ORIGINAL RESEARCH

Blood Neutrophil-to-Lymphocyte Ratio and Urine IL-8 Levels Predict the Type of Bacterial Urinary Tract Infection in Type 2 Diabetes Mellitus Patients

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Background: Extended-spectrum ß-lactamase (ESBL)-producing *Escherichia coli (E. coli)* and *Klebsiella pneumoniae (K. pneumoniae)* are the most common uropathogens causing UTI (urinary tract infection) in type 2 diabetes mellitus (T2DM). Circulatory inflammatory markers such as C-reactive protein (CRP) and neutrophil-to-lymphocyte ratio (NLR) are usually dysregulated during UTI. However, the differential regulation of these inflammatory signatures during *E. coli* and *K. pneumoniae* UTI in T2DM has not been determined.

Methods: A case–control study on 466 patients was performed to investigate the inflammatory signatures indicative of ESBL-*E. coli* and *K. pneumoniae* UTIs in T2DM. Serum CRP levels and blood NLR for these patients were determined and associated with *E. coli* and *K. pneumoniae* ESBL uropathogen using multivariate logistic regression analysis. Urinary interleukin 8 (IL-8) levels were also assessed and associated with these two UTI uropathogens in T2DM. The association of the two ESBL-uropathogens with the survival outcomes of T2DM patients was also analyzed using Cox-proportional hazard model.

Results: T2DM patients with ESBL-*E. coli* UTI had lower serum CRP levels (median, CRP mg/dL 33.7 vs 39.8, respectively; P=0.023) and higher blood NLR (median, NLR 3.2 vs 2.6, respectively; P=0.010) compared to those with *K. pneumoniae* UTIs (*P*<0.001). Moreover, in T2DM, the urinary IL-8 levels was higher in ESBL-*E. coli* compared to those with *K. pneumoniae* UTIs (*P*<0.0001). After adjusting for confounders, including age, gender, serum albumin, hemoglobulin, leukocytes, and platelet counts, T2DM patients with blood NLR ≥ 3.5 were at higher risk for ESBL-*E. coli* UTIs than ESBL-*K. pneumoniae* UTIs (odds ratio [OR], 3.61, 95% confidence interval, Cl, 1.49–8.73; P=0.004). Moreover, T2DM patients with ESBL-*E. coli* UTIs had higher all-cause mortality (hazard ratio [HR], 4.09; 95%, 1.14–14.59) than those with *K. pneumoniae* UTIs.

Conclusion: Serum CRP levels, blood NLR, and IL-8 urinary levels differentiate ESBL-*E. coli* from *K. pneumoniae* UTIs in T2DM.

Keywords: extended-spectrum ß-lactamase, *Klebsiella pneumoniae*, *Escherichia coli*, urinary tract infection, C-reactive protein, survival

Introduction

Escherichia coli (*E. coli*) is the most common cause of community-acquired or nosocomial urinary tract infection (UTI).[1] Extended-spectrum ß-lactamase (ESBL)-producing Enterobacteriaceae such as *E. coli and Klebsiella pneumoniae (K. pneumoniae)*[2,3] are the most frequent isolate in UTIs and are resistant to many classes of antibiotics.[4] Type 2 diabetes (T2DM) patients are at an increased risk for

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UTIs with these uropathogens, and *E. coli* being the most frequent isolate.[5] Almost half of T2DM develop ESBL-positive UTIs that might result in acute kidney injury.[6]

C-reactive protein (CRP) has been used as an early biomarker predicting bacterial infections and is used as a marker to monitor antibiotic treatment of these infections.[7] Recent reports have suggested the use of CRP blood levels to differentiate acute pyelonephritis (upper UTIs) from asymptomatic bacteriuria in pediatric UTIs.[8] Blood neutrophil-to-lymphocyte ratio (NLR) has also been suggested to be used as a diagnostic marker for UTIs.[9,10] Assessment of urine cytokine levels could reflect the type of immune responses induced during UTI, and may be associated with specific types of uropathogens. Elevated levels of urinary IL-8 have been observed in the urine of children with acute pyelonephritis and renal scarring.[11] However, studies that associate these inflammatory markers to the type of uropathogen in T2DM are lacking. Therefore, the objective of our study was to determine the inflammatory markers that may differentiate between ESBL-producing *E. coli and K. pneumoniae* uropathogens causing UTIs during T2DM. The association of these uropathogens with all-cause mortality of T2DM patients was also determined.

Methods
Ethical Considerations
Ethical approval was obtained from the University Hospital Sharjah Research Ethics Board (Reference No: UHS/CS/HERC/F001-2/17). All methods were performed in accordance with the relevant guidelines (Declaration of Helsinki and the Belmont Report). Written informed consent was obtained from all study participants.

