, showed that increased MMP-2 expression was linked to a higher likelihood of a negative biopsy result (OR 1.12 [1.002, 1.252], $P = 0.046$), while increased MMP-9 expression was associated with a higher probability of prostate cancer diagnosis (OR 1.106 [1.002, 1.22], $P = 0.045$). These findings suggest that urinary MMP-9 levels are elevated in PCa patients, though even higher levels in biopsy-negative cases may reflect confounding factors such as benign prostatic inflammation.

Keywords Biomarker, Diagnosis, Hydrolase, Matrix metalloproteinases, Prostatic neoplasms

Prostate cancer (PCa) is the second most commonly diagnosed cancer in men after lung cancer and is the fifth leading cause of cancer-related deaths in men¹. Owing to the wide use of prostate-specific antigen (PSA) blood testing, PCa is being diagnosed at an early stage in countries with active screening programs resulting in rising incidence and improved mortality over the past decade². Despite its shortcomings, including low specificity and elevation in benign conditions such as benign prostatic hyperplasia (BPH), prostatitis, inflammation and increasing age and prostate volume, no serum, urinary, or salivary marker could replace the role of serum PSA in PCa diagnosis³. Available genomic markers for PCa in clinical use mostly detect genetic polymorphisms associated with PCa³. Commercial kits are expensive, and their role is limited to identifying patients who might need a prostatic biopsy, identifying patients at high risk for progression and metastasis and assisting physicians in choosing treatment modalities³. Risk stratification using genomic, tissue and circulating biomarkers remains a significant unmet need.

Matrix metalloproteinases (MMPs), particularly MMP-2 (gelatinase A) and MMP-9 (gelatinase B) have been implicated in cancer progression, through their ability to degrade the extracellular matrix (ECM), facilitating

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angiogenesis⁴, tumor growth, invasion, and metastasis^{5,6}. Animal studies have shown that MMP-2 expression in PCa cells is associated with decreased survival⁷, and MMP-9 expression is associated with higher metastatic rate⁸.

Exosomes, nano-sized extracellular vesicles secreted by cells, have emerged as promising biomarkers in cancer research due to their presence in bodily fluids and their role in intercellular communication^{9,10}. In PCa, exosomes have been shown to influence the behavior of normal cells *in vitro* by leading them to express prostate-specific genes¹¹, and assist metastatic processes by altering the tumor microenvironment¹².

Despite their potential, studies on exosome-derived MMPs in PCa have produced varying results, with some studies suggesting an important role in PCa diagnosis and prognosis^{13,14}, while others highlighting their minor role in PCa risk determination^{15,16}.

This study aimed to explore whether the level of MMPs expression in urinary exosomes correlates with PCa incidence and burden, and to discuss the methodological limitations of previous research, including sample heterogeneity, detection sensitivity, and the challenges of interpreting null findings.

Methods

Study design and participants

After Institutional Review Board approval (IRB approval IM.DM.08, 19 August 2015), patients presenting to the American University Medical Center between 2015 and 2019, for a trans-rectal ultrasound guided (TRUS) biopsy or radical prostatectomy were included and provided written informed consent to contribute pre-procedure urine samples for analysis of the level of MMP in urine-derived exosomes. Patient charts were then reviewed to collect data on patient demographics, date of diagnosis, stage and grade of tumor at diagnosis, PSA level at diagnosis, development of metastasis, total PSA at follow-up and date of disease progression or death. Following recruitment of patients, age-matched male controls were recruited from community volunteers, hospital staff, and companions of patients attending non-urology clinics. All controls had a normal PSA value within the preceding 12 months, no history of urologic symptoms or disease, and no prior diagnosis of malignancy or chronic inflammatory conditions. Screening for eligibility was conducted through medical history and review of recent laboratory results. Controls were selected in a 1:2 ratio to biopsy patients to reduce potential confounding while ensuring statistical power.

