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Immuno-Expression of some Cytokine Associated with some Bacterial Infections among Patients with Bladder Carcinoma

A Thesis

Submitted to the Council of the College of Medicine, University of Babylon, as a Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in science / Medical Microbiology.

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بِسِم اللّٰه الَّرْحَمِن الَّرِحيم

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صدق الله العلي العظيم
سورة الّاسراء: الآّية ٨٥

Supervisor Certification

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Dedication

To..

Those who are unable to describe them....

The one who lights up my path....

my mother and my father

My best friend, who was the source of my strength, the affectionate heart that filled me with love…

my husband Ameer

My lovely sons, source to my hope in this march….

Ryhana and Habeeb

My support and help in life my brothers…..

Ali, Zaid

My second family is the source of my smaile

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Finally, thanks for everyone who taught me a letter or an advised me thanks to all who helped me, even with a word or encouragement.

One ,who did not thank the creature did not thank the Creator.

Zeana

Summary:

The current study was conducted to identify the bacterial infection associated with bladder cancer (BC) as well as to assess the immune status by immune - expression of some parameters as a marker (TLR-2, TLR-4, Th17 and CD14) and comparing its levels in patients with bladder cancer and cystitis. The study included 100 specimens of (70 blood , urine and 30 tissue biopsy) from patients diagnosed with bladder cancer with infection and cystitis in equal number (50 case) , collecting under super version of specialized professional in private clinics in the period from March 2021 to March 2022.

Bladder cancer were determined in patients with both sexes, males were higher than females,(34) 68% and (16) 32%, respectively. Occurrence of BC in all age groups, especially at (56-71) year were most affected.

Bacteriological study occur on (70) specimen of patients urine. The positive results were obtained from (30 case) bladder cancer patients with infection for bacterial culture in a rate of (7) 23.40% , while (23)76.60 % of the specimens were showed no growth, compared to cystitis (40 case), the highest percentage for bacterial culture was (28)70%. The results revealed that, two different isolates associated with the disease during the study: Uropathogenic *Escherichia coli* (UPEC) and *Staphylococcus saprophyticus*. The percentage of *S. saprophyticus* was highest among bladder cancer patients (4)11.8% , while the percentage of *E.coli* was higher among cystitis patients (14) 35.10%.The present findings revealed the presence of both pure and both cultures . The percentage of pure culture for *E.coli* and *S. saprophyticus* species from (35) case positive culture were found to be (15) 42.50% , (6) 16.90% respectively, while the both culture recorded (14) 40.90%. Regarding bacterial culture , it was classified into four groups (no growth, pure

UPEC , *S. saprophyticus* pure cultures and both culture), which were adopted for comparison with the levels of immune markers (TLR-2, TLR-4 and Th17) .

Immunological assessment of urine and blood of patients with bladder cancer with infection revealed a significant effect in increasing the concentration of the immune receptor TLR-2 and there were no significant differences with both TLR-4 and Th17 compared to patients with cystitis that gave opposite results for significant differences and for different age groups. Estimated levels of TLR-2, TLR-4, and Th17 were found, along with a correlation between those levels with the patients' bacterial culture findings.

On the other hand TLR-4 levels, which indicated substantial differences for both Gram-positive bacteria and both cultures in patients with cystitis, the finding revealed that TLR-2 concentrations were highly significant difference in patients with bladder cancer and Gram-positive bacteria compared to cystitis. The Th17 level was significantly increased with Gram-negative bacteria cultures and both cultures in the cystitis group.

Immunohistochemical expression was determined by estimating the CD14 for bladder biopsy (30 case) from patients with both types of bladder cancer and cystitis (Low grade L.G / High grade H.G) (10 case for each group). The level of CD14 expression was higher in L.G group (7)70% , while (2) 20% for H.G group. Expression intensity was investigated by the finding of cell number percentage which take the stain, according to that, it was subdivided into: weak (less than 25%), medium (25-50%) and strong (more than 50%). In addition to determining the type of correlation of CD14 expression between L.G, H.G and cystitis, it was found that there was a significant inverse correlation between L.G and Cystitis.

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List of Abbreviations

Abbreviation	Full term
AMPs	Antimicrobial peptides
ANOVA	Analysis of variance
APS	Allophycocyanin stain
BC	Bladder cancer
BECs	Bladder epithelial cells
CD14	Cluster differentiation 14
CHROM	Chrom agar
CT	Computed Tomography
D.M	Uncontrolled diabetes mellitus
DAMPs	Damage-associated molecular patterns
DCs	Denteric cells
EMB	Eosin Methylin Blue Media
GPI	Glycosyl-phosphatidylinositol
Gr+ve	Gram-positive bacteria
GUE	General urine examination
HG	High -grade
HRP	Horse radish peroxidase
HSP	Heat shock protein
IBCs	Intracellular Bacterial Colonies

IHC	Immunohistochemistry
LG	Low -grade
LPS	Lipopolysaccharide
LPs	Lipoproteins/ lipopeptides
M.S.A	Manitol salt agar
MD2	Myeloid differentiation 2
MDSCs	Myeloid derived suppressor cells
MECs	Mammary epithelial cells
N.A	Nutrient agar
NBF	Neutral buffered formalin
NF-$_k$B	Nuclear factor- kappa B
NK	Natural killer
PAI	Pathogenicity Islands
PAMP	pathogenassociated molecular patterns
PBS	Phosphate buffer saline
PCD	programmed cell death
PE	Phycoerythrin
PGN	Peptidoglycan
Pre CP	Peridinin chlorophyll protein
PRRs	pattern recognition receptors
SCC	Squamous cell carcinoma
SPSS	Statistical package of social science
T regs	Regulatory T cells
TCC	Transitional cell carcinoma
Th	T-helper cells
Th-17	T- helper 17 cell
TILs	Tumor infiltrating lymphocytes
TLR-2	Toll- like receptor 2

TLR-4	Toll- like receptor 4
TLRs	Toll- like receptors
TME	Tumor microenvironment
TNM	Tumor, Node, Metastasis system
TPI	Tumor-promotion infection
TUR	Transurethral resection
TURBT	Transurethral resection of bladder tumor
UBC	Urinary bladder cancer
UICC	Union International Cancer Control
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary tract infection
VH	Variant histology
WHO	World Health Organization
WLC	White light cystoscopy

Chapter One

Introduction

and Literatures Review

1.1 Introduction:

Bladder cancer is the most common pathological conditions among tumors of the urinary system that require a combination of several techniques and immunological tests to reach the type of tumor, and despite the development of pioneering techniques in the field of medicine, the exact diagnosis of the case has not been made without relying on taking a biopsy from bladder tissue (Powles *et al*., 2019). Following the diagnosis of a tumor anywhere in the urothelial tract, the patient is at risk of acquiring a new lesion in the same or a different site, at a comparable or more advanced stage . Much research in the biomedical sciences has been focused on the biochemical process by which normal cells become malignant cancer cells (Thomas *et al*., 2020).

Bladder cancer is linked to an increase in inflammation and is characterized by a disruption in prostanoid metabolism (Bajic *et al*., 2019). Microbial infection has been linked to the development of a variety of malignancies. There is a connection between bacterial infection and tumors . Bladder cancer, is seldom diagnosed in people under the age of 40 year. Medical comorbidities are a common issue in patient care, given the median age upon diagnosis of 73 years (Siegel *et al*., 2019).