Study Patients
The initial cohort included a total of 668 patients from both outpatients and inpatients diagnosed with ESBL-positive UTI (ESBL-UTI), from January 1st 2017 through 15th July 2019. ESBL-UTI diagnosis was based on positive microbiological test results and patient symptoms. Symptoms were typically acute in onset and included dysuria, urinary frequency and urgency, suprapubic or flank pain, hematuria, and/or documented fever. Inclusion criteria included adults both males and females with ESBL-positive UTI test who had complete microbiological test results and laboratory values including complete blood counts and serum CRP value. Exclusion criteria were younger than 18 years, pregnant women, and those with

active malignancy. Out of the 668 ESBL-UTI patients, 466 patients had complete laboratory values and microbiology test results. Around half of these ESBL-UTI patients had T2DM (n=264, 57%). T2DM was determined using laboratory measurements of FBG, HbA1c and patient medical record information. Following the WHO criteria for both FBG and HbA1c; FBG < 6.1 mmol/l was considered normal and ≥7.0 mmol/l indicated diabetes. However, HbA1c <6.5% was considered non-diabetic and ≥6.5% indicated diabetes.[12]

Univariate and Multivariate Logistic Regression Analysis
For the purpose of descriptive analysis, patient's demographic, clinical data, and laboratory test results were associated with ESBL-*E. coli* or *K. pneumoniae* UTIs. For two-way analysis of continuous variables, *t*-test or Mann–Whitney *U*-test was used depending on the skewness of data. Continuous variables were first analyzed without categorization, but a different cutoff value was used in multivariate analysis. Chi-square (X^2) test was used for categorical variables analysis.

To investigate the inflammatory signatures indicative of UTIs in T2DM and non-T2DM subgroups, we determined the serum CRP and blood NLR levels for these patients in association with *E. coli* or *K. pneumoniae* uropathogen. The risk for *E. coli* or *K. pneumoniae* UTI diagnosis for these patients was assessed based on serum CRP values and blood NLR using multivariate logistic regression analysis, after adjusting for various cofounders.

The accuracy of the regression model was evaluated using positive predictive value, and negative predictive value. Sensitivity (percentage predicted positive among all truly positive) and specificity (percentage predicted negative among all truly negative) were also calculated. A sample size of 77 cases and 77 controls were assessed to provide >80% power to detect a significant difference (α = 0.05) across subgroups of ESBL-*E. coli* or ESBL-*K. pneumoniae* UTIs using G*power software.[13]

ELISA Analysis
Urine IL-8 levels for T2DM were assessed using ELISA assay according to the manufacture's instructors (IL-8, Cat # ab46032 Abcam, Cambridge, MA, USA). Each sample was assayed in triplicate and values were expressed as the mean of three measures per sample.

Survival Analysis

The survival time for individual patients was defined as the date from diagnosis of ESBL-positive UTIs to the date of death from any cause. Surviving patients were censored at the end of the study on July 15, 2019 after survival probability was determined using Cox-proportional hazards model adjusted for various cofounders, and the resultant cumulative survival probability curve was plotted. All statistical tests were 2-sided and considered statistically significant at P <.05. The analysis was performed using SPSS Version 26 (IBM Corporation, Chicago, USA) and Graphpad Prism 7 (GraphPad Software Inc., San Diego, USA).

Gene Set Enrichment Analysis

To determine neutrophil-*E. coli* interaction, Gene Set Enrichment Analysis (GSEA) was carried out on the following data set from the NCBI's GEO database; GSE126757,[14] as previously described on NF-B inflammatory pathway using the Kolmogorov–Smirnoff test.[15] The data used were the RNA-seq generated from healthy human neutrophils and *E. coli*-stimulated human neutrophils. The related Figure is included in the supplemental material.

Results

Demographic and Clinical Characteristics of the Patients

A total of 668 patients with a confirmed diagnosis of UTI caused by either ESBL-*E. coli*, or ESBL-*K. pneumoniae* were screened during the study period between January 2017 and July 2019. Of these, 202 patients did not have pretreatment urine samples or complete laboratory test results and were therefore excluded from the primary analysis (Figure 1). The mean (SD) age of study participants was 68 (20). Almost half of the 466 recruited patients had T2DM (n=264, 57%). The mean (SD) of HbA1c in T2DM was 7.4 (3). One third of T2DM had chronic kidney disease (CKD) (n=85, 32%).