For all three groups of patients (presenting for biopsy, presenting for prostatectomy, control), convenience sampling was employed whereby patients presenting to oncology, urology, and primary care clinics were approached and informed about enrollment into the study. Patients who accepted to be enrolled were consented and enrolled, and those who refused were not included. Patients in all groups were excluded if they were <50 years of age, if they have previously been diagnosed with any malignancy, or have previously or are currently receiving local or systemic treatment for any malignancy. In addition, patients in the biopsy group were excluded if they were undergoing a biopsy for reasons other than an elevated PSA or suspicious clinical findings on digital rectal examination concordant with suspicious MRI findings. Patients in the control group were excluded if they had any urologic complaint or have any urologic history including BPH. Urine samples were collected before any PCa-related procedures. Participants were distributed in three groups: (1) Positive biopsy + Prostatectomy; (2) Negative biopsy; and (3) Healthy controls.

Urine collection

Urine was collected from patients who underwent radical prostatectomy or biopsy and from age-matched controls; before any procedure. Urine was collected according to the institutional bioethical guidelines after consent was obtained from patients and control subjects. Urine samples were kept on ice for no longer than 2 h and then stored at -80 °C. Before analysis, urine samples were tested for the presence of blood using Ames Multistix 7 reagent strips (Miles), and specimens containing blood were excluded from subsequent assays.

Exosomes purification from urine

Urine samples were transferred into 15 ml conical tubes and centrifuged for 30 min at 2,000 × g. The supernatant containing the clarified urine was transferred to a new clean 15 ml tube without disrupting the pellet, made up of cells and debris, and an equal volume of total exosome isolation reagent was added; according to the manufacturer's instructions of the Total exosome isolation kit from urine (Cat# 4484452, Invitrogen™, ThermoFischer Scientific, Karlsruhe, Germany). Mixing was performed by gentle vortexing, until the solution became homogenous. The mixture was incubated at room temperature for 1 h, and then samples were centrifuged for 1 h at 10,000 × g. The supernatant was removed without disturbing the pellet and the pellet was re-suspended in phosphate buffered saline (PBS). Isolated exosomes were either stored at -20 °C or fixed in 2% paraformaldehyde (PFA), depending on subsequent assays.

Exosomes characterization by scanning electron microscopy

The exosome pellet was fixed in 2% paraformaldehyde and was left to adsorb on carbon adhesive tabs for 20 min in a dry environment. After one wash with PBS, the tabs were transferred to a solution of 1% glutaraldehyde for 5 min and then rinsed with distilled water for 2 min each, for a total of eight washes. The fixed exosomes were dehydrated by 5-minute immersion in an ascending gradient of ethanol (40%, 60%, 80% and 97%). After evaporation of ethanol, the tabs were left to air dry at room temperature for 24 h on a glass microscope slide. The tabs were then mounted on aluminum specimen mounts and examined by Mira3 LM Scanning Electron Microscope (SEM) (Tescan, Czech Republic) (Fig. 1A).