Gram-negative bacilli were shown to be the most frequently as the causal agents of urinary tract infection (UTI), with a higher incidence in bladder cancer patients than Gram positive bacteria. Most UTIs are ascending in nature ,with bacteria entering the bladder through the urethra from the faecal reservoir, especially in patients who use intermittent or indwelling catheters. Gram-negative bacteria, especially *Escherichia coli*, play an important role in the development of bladder cancer, as it is believed to have the ability to activate a nuclear factor-kappa B (NF-κB) pathway as part of bacterial pathophysiology. NF-κB, which is the main

transcription factor associated with chronic infections **(**Schwitalla *et al*., 2013 **)**. Diagnosing the bacterial species present in cystitis and identifying their characteristics and virulence factors contribute to giving a better picture of the understanding of the inflammation process and how to deal with it. *E.coli* and Staphylococci are the most common type of bacteria (Karla *et al*., 2020).

Cancer cell invasion and metastasis, as well as essential physiologic and pathological processes such as lymphocyte homing, inflammation, hematopoiesis, and recurrent urinary tract infections, have all been linked to immune system failure. Toll-Like receptors (TLRs) and cluster differentiation 14 (CD14) are two immunological molecules implicated in carcinogenesis that have been utilized as markers for tumor aggressiveness in a variety of cancers (Gonzalez *et al*., 2018).

The major immunological tool for analyzing numerous parameters on complicated cell populations is flow cytometry were used in the current investigation to better understand the role of the immune system in the bladder tumor and progressing to cancer stage. The tumor tissue is also relied upon in the Immunohistochemistry technique to determine CD14 expression within the neoplastic environment, indicating its role in the reduction or progression of the bladder tumor (Kim , *et al.*, 2016 ; Boin *et al*., 2017).

Aim of study:

The aim of the study is to investigate the relationship of urine bacterial infection and some immunological parameters as marker in patients with bladder cancer. This is achieved by the following objectives:

1. Collect (100) specimens from patients , included (70 of urine , blood) for bacteriological and immunological study, (30 of bladder biopsy) for immunohistochemistry.

2. Conducting a survey and informational questionnaire on tumor history and their relationship to age and gender.
3. Isolation and identification of bacterial isolated types in urine specimens from patients with bladder tumors and investigated by culturing urine samples.
4. Detection of some immune parameter (TLR4,TLR2 and Th-17) in patient's urine and blood by flow cytometry and ELISA technique.
5. Detection of the (CD14) expression in transitional epithelial cell by immuno-histochemistry (IHC) technique.

1.2 Physiology of bladder cancer:

The urinary bladder is a hollow organ located in the lower abdomen that stores urine from the kidneys (through the ureter) until urination. Urothelial cells, which line the urinary bladder and urinary tract, are specialized transitional epithelial cells that accommodate the volume. Smooth muscle lines the bladder, which may relax to accept larger quantities and contract to discharge urine through the urethra and out of the body under voluntary or reflex control (Andersson and Arner, 2004; Zhou *et al*., 2021)

Tumor is an irregular growth of cells (neoplasia) in any organ of the body. It may not spread and does not threaten life. It is often a benign tumor, with the possibility of spreading to other parts of the body through the blood or lymph, causing new tumors far from the original, whose cancerous cases are difficult to identify, The causes of cancer are still a subject of controversy, despite the possibility of diagnosing cancer for more than 200 year (WHO., 2018(a)). In (2017) Ribatti, described a tumor from an immunological point of view as the failure of the immune systems ability to recognize and eliminate mutated and irregular cells by suppressing their growth. In another meaning, the tumor environment is made up of a diverse mix of immune suppressive cells (Sato *et al*., 2009). Although contentious when it was initially stated by Paul Erlich in the early 1900s, there is presently a substantial quantity of experimental and observational data to support his hypothesis. Implying that the immune system has an active part in the elimination of cells that have been altered (Dunn *et al*., 2019). More lately, there has been an increasing understanding of some of a specific immune cells pro-tumorigenic action, the study of populations has resulted in a more comprehensive cancer theory (Magdalene and Deborah , 2019).

There are many studies that indicate the possibility of the emergence of infections associated with tumors ,Tumor-promotion infection TPI which in turn creates stimulation of the immune defense line represented by macrophage (Qian and Pollard , 2010 ; Ming *et al*., 2015).There is a lot of evidence that indicates the diversity of cells within the tumor through the different shapes of the tumor and its phenotypic characteristics that contribute to the progression of the cancerous case for each type, despite the difficulty of diagnosing cancer in some cases (Ishimoto *et al*., 2011).

1.2.1 Epidemiology of bladder cancer:

Epidemiological studies indicated that there is a wide variation in the prevalence rates of bladder tumors among tumors of the urinary system, according to geographical variation, especially between African and European countries. Bladder cancer is the ninth in terms of prevalence among malignant tumors in the world and the fifth in developing countries, alone accounting for 5% of all diagnosed cancers. In 2020, (573,278) new cases of bladder cancer were diagnosed around the world, causing (200,000) deaths, Bladder cancer is estimated to be tenth most prevalent cancer in worldwide according to GLOBOCAN statistics . (Antoni *et al*.,2017 ; Santoni *et al*., 2018; Ferlay, 2020 ; Sergei, 2020).

Some studies have confirmed that there are noticeable differences between people based on their ethnic affiliations, as the prevalence of bladder cancer in Middle East and Africa is higher than in developed countries, Turkey and Egypt are known for having a high incidence and mortality rate of bladder cancer. From 1999 to 2012, bladder cancer was the second most frequent urologic malignancy in Korea (Dy *et al*., 2017; Woldu *et al*., 2017; Joung *et al* ., 2017).

1.2.2 Prevalence of bladder cancer:

Bladder cancer is more prevalent in white people than in black people, and in males than in women, with a poorer prognosis in black people and women, owing to a greater tumor stage at presentation (Mallin *et al*., 2011). The number of bladder cancer cases is estimated at 550,000 diagnosed cases of the population for 2018, according to GLOBOCAN statistics, which constitutes 3% of all diagnosed cancer cases, compared to a significant increase in central Europe and North America, while the number of bladder cancer increases among women in Lebanon and South Europe, in contrast to its relatively low level in Africa (Ferlay *et al*., 2018).

In Iraq, the sixth most common malignancy is bladder cancer, and males are three times as likely as women to develop it (Iraqi Cancer Board 2018), with males accounting for 80% of cases and females accounting for 20%. Males had an average age of (66year) while females had an average age of (67.8 year) . According to statistics from the last two decades of the century, there was an increase in the incidence of bladder tumors and tumor removal in males whose ages ranged between 55 and 80 year, but the highest rates were recorded in the elderly with an average age of 73 year, according to several studies **(** Siegel *et al*., 2019; Mushtaq and Thurairaja , 2019 ; Rafid *et al*,. 2020) .

Bladder cancer occurs in both sexes and is not limited to males only, but the risk of males is three to four times higher than that of females. For women, bladder cancer-related mortality has reduced, while for males, it has remained same. Bladder cancer claimed the lives of 16,400 people in 2016 (Bray *et al*., 2018).