Patients were categorized into two groups: patients with ESBL-*E. coli* UTIs and those with ESBL-*K. pneumoniae* UTIs. Overall, the two groups were balanced relative to baseline characteristics and were similar with respect to serum albumin levels (Table 1). ESBL-*E. coli* uropathogens had lower level of resistance to β-lactam/β-lactamase inhibitors (sensitivity of 65% vs 52% to amoxicillin-clavulanate and 92% vs 83% to piperacillin-tazobactam for ESBL-*E. coli* UTIs and ESBL-*K. pneumoniae* UTIs, respectively). They were also more resistance to ciprofloxacin (sensitivity of 27% vs 40% for ESBL-*E. coli* UTIs and ESBL-*K. pneumoniae* UTIs, respectively).

Serum CRP Levels and Blood NLR Differentiate ESBL-*E. coli* from *K. pneumoniae* UTIs in T2DM Patients

Elevated serum CRP is a marker for inflammation, and its level increases during bacterial infection including UTI.[7] However, this concept applies for all pathogens, including *E. coli*, which accounts for up to two-thirds of all UTI cases; and is the most frequent isolate in T2DM UTIs.[5] There is no report differentiating the inflammatory profile of UTIs caused by ESBL-*E. coli* versus *K. pneumoniae*, especially in T2DM.
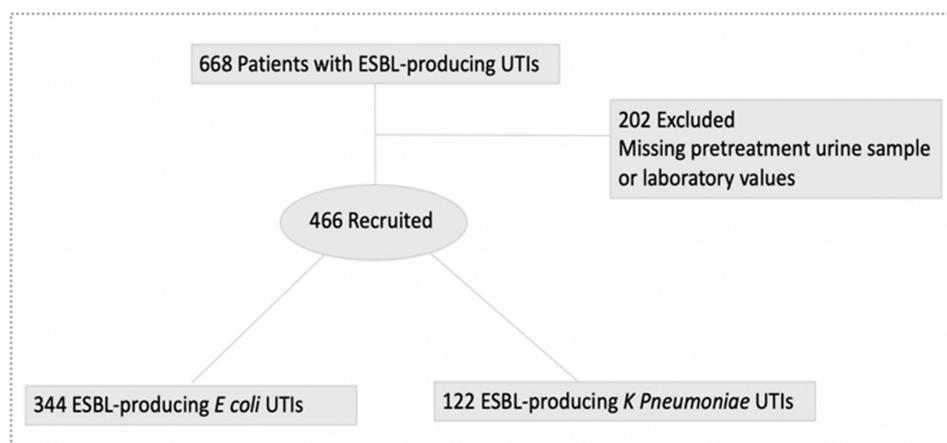

Figure I Patient recruitment and flow through study.

Characteristic	E. coli (n=344)	K. pneumoniae (n=122)	P-value*
Age, mean (SD), y	67 (21)	74 (18)	0.001
Female	210 (61)	72 (59)	0.668
T2DM	179 (63)	85 (77)	0.421
CKD	64 (19)	53 (43)	0.205
Laboratory data			
HbA1C, mean (SD), %	7.25 (3)	7.29 (2)	0.959
Albumin, mean (SD), g/dL	28.54 (5)	28.28 (5)	0.706
Hemoglobin, mean (SD), g/dL	10.94 (1)	10.85 (1)	0.544
Leukocyte, mean (SD)	10.09 (4)	9.76 (4)	0.512
Neutrophils, mean (SD)	7.30 (5)	7.00 (6)	0.741
Lymphocytes, mean (SD)	1.98 (3)	2.82 (6)	0.051
Procalcitonin, mean (SD)	0.16 (0.1)	0.15 (0.1)	0.712
Platelet count, mean (SD)	269 (108)	274 (141)	0.696
Antibiotic sensitivity			
β-lactam/β-lactamase inhibitor			
Amoxicillin-clavulanate	224 (65)	64 (52)	0.015
Piperacillin-tazobactam	315 (92)	101 (83)	0.026
Carbapenem			
Meropenem	336 (98)	119 (97)	0.640
Ertapenem	323 (94)	108 (88)	0.196
Other			
Ciprofloxacin	93 (27)	49 (40)	0.000
Nitrofurantoin	324 (94)	57 (47)	0.000
Gentamycin	218 (63)	95 (78)	0.000
Trimethoprim/Sulfamethoxazole	146 (42)	34 (28)	0.005

Notes: *Statistical significance: $P \leq 0.05$.