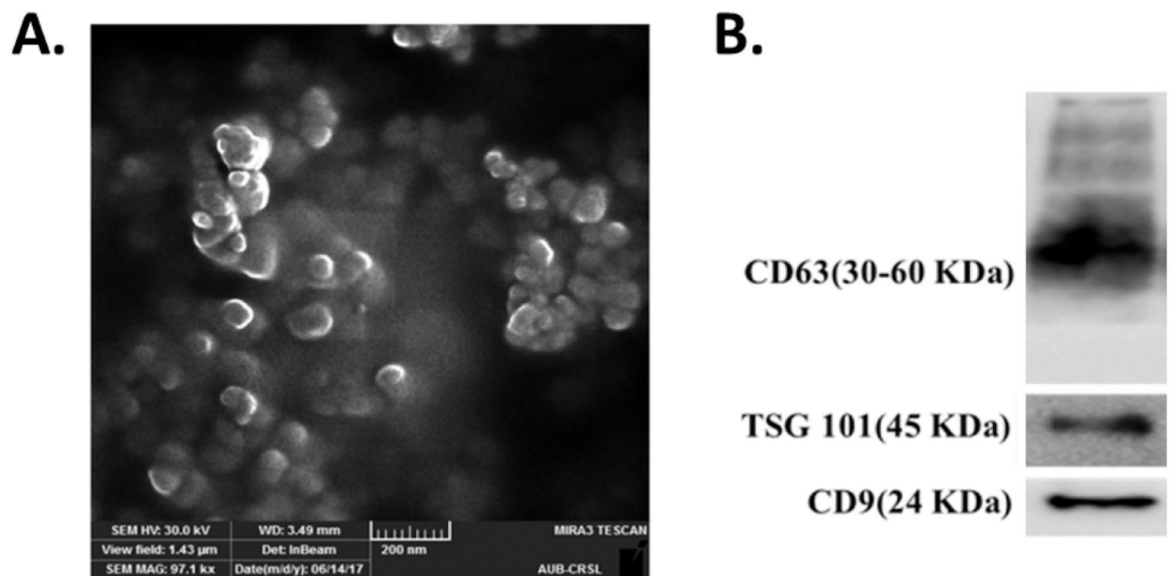


Fig. 1. Characterization of exosomes derived from urine. (A) Scanning electron micrograph of exosomes isolated from urine, scale bar 1 μ m. (B) A representative western blot showing the protein expression of TSG-101, CD9, and CD63 in the lysates of exosomes isolated from urine samples.

Immunoblot analysis of exosomes

To confirm the identity of extracted exosomes, western blot analysis was performed. 50 μ g of proteins were loaded onto a 10% separating gel, to detect the protein expression of exosomal markers such as TSG101, CD63 and CD9. The different antibodies used were: anti-TSG101 (Cat# ab125011, Abcam, Waltham, Massachusetts, USA), anti-CD63 (Cat# ab59479, Abcam, Waltham, Massachusetts, USA) and anti-CD9 (Cat# 10626D, ThermoFischer Scientific, Karlsruhe, Germany), diluted to the working concentration of 1 μ g/ml. The appropriate horseradish peroxidase-conjugated antibodies were detected using chemiluminescence. Bands were visualized using the ChemiDoc MP Imaging System operated by the Image Lab software (Bio-Rad, Hercules, California, USA) (Fig. 1B).

Image acquisition and presentation

All gels and blots were imaged using the ChemiDoc MP Imaging System operated by Image Lab software (Bio-Rad, Hercules, California, USA). Images presented in the main figures were cropped to improve clarity and focus on the bands of interest, without any modification of contrast, brightness, or exposure. To ensure compliance with digital image integrity policies, the original uncropped full-length gels and blots, including visible membrane edges, are provided in Supplementary Fig. 1S. These uncropped images represent the same experiments shown in Fig. 2.

Metalloproteinases activity detection by gelatin zymography

To assess the expression of MMPs in the urinary PCa derived exosomes, gelatin zymography was performed. 50 μ g of protein extracted from the urinary exosomes were loaded into a 4% stacking gel and migrated in a 10% polyacrylamide resolving gel containing 4% gelatin. FBS at 0.5% was used as a positive control to detect MMP-2 and MMP-9 enzymatic activities at different molecular weights. The gel was then washed twice with PBS containing 0.025% Triton X-100 for 20 min and incubated in a substrate buffer containing Tris-HCl, CaCl_2 and sodium azide at 37 $^{\circ}\text{C}$ overnight. Gels were then stained with 0.5% Coomassie Brilliant Blue stain for 1 h followed by a destaining step using 30% ethanol, 10% acetic acid and 60% water. Finally, the gels were visualized by a Bio-Imaging System. Enzymatic activity is detected by white bands on a blue background. Quantification of the bands was achieved using the ChemiDoc MP Imaging System operated by the Image Lab software (Bio-Rad, Hercules, California, USA).

Statistical analysis

Univariate and multivariate analyses were carried out to explore the relationship between the level of MMP expression and the presence of PCa, total PSA at diagnosis, clinical stage at diagnosis and Gleason score (GS). Descriptive analysis was performed for all disease-related variables among patients with PCa and healthy subjects who provided urine (controls). Quantitative variables were reported as median and interquartile range (IQR). Qualitative variables were reported as count (frequencies) and percentages. Bivariate analyses were carried out to explore the relationship between the level of urine exosome expression and other disease-related variables, across the three population groups. Since the distribution of exosome expression among study participants is skewed, non-parametric tests (Kruskal-Wallis) were used to compare the means of MMP expression among the three patient groups. Subsequently, the Mann Whitney U test was used to discern the relationship between two