Ferlay, *et al* (2012) explained in his study, bladder cancer is the seventh most often diagnosed cancer in men worldwide, but it ranks eleventh when both genders are taken into account .The global age-standardised incidence rate (per 100,000 person / years) for men is 9.0 and for women it is 2.2 (Ferlay *et al*., 2018). Finally,the diverse approaches utilized and the quality of data collection are partly to blame for the discrepancies in bladder cancer incidence and fatality rates. In some registries, the incidence and mortality of BC have decreased, probably because of the reduced impact of causative factors (Chavan *et al*., 2014).

1.2.3 Type of bladder cancer:

Transitional cell carcinoma (TCC) is the most common type about (97%) , followed by squamous cell carcinoma (SCC) about (2%) and the lowest frequency is adenocarcinoma as (1%). Its divided into invasive, extending to the deep layers of the bladder, and non-invasive, extending to the superficial layers only (Ralston *et al*., 2018 ; Babjuk *et al*.,2022). Depending on morphology, bladder tumor can be divided into papillary, solid and mixed type (Moch, 2016). More than 70% of patients diagnosed have the type associated with bladder well-differentiated superficial papillary tumors (Hla *et al*., 2021). In pathological terms, papillary development is frequently related with a low stage (invasive depth) and grade . When compared to solid invasive cancers, evaluation (Sjodahl, 2013).

Solid tumors represent a complex mass of cells that interfere with each other to stimulate a wide range of immunological markers that play a role in the diagnosis of microenvironment (Eruslanov *et al*., 2012 ; Witjes *et al*., 2021).

1.2.4 Tumor stage and grade:

There are many methods used to classify tumors, some of them are clinical stage, and some depend on the results of the pathologic stage.

1.2.4.1 Staging of bladder cancer:

Staging in any case, the internationally approved classification is Tumour, Node, Metastasis (**TNM**) system: (Ooms *et al*., 1983 ; Kurpad *et al*., 2011) .

- **T:** size and extent of the tumor in the bladder wall, T1 tumor in the superficial layer lining the bladder (transitional cell layer), T2 tumor in the muscle layer, T3 tumor in the fat layer that surrounds the muscle, T4 tumor has reached organs and tissues outside the bladder.
- **N**: The case of adjacent lymph nodes, N0, the tumor has not invaded any lymph nodes, N1 the tumor has spread to one lymph node in the true pelvis, N2 refers to the tumor spreading to two or more lymph nodes in the true pelvis, the tumor has spread to lymph nodes around the common iliac artery.
- **M**: The tumor has spread to other organs, M0 has not moved to another than the bladder, M1 the tumor has moved to other organs or distant lymph nodes, and the most common transitions of bladder tumors are to the bones, liver and lung.

1.2.4.1.1 Staging groups:

According to the previous data, bladder tumors are classified into four stages: (Sobin and Wittekind, 2009 ; Moch, *et al*., 2016) .

- **Stage.I** : T1, N0, M0

- **Stage.II**: T2, N0, M0
- **Stage.III**: T3, N0, M0
- **Stage.IV**: T4, N0, M0 or any T, N1-2-3 or any T, any N, M1.

The Union International Cancer Control (UICC) authorized TNM classification in 2009, which was modified in 2016 (8th Edn.) with no changes in reference to bladder tumors. (**Table 1-1**) (Sobin *et al*., 2009).

Table1-1: Stages of urothelial carcinoma pathology

Tumor1 stage	**Description**
T x	Primary tumor can not be assessed
T 0	N o evidence of primary tumor
T a	Noninvasive papillary carcinoma
T is	Carcinoma in situe: "flat tumor "
T 1	Invades subepithelial connective tissue
T 2	Invades muscularis propria
T2a	Invades superficial muscularis propria (inner half)
T2b	Invades deep muscularis propria (outer half)
T3	Invades perivesical tissue
T3a	Microscopically
T3b	Macroscopically (extravesica mass)
T4	Invades surrounding tissue/ organ
T4a	Invades prostate , uterus , vagina
T4b	Invades pelvic wall , abdominal wall

(Sobin *et al*., 2009).

1.2.4.2 Grading of bladder cancer:

In 2004, the World Health Organization and the International Society of Urological Pathology announced a new histological classification of urothelial carcinomas, which differs from the older WHO classification of 1973 in terms of patient stratification between individual categories

(Sauter *et al*., 2004). Recently an update of the 2004 WHO grading classification was published (Moch *et al*., 2016) . However, because most published data relies on these two classifications, the following rules are still based on the 1973 and 2004 WHO classifications. (**Tables 1-2**) (Mostofi *et al*., 1973 ; Sauter *et al*., 2004 ; Soukup *et al*.,2017).

Table 1-2: WHO grading in 1973 and1 in 2004

1973 WHO grading
Grade.1: well differentiated Grade.2: moderately differentiated Grade.3: poorly differentiated
2004 WHO grading system (papillary lesions)
Papillary urothelial neoplasm of low malignant potential (PUNLMP) Low -grade (LG) papillary urothelial carcinoma High -grade (HG) papillary urothelia1 carcinoma

1.2.5 Etiology and risk factors of bladder cancer:

Most bladder cancer is caused by unknown factors, according to researchers. However, they have discovered certain risk factors and are beginning to understand how they drive bladder cells to become cancerous. Many biological and environmental factors interfere with the development of the cancerous condition, In addition to the role of several live style and nutritional factors (Marilyn, 2019). Bladder cancer represents the next step in the case of cystitis, and the causes of inflammation are many, including bacteria, parasites, and sometimes viruses. In all cases, the tumor usually occurs after infection of the urinary system and the failure of the immune system to identify it (Rafid *et al*., 2020). Depth of invasion into the bladder wall and the degree of differentiation of the tumor are the two most important prognostic markers

in bladder cancer. However, there is no reliable parameter predicting the risk of recurrence or progression (Burak *et al*.,2009 ; Cui *et al*., 2019).

Previous studies confirmed that advancing age is one of the factors related to the progression of the cancerous condition, as approximately 90% of bladder cancer cases occur at the age of more than 55 years and 80% at the age of 65 years, and the average age at which this disease was diagnosed is 73 year. Bladder cancer is also rare in children and adolescents, and if it is found, it is a low-grade non-invasive tumor (Siegel *et al*., 2019; Kalyan *et al*., 2020).

Personal history increases the risk of bladder cancer and is mainly associated with early onset, however, there is less information on genetically inherited bladder cancer, Although the results of studies have mentioned about risk factors and their relationship to bladder cancer, it does not mean that all people who have these factors are affected (Colombel *et al*., 2018 ; Saginala *et al*., 2020).

Urothelial carcinomas can develop in a variety of places in the bladder, including the lining of the ureters, and the urethra. If patient have cancer in the lining of urinary system, sporting chance likely to get another type of cancer, either at the same location or in a different region of urinary tract. This holds true even if the initial tumor is entirely eliminated. For this reason, people who have been diagnosed with bladder cancer require careful to check for future malignancies (Cumberbatch, 2018).