Therefore, the difference in serum CRP and Blood NLR between these two ESBL-uropathogens was evaluated in UTI patients (Figure 2). T2DM patients with ESBL-*E. coli* UTIs had lower serum CRP levels (median, CRP mg/dL 33.7 vs 39.8, respectively; P=0.023) and higher blood NLR (median, NLR 3.2 vs 2.6, respectively; P=0.010) compared to patients with ESBL-*K. pneumoniae* UTIs. However, the reverse pattern was observed for non-T2DM patients as they had higher blood NLR levels with ESBL-*K. pneumoniae* UTIs, but not with ESBL-*E. coli* UTIs, although not to a significant level (median, NLR 2.8 vs 4.1, respectively; P=0.538).

The risk of serum CRP ≥ 35 mg/dL, and blood NLR ≥ 3.5 was then evaluated for ESBL-*E. coli* and *K. pneumoniae* uropathogens in T2DM, and non-T2DM patients. As demonstrated in Figure 3, after adjusting for confounders, including age, gender, serum albumin, hemoglobulin,

leukocytes, and platelet counts, T2DM patients with blood NLR ≥ 3.5 were at higher risk for ESBL-*E. coli* UTIs [OR, 3.61 (95% Cl, 1.49–8.73; P=0.004)] than for ESBL-*K. pneumoniae* UTIs. The model showed a good predictive ability for NLR ≥ 3.5 (n=466, sensitivity (%) of 88, specificity (%) of 73, positive predictive value (%) of 93, and negative predictive value (%) of 24).

Moreover, although not to a significant level, non-T2DM patients with NLR ≥ 3.5 were at higher risk for ESBL-*K. pneumoniae* UTIs, but not for ESBL-*E. coli* UTIs [OR, 6.11 (95% Cl, 0.94–39.67; P=0.058)]

Elevated Urine IL-8 Levels as a Marker of ESBL-*E. coli* UTIs

Urinary IL-8 levels were determined for the two groups of T2DM UTI patients using ELISA assay. T2DM patients with ESBL-*E. coli* UTIs had significantly higher urinary IL-8 levels compared to those with ESBL-*K. pneumoniae* UTIs (median, IL-8 pg/mL 2120 vs 668.5, respectively; P<0.0001) (Figure 4).

Survival Analysis

The risk of all-cause mortality was evaluated for UTI with ESBL-*E. coli* and *K. pneumoniae* uropathogens and for T2DM, and non-T2DM subgroups. After adjusting for age, gender, and serum albumin, cox proportional hazards regression analysis revealed that T2DM with ESBL-*E. coli* had higher risk of all-cause mortality than those with ESBL-*K. pneumoniae* (Hazard ratio [HR], 4.09; 95%, 1.14–14.59). The risk was non-significant for ESBL-*E. coli* and *K. pneumoniae* uropathogens in non-T2DM (Figure 5).

Discussion

This study showed that circulatory inflammatory markers such as serum CRP and blood NLR could differentiate between the type of uropathogen causing UTI in T2DM patients. Elevated blood NLR was shown to correlate with ESBL-*E. coli* UTIs, but not with ESBL-*K. pneumoniae* UTIs during T2DM. The reverse trend was observed in non-T2DM, as elevated blood NLR was associated with ESBL-*K. pneumoniae* UTIs. Subsequently, T2DM patients with ESBL-*E. coli* UTIs had elevated urinarily IL-8 levels, and higher risk of all-cause mortality compared to those with ESBL-*K. pneumoniae* UTIs.