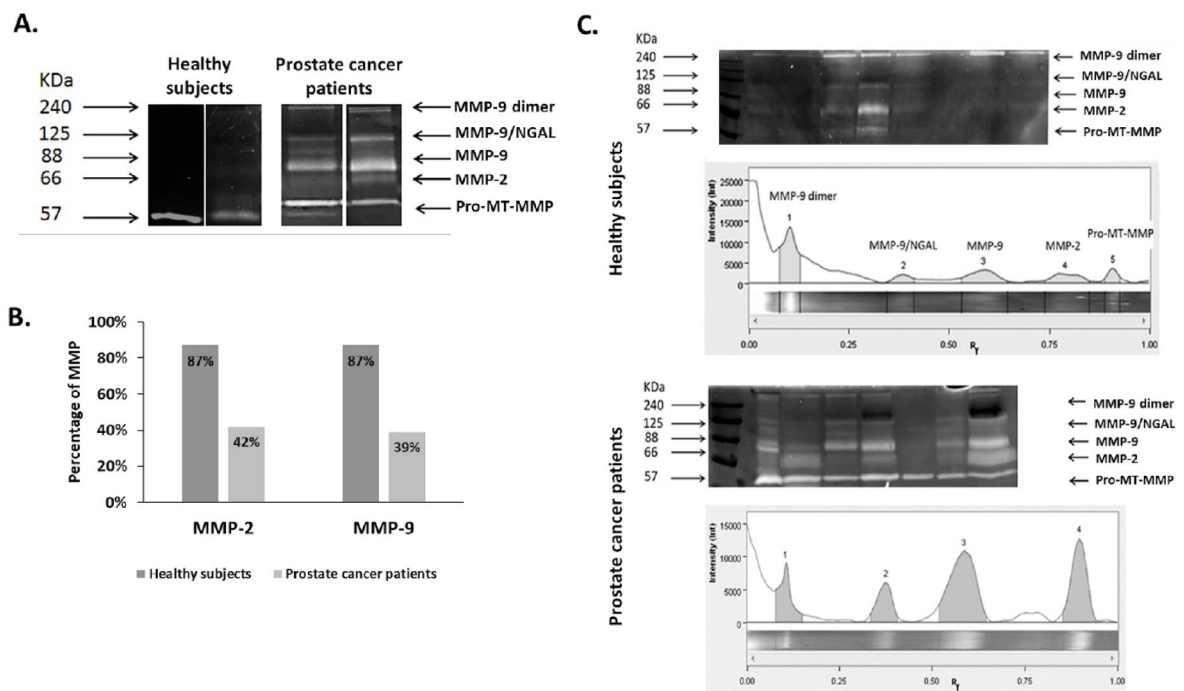


Fig. 2. Tumor-specific fingerprinting of urinary MMP. **(A)** Zymograms of MMPs in representative urine samples from healthy subjects and PCa patients. **(B)** Positive expression of MMP-9 and MMP-2 in urine exosomes from healthy subjects and PCa patients. **(C)** Gelatinase species in urine of healthy subjects (upper panel) and PCa patients (lower panel). Representative urine samples were analyzed by zymography with their respective band profiles. Uncropped full-length gels corresponding to these panels, with membrane edges visible, are provided in Supplementary Fig. 1S. * $P < 0.001$ for each, Fisher's exact test. MMP: matrix metalloproteinase; NGAL: neutrophil gelatinase-associated lipocalin.

groups at a time. Moreover, to decrease the effect of outliers, the log base 10 of MMP-2 and MMP-9 expression was used in constructing Fig. 3. An exploratory univariate logistic regression analysis was performed on all available patient factors, and odds ratios (OR) with 95% confidence intervals (CI) and P -values were determined. Variables that had a P value < 0.1 at the univariate level were considered in the multivariate logistic regression analysis. The eligible variables were then entered simultaneously at the first step, and a stepwise multivariate logistic regression analysis was performed. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) Software, v.26 (IBM Corp., Armonk, New York, USA). Statistical significance was set at the alpha level of 0.05.