Bladder cancer development influenced by three general factors: Abnormalities of the genetic and molecular nature (specific oncogenes and tumor suppressor of bladder cancer is genes) Chronic irritation caused by chemical or environmental exposures (most notably cigarette smoke)

(such as pelvic irradiation or indwelling catheters) (Kaufman, 2006 ; Cumberbatch *et al*., 2018 ; Rebecca *et al*., 2019).

While studies have failed to find large germ line genetic factors driving sporadic bladder cancer, genome wide association studies have shown several genetic sites that have a moderate relationship with increased bladder cancer risk (Gu and Wu, 2011; Palmer *et al*., 2018). The majority of gene changes linked to bladder cancer occur during a person's lifetime rather than being inherited at birth. Changes in the DNA of normal bladder cells can lead them to develop abnormally and become tumors. However, these mutations are caused by a variety of factors (Mucci *et al*., 2016). Drinking fluid may be contaminated with bladder carcinogens such as chlorination byproducts or arsenic, which may have an influence on the development of bladder cancer. A high consumption of these carcinogen-contaminated liquids may increase the risk of bladder cancer (Morales *et a l*., 2000 ; Letasiova *et al*., 2012 ; Etemadi *et al*., 2020)).

Environmental factors are combined as an etiology of bladder cancer, and the second greatest preventable risk factor. Workplace exposure to certain chemical substances, pelvic radiation, usage of certain medicines (cyclophosphamide), persistent urinary tract infection (UTI), and cigarette smoking are all environmental risk factors (Zeegers *et al*., 2001(a,b) ; Iraqi Cancer Board, 2018). Association between bladder cancer and environmental carcinogens is first discovered in 1895 (Behnam , 2021). In a recent comprehensive investigation , gene-environment interactions have been proposed as the key etiological risk factors in bladder cancer (Charostad *et al*., 2019).

The majority of research found a strong link between the tobacco smoking factor and a variety of hazardous behaviors that people engage in

throughout their lifestyle and their effective relationship with the development of bladder cancer. According to (Silverman *et al*., 2006 ; Wojtczyk and Schlichtholz, 2019), approximately half of people with bladder cancer are smokers. The risk of smoking doubles when there are previous cases of cancer in the family history as a result of chemotherapy and radiation, as the risk of developing bladder cancer increases by at least three times (Mikhaleva *et al*., 2021). According to a study by (Van Osch *et al*, 2016) . Smokers have a higher risk of developing bladder cancer dependent on their daily tobacco consumption, since there is a clear link between the amount of cigarettes taken per day and the high prevalence of infection during the previous 50 years (Abdolahinia *et al*.,2021).

Alcohol is one of the common substances in affecting the transformation of tumors into malignant tumors, as it is one of the carcinogenic factors, but there is no significant relationship with the development of bladder cancer (Masaoka *et al*., 2017 ; Huang *et al*., 2021). Recently, physical risk factors have been known within the urinary system when exposed to continuous abrasion resulting from kidney and bladder stones and long-term urinary catheters, and other causes of chronic (ongoing) bladder irritation have been linked to bladder cancer (especially squamous cell carcinoma of the bladder). However, it's unclear if they truly cause bladder cancer (Letašiová *et al*., 2012). As well as infection with schistosomiasis , bacteria that produce carcinogens such as N-nitroso compounds have been shown to infect the bladder when worms infect it, schistosomiasis disease appears to progress much more quickly than that caused by tobacco or chemical exposure, , which are common in the Middle East and Egypt , and it can be avoided by controlling parasites with the anti-helminthic drug praziquantel (Barnett , 2018; Nacif-Pimenta *et al*., 2019). Microorganisms have an impact on human physiological

activities such as metabolism, immunity, and hematopoiesis (Guoqin, 2019). It is also the most expensive cancer per patient from diagnosis to death due to its extraordinarily high recurrence rate (Barocas *et al*., 2012).

1.2.6 Clinical finding of bladder cancer :

Asymptomatic hematuria is the most frequent presenting sign of bladder cancer, occurring in roughly 85% of patients with asymptomatic microscopic hematuria, while Gross hematuria has been correlated with a malignant tumor at 20%. The second most frequent symptom combination of bladder cancer is bladder irritation along with urine frequency, urgency, and dysuria (Minton *et al*., 1964 ; Davis *et al*., 2012 ; Sell *et al*., 2019).

Other signs and symptoms of bladder cancer include ureteral obstruction-related flank discomfort, lower extremities edema, and a palpable pelvic mass. Patients with advanced illness signs, such as weight loss and stomach or bone discomfort from distant metastases, appear seldom. These symptoms, on the other hand, usually never arise in the absence of microscopic or macroscopic hematuria. (Toll and Ali, 2013; Liu *et al*., 2019).

1.2.7 Bladder tumor diagnosis:

Urinary cytology can be used to diagnose and monitor bladder tumors in addition to standard screening and evaluation of hematuria. The preferred imaging modality during the work-up for bladder tumor is computed tomography (CT) or magnetic resonance imaging, which is verified by direct viewing of the tumor and associated mucosal abnormalities with endoscopic excision utilizing cystoscopy and

transurethral resection of bladder tumor (TURBT) (**Figure1-1**) (Sharma *et al*., 2009 ; Chang *et al*., 2016).

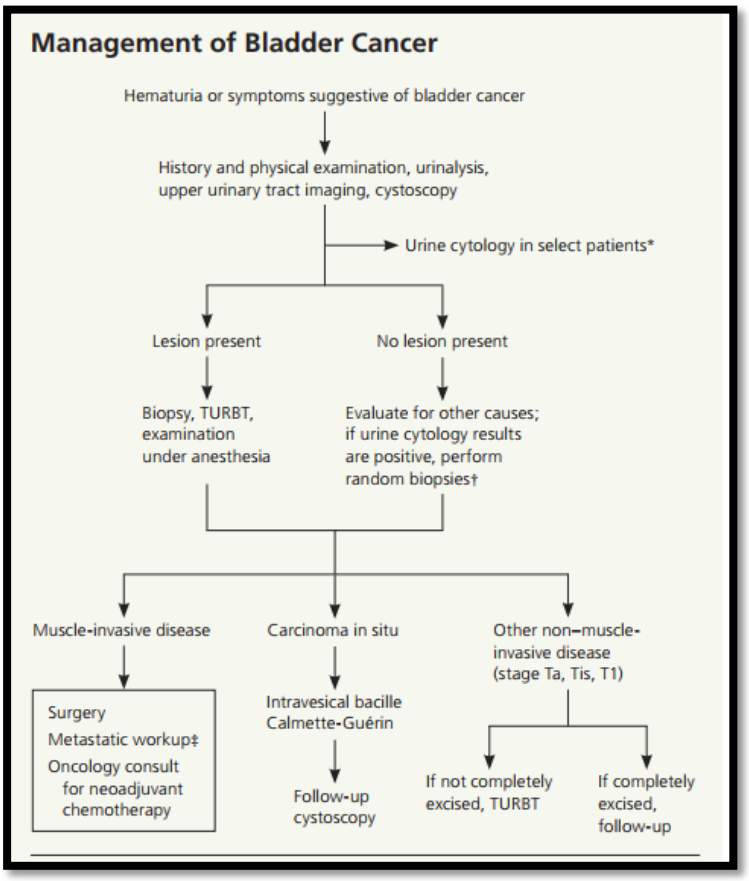


Figure 1-1 : Algorithm for the management of bladder cancer . (CT = computed tomography ; TURBT = transurethral resection of bladder tumor (Sharma *et al*., 2009).