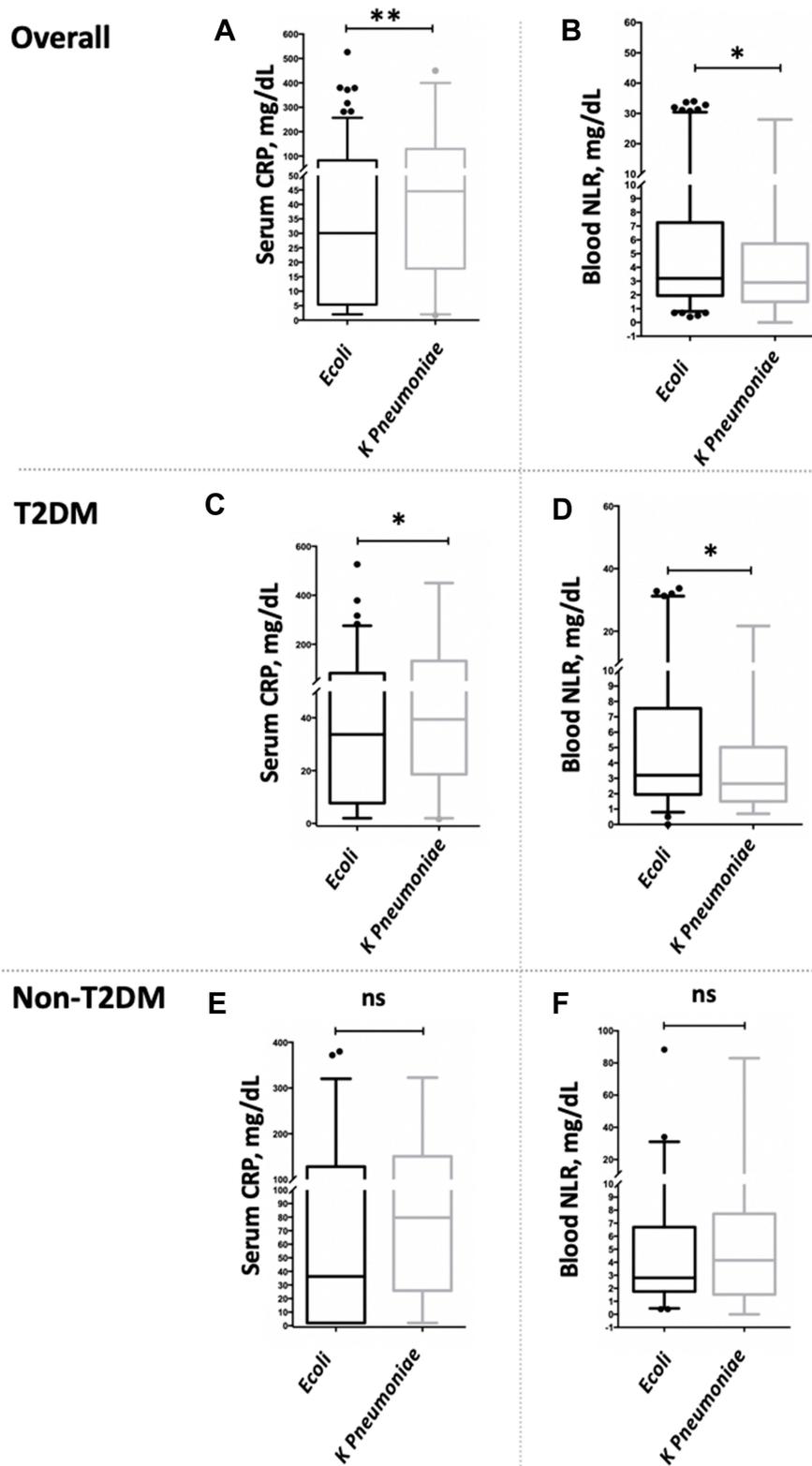ESBL-producing *E. coli* is the causative agent for the majority of UTIs, while only one in four UTIs is caused by

Figure 2 Comparison of circulatory inflammatory markers, including CRPs and NLR between two ESBL-positive UTI groups in accordance with T2DM status. (**A, B**) Serum CRP and blood NLR levels between *E. coli* and *K. pneumonia* in all ESBL-UTI cases. Representative data showing that patients with ESBL-*E. coli* UTIs had lower serum CRP levels and higher blood NLR compared to ESBL-*K. pneumoniae* UTIs. (**C, D**) Serum CRP and blood NLR between *E. coli* and *K. pneumonia* in T2DM ESBL-UTI cases. Representative data showing that T2DM patients with ESBL-*E. coli* UTIs had lower serum CRP levels and higher blood NLR compared to ESBL-*K. pneumoniae* UTIs. (**E, F**) Serum CRP and blood NLR between *E. coli* and *K. pneumonia* in non-T2DM ESBL-UTI cases. Representative data showing that non-T2DM patients with ESBL-*E. coli* UTIs had both lower serum CRP levels and blood NLR compared to ESBL-*K. pneumoniae* UTIs. The values of serum CRP and blood NLR levels were reported in median and IQR. Two-way comparison was done using Mann–Whitney test. * $P<0.05$, ** $P<0.001$, ns= non-significant.
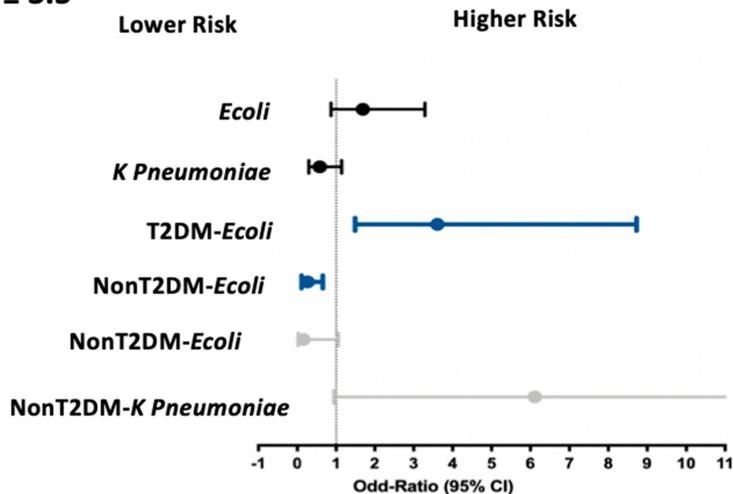
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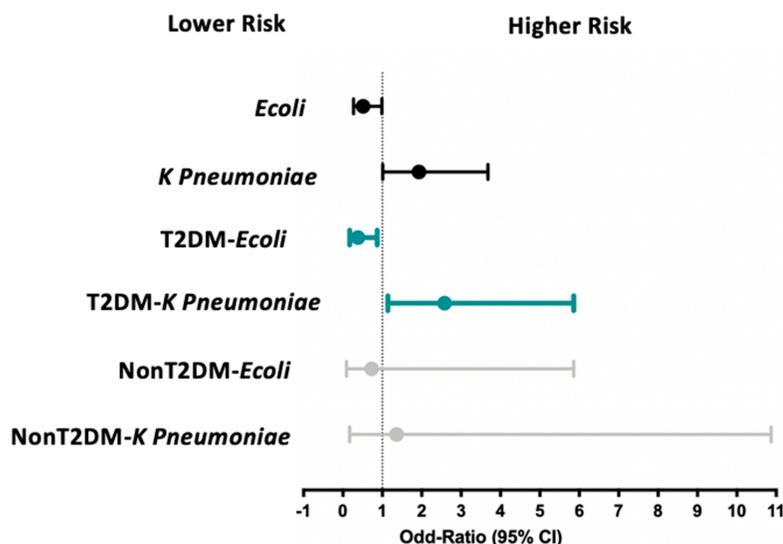
B Blood NLR ≥ 3.5