Results

Characteristics of study participants

Out of 200 patients who were approached for enrollment into the study, a total of 147 participants, with a mean age of 61.7 years, consented and provided urine samples. The participation rate was 73.5%. Urine samples were collected before PCa-related procedures: 62 urine samples prior to TRUS-guided biopsy (42.2%), 37 prior to prostatectomy (25.2%) and 48 were from healthy controls (32.6%). Out of 62 patients who had a biopsy, 21 did not have PCa (negative biopsy), making the total number of patients with PCa 78 (53.1%) versus 69 (46.9%) PCa-free patients. Median total PSA level at enrollment was 6.7 ng/mL (IQR: 4.9–9.6 ng/mL) for the patients with PCa vs. 6.5 (IQR: 5.4–9.3 ng/mL) for the patients with negative biopsy. Control patients did not have PSA values. At diagnosis, 53 patients out of 78 PCa patients (83.4%) had a disease with clinical stage earlier than T3 and 57 patients (80.3%) had GS below 8. Three patients (3.8%) had distant metastasis at diagnosis, and 6 patients (7.6%) developed distant metastases during follow-up. In total, 52 patients (75.4%) were then treated with prostatectomy (Table 1). Only one patient passed away during the 5 years of follow-up.

Isolation and characterization of urine exosomes

Exosomes were isolated from the participants' urine samples and processed for SEM. Figure 1A displays a representative electron micrograph of urine exosomes. Figure 1B shows the expression of exosome-specific biomarkers; proving that the membrane-bound vesicles detected by SEM are exosomes.

MMP expression in urine exosomes

Urinary MMP-2 and MMP-9 were mapped in exosomes isolated from healthy controls, patients with PCa (prostatectomy or positive biopsy), and patients with negative biopsy. Zymography results showed gelatinase activity in the urine of patients with PCa at 125 KDa and > 220 KDa, corresponding to MMP-9/NGAL complex