White light cystoscopy (WLC) is the gold standard to take a biopsy for diagnosing and monitoring bladder cancer, with approximately 2 million cystoscopies conducted each year in the United States and Europe (Svatek *et al*., 2014 ; McKibben *et al*., 2016). When WLC is difficult to diagnose, doctors may utilize blue light cystoscopy to diagnose some cases (Mateu *et al*., 2019). Identifying the cancer cells themselves is the most effective method of detecting a bladder neoplasm. Currently, only well-established

method of achieving this aim is to visit a pathologist who will analyze tissue specimens or urine samples (Mark *et al*., 2005).

Finally, Patients with aberrant cytology results of the bladder wash or tissue pathology should have the bladder tumor transurethrally resected (TURBT). This technique offers crucial histopathologic data for conclusive diagnosis, staging, and grading, as well as the removal of visible tumors and sample of adjacent muscle to determine depth to avoid missing (Saini *et al*., 2019).

The process of diagnosing bladder cancer appears to be a complex matter, as each tumor is characterized by a specific way of expressing through markers and signals that may be easily identified within the affected organ or difficult to interfere with other injuries. While this tumor-specific mark adds levels of complexity to our knowledge of cancer biology, it also opens the door to developing targeted medicines for early detection, diagnosis, and treatments (Le and Laakkonen, 2018).

All patients with bladder cancer should have their blood urea nitrogen and keratinize levels checked to see if they have renal impairment. A complete blood count and comprehensive metabolic analysis, including alkaline phosphatase level and evaluation of liver function, are recommended if metastatic illness is suspected (Davis *et al.*, 2012). Ami *et al* was the first to succeed in giving an accurate description of micro papillary bladder cancer in 1982 during an article he published in which he outlined the pathological symptoms of the condition (Ploeg *et al*., 2009 ; Pierre *et al*., 2009). Despite the development of medicine and the multiplicity of modern medical technologies in the field of diagnosis, it is not possible to rely on a single parameter for diagnosing a tumor and determining its type, so it is necessary to rely on a set of analyzes such as

renal function testing and upper urinary tract imaging, particularly with computed tomography (CT) urography in addition to cross-sectional images of tissue (Clark *et al*., 2013).

On the other hand, many studies focused on the diagnosis of bladder tumors based on immune markers, including those free circulating in blood and body fluids or associated with the surface of tumor cells alike. These markers, according to Bardelli and Pantel (2017), can be tumor cells or tumor related products that can be detected in the blood and other body fluids by liquid biopsy, such as free molecular and immunological markers. It is known that increased production of some markers by tumor cells causes local immunosuppression, which in turn promotes malignant transformation and tumor progression, facilitating early detection of bladder cancer (Sinha *et al*., 2007 ; Stolina *et al*., 2000 ; Liao *et al*., 2019) .

Advanced tumors can suppress local immune responses and create a tolerant milieu by secreting inhibitory substances and activating negative regulatory pathways (Evgeniy *et al* ., 2011).Toll-like receptors (TLRs) are one of the immunological indicators that have been studied, TLR 2, TLR 4, TLR -7, and TLR- 9 are receptors produced by peripheral immune cells and the urinary bladder epithelium, and their function in triggering the anti-bladder tumor immune response has been described (LaRue *et al*., 2013; Rasha *et al* ., 2019). The most frequent use of immunostaining for the identification of solid tumors is immunohistochemistry (IHC). CD14 tumor infiltrating lymphocytes can be detected immunohistochemically and utilized as a possible predictor factor in the diagnosis of urothelial cell carcinoma of the bladder tissue, It relies on both color and morphology to differentiate between benign and malignant cells in cytology specimens (Ming *et al*., 2015).

1.2.8 Prevention of bladder cancer recurrence:

Avoiding smoking and carcinogenic chemicals is the most important factor in the prevention of malignant bladder tumors, Although the extent to which a fruit and vegetable-rich diet might help prevent bladder cancer . Smoking cessation has been proven to lower the risk of bladder cancer by 40% in as little as 1–4 years, and to revert to baseline risk in as little as 20 years (Freedman *et al*., 2011 ; Al-Zalabani *et al*., 2016) .

Physical exercise has been linked to a reduced risk of bladder cancer. That is linked to physiological processes that can directly affect cancer development, which is one possible explanation. Immune function is improved, chronic inflammation is decreased, carcinogen detoxification is raised, DNA repair is improved, and cell proliferation, differentiation, and apoptosis are altered (Friedenreich *et al*,. 2010).

1.2.9 Anatomy of bladder cancer:

Microscopic anatomy of the bladder separated into three primary sections: dome, mid-portion and the base.

On its outside surface, the dome is bordered with peritoneum and is placed superiorly. The apex, or tip, of the dome is placed anterior-superiorly. The bladder apex is connected to the median umbilical ligament, which is a vestige of the embryonic urachus. The urachus is a tube that joins the bladder and the umbilicus throughout fetal development. The bladder's base is posteriorly and inferiorly positioned. The trigone is a section of the base that is located within the base. The bladder's midportion, which is between the dome and the apex, takes up the majority of the bladder's space. The anterior and posterior walls, as well as the lateral (left and right) walls, make up the midsection (Zhou *et al*., 2021) .

1.2.10 Histopathology of bladder cancer:

Tumors having a different histology are identified at a lower rate. Squamous cell carcinomas of the bladder are the most common non-urothelial tumors seen in the bladder (Tania *et al*., 2020). Squamous differentiation is defined by the presence of intercellular bridges or keratinization and is seen in up to 40% of urothelial carcinomas of the bladder (Magers *et al*,. 2019). More than 95% of bladder cancers are epithelial in nature. The most prevalent histology of bladder cancer is pure urothelial carcinoma, which accounts for around 75% of all cases. Variant histology (VH) is used to represent the remaining instances, which are classified into urothelial and non-urothelial categories (Gordetsky and Epstein, 2015 ; Renshaw and Gould, 2017).

The histological damage of the bladder tumor is determined by sending biopsy samples for fixation and histological examination. (Lopez-Beltran *et al*,. 2017 ; Paner *et al*., 2019).