Figure 3 Association of circulatory inflammatory markers, including CRPs and NLR, in the two ESBL-positive UTI groups in accordance with T2DM status. (**A**) Risk of CRP ≥ 35 mg/dL in all ESBL-UTI cases, T2DM, and non-T2DM. Representative data showing that T2DM patients with serum CRP ≥ 35 mg/dL were at higher risk for ESBL-*E. coli* UTIs than for ESBL-*K. pneumoniae* UTIs. (**B**) Risk of NLP ≥ 35 mg/dL in all ESBL-UTI cases, T2DM, and non-T2DM. Representative data showing that T2DM patients with blood NLR ≥ 3.5 were at higher risk for ESBL-*E. coli* UTIs than for ESBL-*K. pneumoniae* UTIs. Adjusted for age, gender, serum albumin, hemoglobulin, leukocytes, and platelet counts.

ESBL-producing *K. pneumoniae*.[1] It is, hence, crucial that we understand its role in the subversion of host innate immunity. We found that uropathogenic *E. coli* was associated with lower serum inflammatory markers measured as serum CRP level compared to *K. pneumoniae*. This difference in the level of CRP between the two uropathogens is indicative of the difference in innate-immune responses initiated against these uropathogens. Recently, it has been reported that uropathogenic *E. coli* subverts the host innate-

immune system by causing zinc mediated toxicity to persist and disseminate within the host.[16] An inverse relationship between serum CRP levels and zinc was acutely reported in UTI patients.[17] Therefore, our finding that CRP levels are downregulated during *E. coli* UTI compared to *K. pneumoniae* is in line with the reported pathogenesis mechanisms of *E. coli*. Clinically, *K. pneumoniae*, which cause the highest acute inflammatory response measured as elevated CRP may rarely cause renal scars.[18,19]
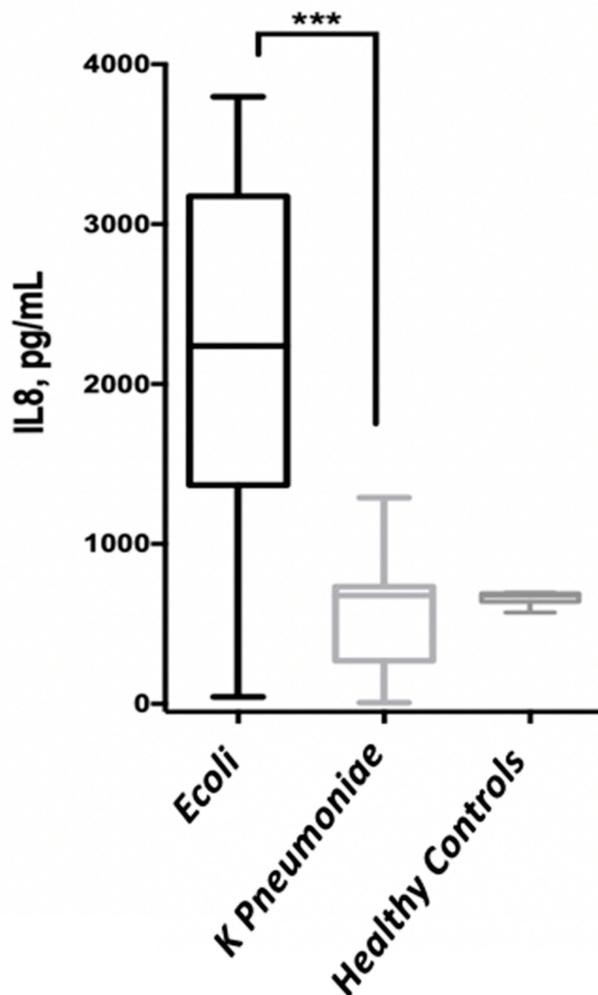
Figure 4 Comparison of urinary IL-8 level between the two ESBL-positive UTI groups in T2DM. *** P<0.0001.