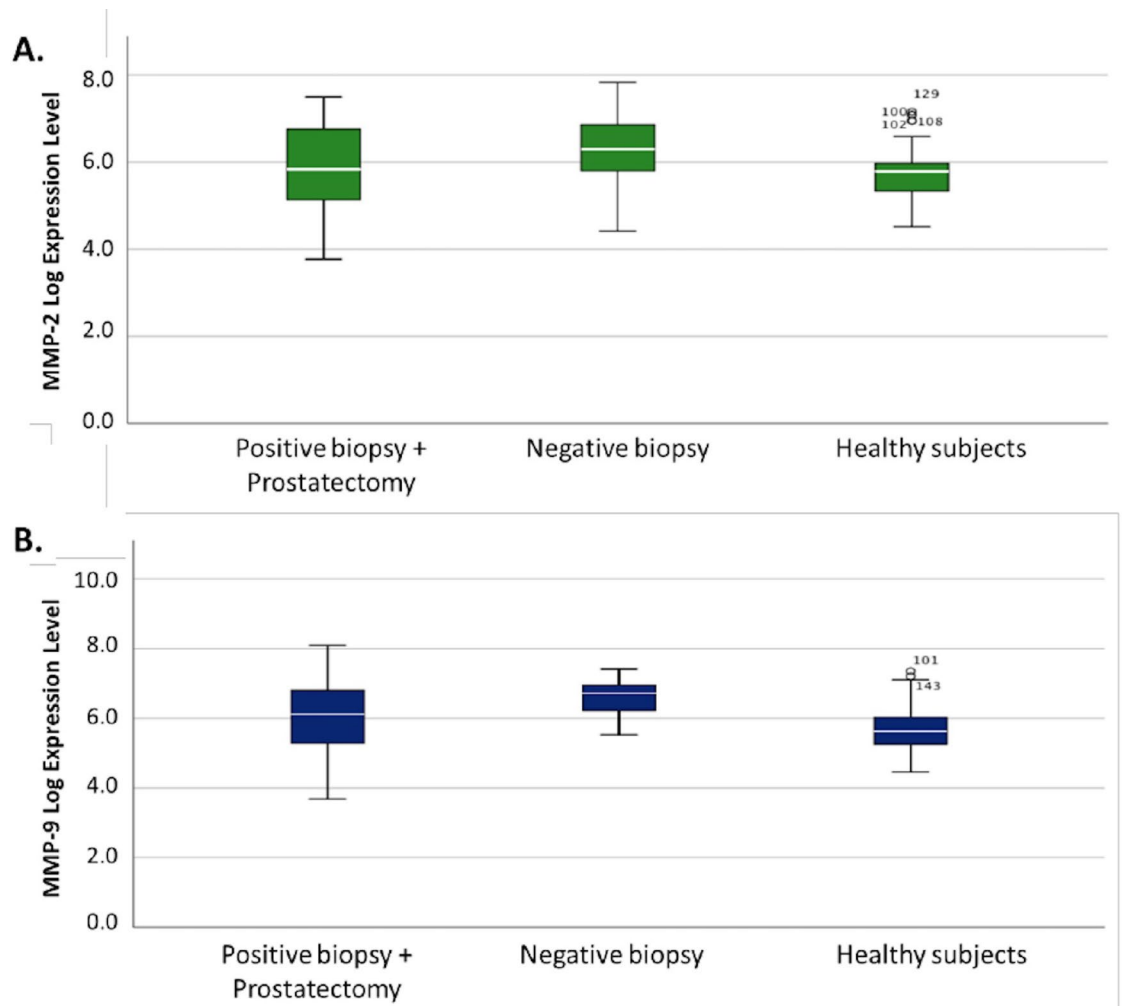


Fig. 3. Distribution of MMP expression in population subsets. **(A)** MMP-2 expression in urine exosomes among patients with PCa on biopsy (positive biopsy), patients with no cancer on biopsy (negative biopsy), and healthy subjects. **(B)** MMP-9 expression in urine exosomes among patients with PCa on biopsy (positive biopsy), patients with no cancer on biopsy (negative biopsy), and healthy subjects.

and MMP-9 dimer, respectively (Fig. 2A). MMP-2 and MMP-9 expression and activity were detected in 87% of patients with PCa, compared to 42% and 39% of healthy controls ($P < 0.001$, Fig. 2B). Figure 2C shows gelatinase species in the urine of healthy controls and PCa patients. Band quantification underscores the increased activity of MMP-2 and MMP-9 species and complexes in PCa patients, compared to their healthy counterparts.

The expression of MMP-2 and MMP-9 was lower in the exosomes isolated from healthy subjects, compared to PCa patients. To evaluate whether urinary MMP expression can be used as a PCa biomarker, we looked at MMP expression in the exosomes extracted from subjects who had a negative biopsy. Figure 3A shows that MMP-2 expression was similar in patients with PCa and healthy controls ($P = 0.714$), but significantly higher in those whose prostate biopsy turned out to be negative for PCa ($P = 0.015$). On the other hand, MMP-9 expression was found to be higher in PCa patients compared to healthy subjects ($P = 0.016$). Patients whose biopsy was negative for PCa had an even higher MMP-9 expression level ($P = 0.014$) (Fig. 3B). Overall, analysis showed that MMP-2 and MMP-9 expression was different in patients who underwent prostate biopsy after providing urine samples and healthy subjects.

Univariate regression analysis showed that MMP-2 and MMP-9 expression did not correlate with age ($P = 0.59$ and $P = 0.90$, respectively), total PSA at diagnosis, stage at diagnosis and GS. On multivariate logistic regression and after adjusting for age, with the control group as reference, an increase in MMP-9 expression by 1×10^6 was associated with increased odds of being diagnosed with PCa (OR = 1.106 [1.002, 1.22], $P = 0.045$), whereas an increase in MMP-2 expression by 1×10^6 was associated with increased probability of negative biopsy (OR 1.12 [1.002–1.252], $P = 0.046$) (Table 2). MMP-2 expression did not significantly impact the probability of being diagnosed with PCa (OR 1.08 [0.971–1.202], $P = 0.156$), and MMP-9 expression did not significantly impact the probability of negative biopsy (OR 1.097 [0.990–1.214], $P = 0.076$) (Table 2).