1.3 Bacterial infection and bladder cancer:

1.3.1 Cystitis:

One of the most prevalent urinary tract illnesses is cystitis. The word cystitis was developed to describe the infection's location, which termed to bladder infection, it is usually caused by bacteria from intestinal flora (Ki *et al.*, 2017 ; Huether, 2019) . It may be divided into two categories: simple and complex. Uncomplicated cystitis is a lower urinary tract infection (UTI) in men or non-pregnant women who are otherwise healthy. Complicated cystitis, on the other hand, is linked to variables that raise the likelihood of infection or failure to respond to antibiotic treatment (Karamali *et al*., 2019). The most prevalent cause of acute cystitis is a

bacterial infection of the urinary bladder. In both males and females, *Escherichia coli (E. coli)* is the most prevalent bacterial infection, accounting for 80 %- 90% percent of cases with uncomplicated cystitis, However, species like as *Enterobacter*, *Citrobacter, Serratia*, *Pseudomonas*, enterococci, staphylococci, and even fungus are among the microbial pathogens that can cause a complex cystitis. Many additional organisms, such as lactobacilli, Group B streptococci, coagulase negative staphylococci, and enterococcus, are typically regarded pollutants unless there are extremely large quantities of a single organism in which an infection is feasible (Hooton *et al*., 2013 ; Conner *et al*., 2019). Patients with cystitis suffering from dysuria that have pyruia, bacteriuria and positive urine cultures .The signs and symptoms of cystitis in men is similar to those in women. Cystitis is a condition caused by bacteria from the feces or vaginal flora invading the periurethral mucosa and ascending to the urine bladder. Uropathogens may have virulence properties that allow them to get past the host's defenses and into the tissues of the urinary system (Tyagi *et al*., 2018). When cystitis escapes the immune system's control and does not react to antibiotics, inflammation recurs with the same clinical criteria and signs. It may lead to genetic and physiological changes that participate in the formation of a bladder tumor that can later develop into cancer (Bonkat *et al*., 2020; McKertich , 2021).

1.3.1.1 Uropathogenic *E.coli* :

Escherichia coli coexist in the gastrointestinal tract without causing disease except in the special cases such as changing the natural environment in which lives , when moving to the urinary tract causes inflammation (Martínez-Delgado *et al*., 2020). About 80–90% of community-acquired UTIs are caused by Uropathogenic *Escherichia coli* (UPEC). It is responsible for around 90% of urinary tract infections, and it

may readily spread from the anal region to the urinary system and bladder. It is 14 times more frequent in females than in males due to females' shorter urethra (Yuan *et al*., 2021).

Based on the presence of genomic Pathogenicity Islands (PAI) and the expression of virulence factors such adhesions, lipopolysaccharide (LPS), toxins, surface polysaccharides, flagella, and iron-acquisition systems, four major *E.coli* phylogroups (A, B1, B2, and D) have been discovered (Bien *et al*., 2012).Usually, many of these virulence factors are often necessary for *E.coli* to colonize the bladder and cause UTI (Byron , 2019). To successfully colonize the bladder, *E.coli* must be capable of overcome the innate and immune response and evading these mucosal barriers to facilitate bladder colonization or they will not be able to persist , Type 1 fimbriae enhance bacterial attachment to the uroplakin complex matrix on the surface of superficial bladder epithelial cells (BECs) (Bucevic *et al*., 2018; Andolfi *et al*, 2020). As well as that BECs itself have important role in bacterial colonization by major receptors for *E.coli* adherence factor. The nature of this binding interaction is such that it gets even tighter when subjected to the shear forces of urine known as Intracellular Bacterial Colonies (IBCs) (Habuka *et al*., 2015; Kleina *et al*., 2020). *E.coli* have adapted to an intracellular route by actively inhibiting immunological signaling in urothelial cells, including TLR4 signaling, NF-jB activity, and proinflammatory cytokine secretion, which affects apoptosis and tumor progression (Finucane, 2017 ; Yeh *et al*., 2019).

The immune system recognizes chronic and repeated bacterial infections as carcinogens, and the immune response to them is represented by many mechanisms to neutralize the bladder's internal environment, minimize damage, and trigger a sequence of subsequent immune molecular signals (Picardo *et al*., 2019). Bacteria have sophisticated and

effective mechanisms to escape the immune equation by progressing an inflammatory state into a carcinogenic environment, which is caused by bacterial translocation through bladder tissues, which stimulates multiple local immune cells and increases TLR expression as a regulatory response to MAMPs. As a result of the combination between bacterial virulence factors and an overly aggressive immune response, the inflammatory environment is transformed into an uncontrollable tumor (Bajic *et al*., 2019 ; Huang *et al.*, 2021) .

Urinary tract bacteria (*E.coli*) is more frequent in children, and the risk of infection rises while using urinary catheters, which reach up to the bladder through the urethra, since bacteria are introduced into the urethra. Infection rates vary by geography and health status, with infection rates increasing with poor health and malnutrition (Hadi *et al.*, 2017).

1.3.1.2 *Staphylococcus saprophyticus*:

In the early 1970s, *S.saprophyticus* was discovered to be the second most prevalent cause of cystitis, behind *E. coli*. It has unique urotropic and ecological characteristics that distinguish it from other staphylococci and *Escherichia coli*. Simple bladder infections in young women are caused by Gram-positive and coagulase-negative cocci (Widerström *et al* ., 2012). In males, it can also induce urethritis, epididymitis, prostatitis, and nephrolithiasis of all ages, however this is uncommon in hospitalized men (Dutta *et al*., 2020) . *S. saprophyticus* can have a polysaccharide capsule that increases virulence in an animal model but has no effect on the rate of internalization in human bladder cells (Loes *et al*., 2014) . Implying that the capsule alone isn't necessary for infection, while some strain can create biofilms that are polysaccharide-dependent. Biofilms can obstruct antibiotic activity by limiting the human defense system's access to

Staphylococcus. However, whether biofilms generated by these bacteria are also linked to cystitis or urinary stones has yet to be established (Karla *et al*., 2020). According to an analysis of 169 *S. saprophyticus* strains, 70% of them had the potential to produce biofilm. Furthermore, the development of biofilms enhances resistance to some antibiotics (vancomycin, oxacillin, trimethoprim/sulfamethoxazole, ciprofloxacin, and norfloxacin) (Aguila-Arcos *et al*., 2018 ; Martins *et al*., 2019). *S. saprophyticus* causing UTI in people belonged to two basic clonal lineages (G and S), which developed in food/production animals and humans, respectively, according to a recent research (Lawal *et al*., 2021).

Inflammation represented two face in tumor biology; it has the ability to activate an antitumoural immune response that kills tumors, but it may also promote carcinogenesis if left unchecked. Inflammation is a host response triggered by a variety of causes such as proinflammatory mediators, environmental pollutants, tissue damage, and persistent infection (Hourigan *et al*., 2020).

1.3.2 Bladder cancer relationship of bacterial infection:

The development of tumors and their transformation into malignancies is occasionally under the influence of a group of microorganisms. Bacteria, which are one of the major causes, accounting for more than 20% of human malignant tumors (Garrett, 2015 ; Urbaniak *et al*., 2016).

The tumor microenvironment (TME), which consists of extracellular matrix (ECM) , stromal cells, immune/inflammatory cells, and secreted proteins, has been found to be substantially linked with cancer development and treatment responses (Cai *et al.,* 2020). The tumor environment is characterized by containing a high percentage of pathogenic bacteria as a result of the internal environment suitable for the

growth of microorganisms that are rarely available in normal tissues, tumor act as forgin body (Laliani *et al*., 2020). Therefore, it is important to know the relationship between them in order to reach a strategy for the tumor and the mechanism of its transformation into cancer in the presence of bacteria (De Martel *et al*., 2012; Zhou *et al*., 2018).