T2DM patients with ESBL-*E. coli* UTIs were associated with higher serum NLR and elevated urinary IL-8 level compared to those with ESBL- positive *K. pneumoniae* UTIs. During infection with uropathogenic *E. coli*, mature neutrophils circulate through the blood and enter infected uroepithelium where they impose their neutrophil-mediated bactericidal activity, or phagocytosis,[20,21] and induce the release of IL-8 for surrounding tissue cells. IL-8 was also shown to be released by mature cells themselves upon phagocyting bacteria.[22]

Furthermore, gene-set enrichment analysis (GSEA) of human neutrophils incubated with *E. coli* indicated that this uropathogen induced an upregulation of IL-8 signaling within neutrophils (Figure S1). This suggested that neutrophils could be an important source of its own chemoattractant. In T2DM, the increase in blood NLR and urinary IL-8 is of concern as high IL-8 can increase the progression of renal disease.[23,24]

Renal damage and scarring are reported to be due to cytotoxic products of neutrophils.[25] A positive correlation between NLR and diabetic peripheral neuropathy has been recently suggested.[26] Therefore, correlating NLR to urinary IL-8 levels could suggest that these markers could be used as an independent risk factor for diabetic nephropathy. Furthermore, IL-8-induced by *uropathogenic E. coli* could exacerbate urinary tract tissue inflammation of T2DM patients; resulting in tissue damage and lower overall survival.

On the contrary blood, NLR were not elevated in non-T2DM during *E. coli* UTI (uropathogenic *E. coli* caused a reduction in blood NLR in non-T2M). Clinically, there is a need for a diagnostic biomarker for UTI, especially in T2DM. Other diagnostic biomarkers of bacterial UTIs such as serum procalcitonin[27] and CRP[28] are either non-specific for uropathogens, or cannot differentiate between them. The reverse pattern of blood NLR in non-T2DM patients supports the predictive ability of this blood marker for *E. coli* UTI in T2DM.

Uropathogenic *E. coli* and *K. pneumoniae* can develop antibiotic resistance that may lead to end-organ damage, especially in immunocompromised patients such as in T2DM.[29] The fact that uropathogenic *E. coli* and *K. pneumoniae* subvert the innate-immune system via different mechanisms suggest that the pathological damage they induce could also be different. ESBL-*E. coli* UTIs was associated with higher all-cause mortality specifically in T2DM patients. The observed blood NLR as well as IL-8 levels could contribute, besides bacterial persistence, to enhanced nephrotic inflammations, and the observed higher mortality in T2DM patients. This suggest that using immune suppressive agents, besides antibiotics, may improve the clinical outcome of these diabetics, and reduce their mortality rate.