	N = 147
Age, median (IQR) in years	61.7 (56–69.7)
Urine specimen type, n (%)	
Prebiopsy	62 (42.2%)
Pre-prostatectomy	37 (25.2%)
Healthy subjects	48 (32.7%)
Total PSA at enrollment (PCa patients), median (IQR) in ng/ml	6.7 (4.9–9.6)
Total PSA at enrollment (negative biopsy patients), median (IQR) in ng/ml	6.5 (5.4–9.3)
PCa status, n (%)	
PCa diagnosis	78 (53.1%)
No PCa diagnosis	69 (46.9%)
Stage at diagnosis, n (%)	
< T3	53 (83.4%)
≥ T3	5 (8.4%)
N1M0	2 (3.3%)
N1M1	3 (5%)
Missing	15
Gleason score at diagnosis, n (%)	
< 8	57 (80.3%)
≥ 8	14 (19.7%)
Missing	7
Metastasis at diagnosis, n (%)	
None	96 (97%)
Presence of metastasis	3 (3%)
Missing	48
Development of distant metastases, n (%)	
None	93 (93.9%)
Development of metastatic lesions	6 (6.1%)
Missing	48
Initial treatment received, n (%)	
Robot-Assisted Radical Prostatectomy	52 (75.4%)
Androgen deprivation	9 (13%)
Active surveillance	4 (5.8%)
Radiotherapy	4 (5.8%)
Missing	9
Status at the 5-year follow-up, n (%)	
Alive	73 (76.8%)
Lost to follow-up	21 (22.1%)
Deceased	1 (1.1%)
Missing	52

Table 1. Characteristics of enrolled patients. IQR: interquartile range; PCa: prostate cancer; PSA: prostate-specific antigen; TNM: tumor size (T), number of lymph nodes involved (N) and metastatic lesions (M)

Patient group	Variable	OR (95%CI)	P-value
Positive biopsy for PCa AND prostatectomy	MMP-2/10 ⁶	1.08 (0.971–1.202)	0.156
	MMP-9/10 ⁶	1.106 (1.002–1.220)	0.045
	Age	1.06 (1.016–1.106)	0.006
Negative biopsy for PCa	MMP-2/10 ⁶	1.12 (1.002–1.252)	0.046
	MMP-9/10 ⁶	1.097 (0.990–1.214)	0.076
	Age	1.018 (0.961–1.078)	0.55

Table 2. Multivariate logistic regression for the impact of MMP expression and age on PCa diagnosis after biopsy. CI: confidence interval; MMP: matrix metalloproteinase; OR: odds ratio For OR calculation, control group was used as reference.

Discussion

There is a growing interest in investigating the role of urinary biomarkers for PCa as a non-invasive and relatively cost-effective method for diagnosis and design of a therapeutic plan in patients with PCa¹⁷. MMP-2 and MMP-9 are examples of urinary biomarkers that show a great potential for risk stratification of patients for screening and diagnosis of PCa.

Similar to our findings of increased MMP-9 expression in urine of PCa patients compared to controls, several studies have found higher MMP-9 expression in serum and prostatic tissue of PCa patients compared to patients with benign prostatic hyperplasia (BPH)^{18–20}. However, our data shows significant levels of expression of urinary MMP-9 in patients who have a negative prostatic biopsy. Those patients presented with elevated PSA and suspicious lesions on prostatic MRI. We postulate that the reason behind elevated MMP-9 expression, in this subset of patients, is related to inflammatory changes in the prostate, possibly related to infections. In fact, a study by Sun et al. showed increased serum expression of MMP-9 in patient with prostatitis²¹. Besides its role in

PCa, MMP-9 was shown to have an important role in the pathophysiological mechanisms of multiple diseases in the nervous, respiratory and cardiovascular systems and its expression in serum and urine is affected by multiple inflammatory conditions in the body including autoimmune and idiopathic inflammatory diseases²². Thus, its use in the diagnosis and management of PCa can be jeopardized by other confounders that lead to MMP-9 level elevation in urine, blood and prostatic tissue.

In this study, MMP-2 expression was found to be similar between patients with PCa and healthy subjects. In comparison, a systematic review and meta-analysis by Xie et al. showed that increased MMP-2 expression is associated with PCa. The even higher level of MMP-2 expression in patients with no PCa on biopsy can be attributed to reasons similar to those mentioned above for MMP-9 expression. Moreover, our multivariate analysis showed a contradictory protective role of MMP-2 expression in urine²³. This can be due to the limited sample size, or it can be due to a different genetic behavior of PCa among the population from which our cohort was obtained. In fact, it has been well established that cancers including PCa have different genetic behaviors among different races and ethnicities²⁴, and most studies investigating genetic susceptibilities are performed in Western countries with scarcity of data from underdeveloped or developing countries, which precludes our ability to make meaningful comparisons. Our data shows no significant correlation between MMP-2 and MMP-9 expression and GS, PSA at diagnosis and stage at diagnosis. This contradicts available data which shows higher GS and higher stage at diagnosis in patients with elevated MMP-2 and MMP-9 expression, but goes in line with available data suggesting no correlation between their level and PSA level at diagnosis^{7,24,25}.