Microbial infection has been linked to the development of a variety of cancer (Mousa *et al.,* 2017). As well as, the severity interaction between bacteria and their host in one way or another, affects the formation of tumors, which in turn inhibits the process of responding to anticancer drugs, leading to the progression of tumors (Maria *et al*,. 2017; Al-Hilu and Al-Shujairi , 2020). Inflammation has been linked to higher tumor grades and a tumor progresis, despite the fact that it is a nonmutational driver of tumor growth and progression (Hibino *et al*., 2021). It is noteworthy that bacteria sometimes have a direct toxic effect on bladder cancer through the secretion of bacterial genotoxins, in addition to their metabolic processes release many carcinogenic factors such as dietary nitro amines, acetaldehyde and others that increase the effectiveness of genotoxins (**Figure1-2**) (Huang *et al.*, 2021) .

Finally, can say that bacteria can cause cancer in human cells in three ways: by generating chronic inflammation, acting as an antiapoptotic agent, and manufacturing cancer-causing biological (Tomasz , 2019).

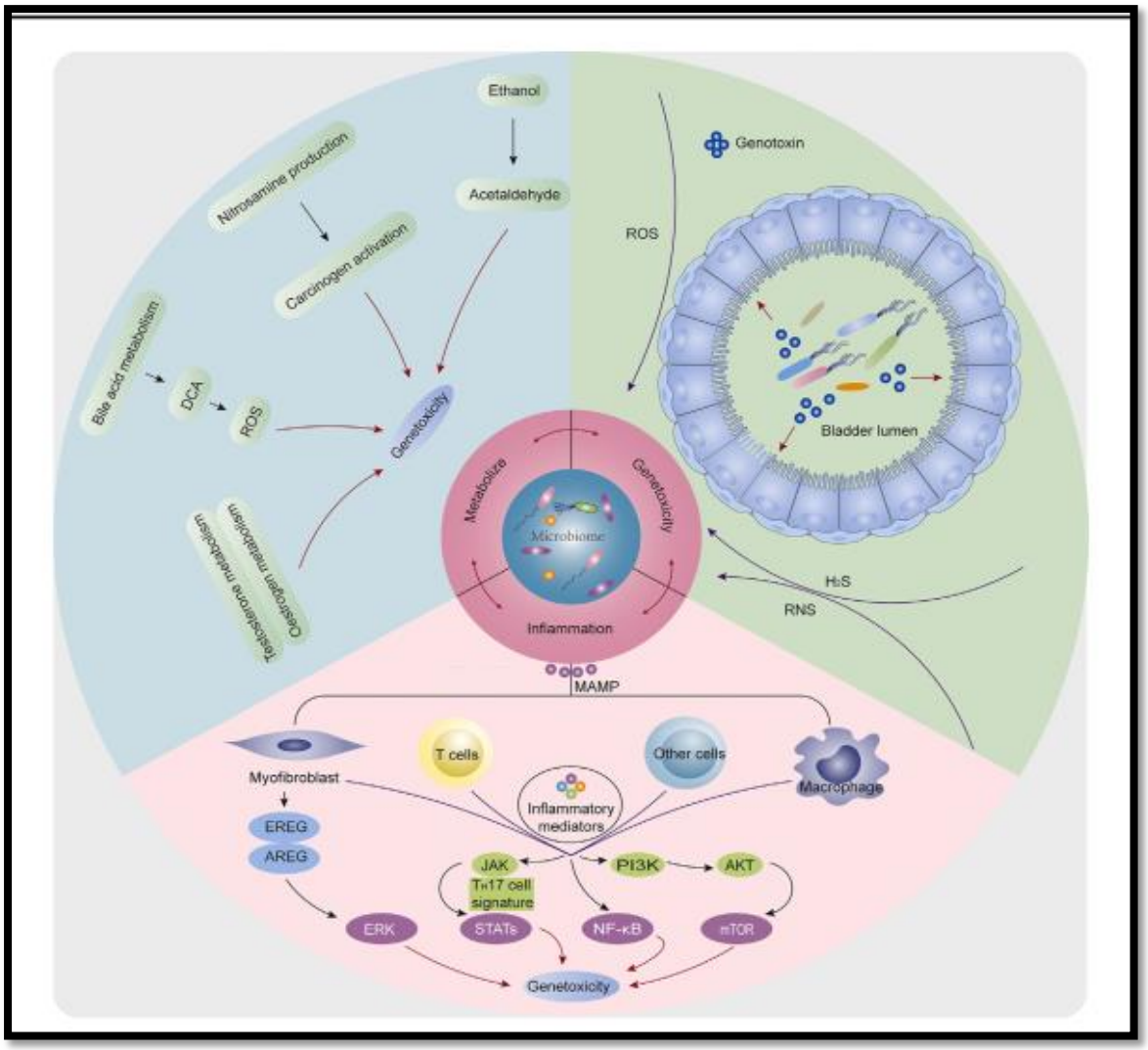


Figure 1-2: Bacterial mechanisms influence bladder cancer development.

1.4 Immune system and bladder tumor:

The immune response accompanying tumors includes both humoral and cellular types in an integrated manner, and therefore it is noted that both innate and adaptive immunity are involved to limit tumor development (Petitprez *et al*., 2020).

Tumors are one of the most common triggers for the immune response because they provide a useful model for the expression of markers and immune receptors, as tumor cells work to initiate a local response that attracts and activates lymphocytes and increases blood vessel

permeability, increasing blood flow to the tumor stroma. (Schnell *et al*., 2018). Immune cells can be harmed by cancer and are frequently shown to be dysregulated in patients (Fridman *et al*., 2017). Tumors can evade immune system through a variety of ways, the capacity of malignant cells to evade the immune system is a key feature that promotes tumor survival, growth, and spread. Therefore identifying particular immune evasion pathways might aid in the treatment of existing malignancies (Alegrezza and Conejo-Garcia, 2017 ; Roumenina *et al*., 2019).

Bladder tumor represents microenvironment rich in growth factors and some immune markers that stimulate the growth and development of tumor cells to reduce the processes of programmed cell death of cells (Shapour *et al*., 2019), so recent studies have directed to study these stimuli and measure their concentrations to determine the type of immune response for early detection of the tumor (Audisio *et al*., 2022). The human bladder already contains certain key immune cell types, such as dendritic cells, and additional can be recruited from the circulation. Neutrophils, regulatory T cells (T regs), and myeloid-derived suppressor cells are among the others (MDSCs), are recruited completely from the bloodstream in response to substances produced by the tumor or nearby immune cells (Mithunah *et al*., 2021).

Cellular immunity is responsible for the majority of the immune response to bladder tumors by predicting the concentration of tumor infiltrating lymphocytes (TILs). Many research have looked at TILs as a possible prognostic factor in various kinds of cancer (Joseph and Enting, 2019 ; Rubio *et al*., 2019). Overall survival has been linked to the degree of inflammation and tumor-infiltrating lymphocytes, indicating that tumors with low levels of inflammation may be a good place to initiate the immune response (Wu *et al*., 2018; Tomoe and Yamashita, 2019).

Inflammation is controlled by feedback systems under normal condition by immunological markers, When these feedback mechanisms are dysregulated, such as in tumor, chronic inflammation ensues and then progresses to cancer (Tao *et al*., 2018 ; Hibino *et al*., 2021) .