An intrinsic limitation of this study is not a randomized clinical trial. By using a multivariate logistic regression model, we were able to adjust for the observed confounders and assess the inflammatory signatures indicative of the two uropathogens. However, there might be a number of unobservable factors that could only be controlled with a randomized controlled trial. Another limitation of this study is the small sample size, which is due to the single-institutional nature of the study. Clinical validation needs to be carried out on a larger cohort. Finally, in this study, we were unable to investigate the molecular mechanisms regulating the observed difference in inflammatory profiles between the two groups, or their molecular contribution to the overall pathogenesis and clinical outcome of the two UTI pathogenic groups. This is mostly because we did not have access to blood samples from these patients. Future studies addressing
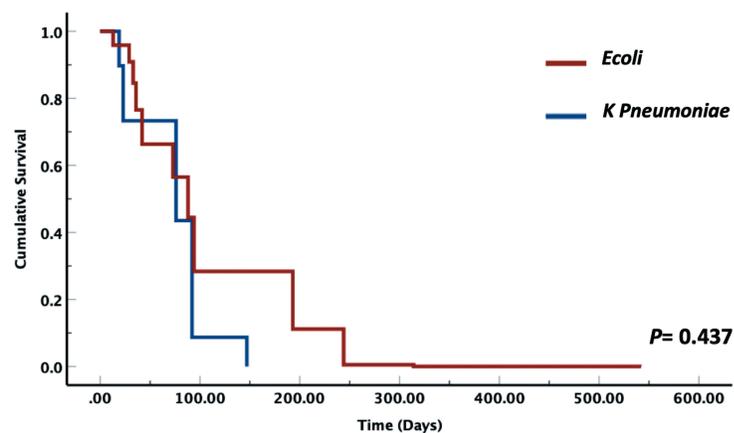
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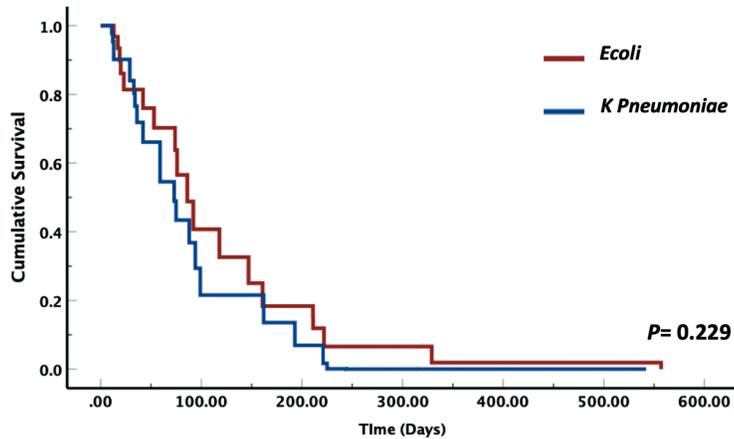
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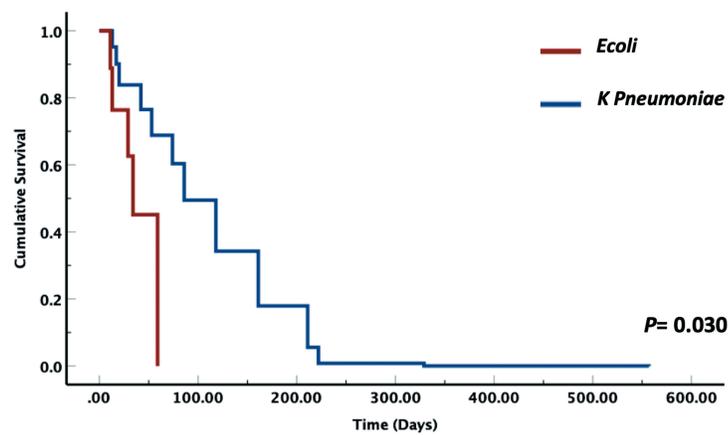
C non-T2DM

Figure 5 Hazard ratios and cumulative survival curve in the two ESBL-positive UTI groups in accordance with T2DM status. (**A**) Risk estimate for all-cause mortality between *E. coli* and *K. pneumonia* in all ESBL-UTI cases. Representative data showing that the risk was non-significant for ESBL-*E. coli* and *K. pneumoniae* uropathogens in all ESBL-UTI cases. (**B**) Risk estimate for all-cause mortality between *E. coli* and *K. pneumonia* in T2DM ESBL-UTI cases. Representative data showing that T2DM with ESBL-*E. coli* had higher risk of all-cause mortality than those with ESBL-*K. pneumoniae*. (**C**) Risk estimate for all-cause mortality between *E. coli* and *K. pneumonia* in non-T2DM ESBL-UTI cases. Representative data showing that the risk was non-significant for ESBL-*E. coli* and *K. pneumoniae* uropathogens in non-T2DM.

these mechanisms may highlight important therapeutic targets that could lead to better control of UTIs, especially in immunocompromised T2DM patients.

In conclusion, we have shown that in T2DM patients, a combination of serum CRP, blood NLR, and urinary IL-8 levels could serve as a biomarker for ESBL-*E. coli* UTI. Moreover, combining antibiotic treatment with immunosuppressive drugs might improve the clinical outcomes as well as the survival of T2DM patients with ESBL-*E. coli* UTIs.

Funding

Disclosure

Rabih Halwani and Rifat Hamoudi are co-senior authors. All authors declare that they have no competing interests.

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