Similar to our results that highlight how the subgroup of patients with a negative biopsy have even higher MMP expression, several studies in biomarker research have referred to how omission of well-defined control groups that account for benign inflammatory conditions may affect the validity and generalizability of results^{21,22,26}. In fact, one study highlighted that on the molecular level, MMP-9 over-expression occurs in the inflammatory cells that surround prostate cancer cells, and not prostate cancer cells specifically²⁷, which could, in part, explain how MMP expression is elevated in PCa patients but even higher in patients with no PCa on biopsy, as this subset of patients may have an inflammatory condition causing the rise in PSA that prompted the biopsy.

Many studies, while establishing correlations between biomarkers and cancer, fail to differentiate between inflammatory-driven elevations and those specifically caused by malignancy. This is particularly critical given that inflammation can contribute to elevated levels of certain biomarkers, such as matrix metalloproteinases (MMPs). Biomarker studies that fail to account for benign inflammatory conditions in control groups risk overestimating the specificity of these biomarkers in multiple types of cancer including but not limited to pancreatic²⁸, and breast cancer²⁹. Therefore, it is imperative that future biomarker studies include carefully selected control groups, not just healthy subjects or PCa-negative patients, but also patients with benign prostatic inflammatory conditions, to better delineate the true diagnostic value of potential biomarkers like MMPs.

Our study has several limitations. We were not able to compare patients with PCa to patients with BPH as we lacked the data on BPH history in our cohort. Additionally, our relatively small sample size could have prevented us from reaching significant results regarding MMP expression and its correlation with PSA, GS and stage at diagnosis. It is widely known that small sample sizes reduce statistical power, increase the risk of both type I and type II errors, and may lead to inflated effect size estimates, thereby limiting the reliability and generalizability of our study findings³⁰. Moreover, other comorbidities including inflammatory disorders, use of steroids, smoking status, race/ethnicity, family history of prostate cancer and other medical conditions were not included in our analysis and they could potentially contribute to elevated MMP expression among some of our patients. Another important limitation is the lack of information on history of previous UTI/prostatitis, since many of the patients had missing data. Furthermore, no information regarding whether healthy subjects had or did not have PCa as none of the participants in this group had a prostate biopsy performed. Finally, our study may be subject to non-response bias, as individuals who declined participation could differ systematically from those who agreed to enroll, potentially affecting the representativeness of our sample.

Conclusion

In conclusion, our data suggest that MMP-2 and MMP-9 urinary expression use as biomarkers for PCa diagnosis and management planning should be evaluated with caution. Multiple confounding variables can affect their level of expression and thus impede their use as reliable indicators of malignancy. However, the urine of patients requiring a prostate biopsy had higher levels of MMPs (especially MMP-9 and to a lesser extent MMP-2); which means that urine MMP levels could indicate the need for prostate biopsy. Future studies evaluating the role of MMP-2 and MMP-9 urinary biomarkers should include appropriately powered control groups with non-malignant prostatic inflammatory disease.

Data availability

Data is available with the PI on a password-protected computer at his office at the Faculty of Medicine of the American University of Beirut. The data is available upon request from Marwan El-Sabban [e-mail: Email: [me00@aub.edu.lb] ; Tel.: +961 (1) 350000].

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Author contributions

JN: Data collection, Data analysis, Manuscript writing; LH: Data collection, Manuscript writing; OH: Data collection, Data analysis, Manuscript writing; TT, CD, MH: Data collection, Manuscript writing; AH: Conceptualization; Manuscript proofreading; WA: Conceptualization; Manuscript proofreading; DM: Project supervision; Manuscript proofreading; MES: Corresponding Author, Conceptualization, Data collection, Project supervision and Manuscript writing/reviewing.

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Declarations

Competing interests

The authors declare no competing interests.

Ethics approval and patient consent

The institutional review board at the American University of Beirut (IRB approval IM.DM.08, 19 August 2015) has approved the study and all patients and healthy subjects provided written informed consent. All methods were carried out in accordance with relevant guidelines and regulations.

Additional information

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