Bladder cancer is correlated to the severity of inflammation and appears as a result of abnormal prostanoid metabolism. When the bladder is infected with Gram-negative bacteria, particularly *Escherichia coli*, bladder cancer develops. Uroepithelial cells are activated by lipopolysaccharide (LPS), the main component of bacterial outer membrane. To react to LPS, bladder epithelial cells employ pathogen recognition receptors such as Toll-Like receptor 4 (TLR-4) and CD14 and trigger LPS-induced signaling by binding of LPS with co-receptor CD14 (Zhang *et al*., 2019 ; Lu *et al*., 2021). CD14 is also used by Gram-positive bacteria such as streptococci for immunological recognition. Peptidoglycan (PGN) and lipoteichoic acid have been demonstrated to activate macrophages in a CD14 dependent manner (Cheah *et al*., 2015 ; Rubio *et al*., 2019; Crispen and Kusmartsev, 2020)**.**

1.4.1 Innate immune response to tumor:

1.4.1.1 Toll like receptors (TLRs):

An immune surveillance molecules family, TLRs are set of germline-encoded receptors , which are called pattern recognition receptors (PRRs) as proper function of innate immunity dependent ,that bind pathogen-associated molecular patterns (PAMP) and are an important part of the innate immune system's defense against invaders. TLRs also identify damage-associated molecular patterns (DAMPs), which are proteins or nucleic acids produced during necrosis, in addition to PAMPs (Vigneron, 2015; Mokhtari *et al*,. 2021). TLRs have emerged as key players in the

formation of the tumor microenvironment in cancer, as they mediate both pro- and antitumorigenic pathways as well as apoptosis response (**Figure 1-3**) (Chuang *et al*., 2020; Shi *et al*., 2020).

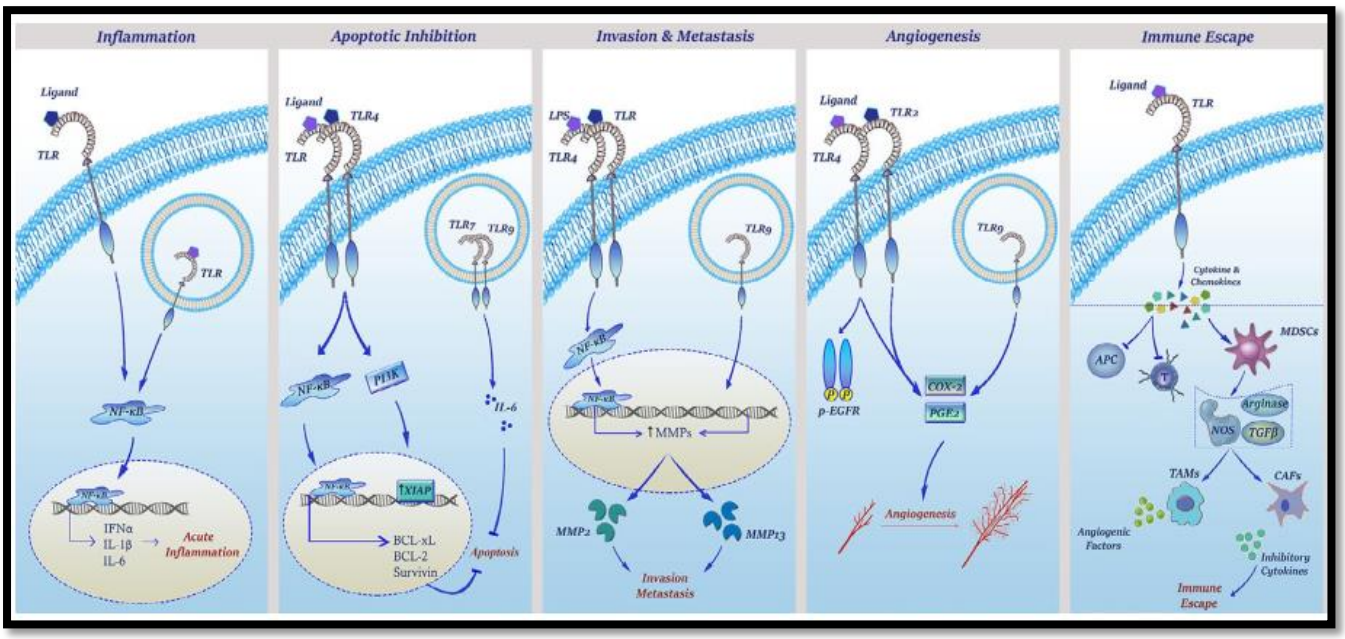


Figure 1-3: TLRs coordinate pro-tumor responses through a variety of methods. TLR activation initiates the NF-B cascade, which enhances inflammatory cytokine transcription such as IFN-, IL-1, and IL-6. The NF-B axis is activated, which increases anti-apoptotic proteins (Bcl-XL, Bcl-2, and survivin) as well as MMPs, which are responsible for extracellular matrix breakdown. TLR4. TLR stimulation in the tumor microenvironment can cause cancer cells or tumour-infiltrating cells to generate suppressor cytokines and chemokines that inhibit immune cells while also attracting additional cells to the tumor microenvironment (Keshavarz *et al*., 2020).

If uropathogens break the physical boundaries of bladder epithelium, they are identified by TLR subfamilies group such as TLR2 (recognizes staphylococci by their lipoteichoic acid or lipoprotein), TLR3 (recognizes double stranded RNA), TLR4 (recognizes lipopolysaccharides (LPS) of *E.coli*), TLR5 (recognizes flagellin), TLR9 (recognizes unmethylated CpG DNA of bacteria and viruses), and TLR11 (recognizes profiling of parasites) (Spencer *et al*., 2014 ; Ohadian Moghadam and Nowroozi , 2019).

TLRs are transmembrane proteins rich with repetitive leucine in their extracellular domain discovered in the 1990s with 13 different species, including 10 human TLRs (TLR1-10) and 12 mouse TLRs (TLR1-9, TLR11and TLR12) (Kawasaki and Kawai, 2014; Babjuk *et al*., 2020). TLRs have been discovered in that have a persistent, extremely powerful role in PAMP recognition. They are released on the surfaces of urothelial cells. TLR activation by uropathogens stimulates multiple signaling pathways that result in interferon signaling, pro-inflammatory cytokines and chemokine expression, and the synthesis of antimicrobial peptides, all of which contribute to a powerful immune response by mediating immune suppression and reducing tumor surveillance. Therefore, activation of TLRs is becoming or has been a target for cancer treatment (Cen *et al* ., 2018 ; Kashani *et al*., 2020). Despite TLRs have important role in DAMP and PAMP recognition, it was recently discovered that Toll-like receptors expressed not only in immune cells, but also in tumor microenvironment (TME) is involved in inhibiting immune defenses, caused dysfunctional immunity within the tumor microenvironment and increasing the induction of tumor immune tolerance via survival signals , antitumor-suppressing molecules and further release aberrant cytokines and chemokines to maintain cellular growth and tumor survival. As a feedback mechanism involved (Petitprez *et al*., 2020 ; Lu *et al*., 2021; Fiorentino *et al*., 2022) .

It is difficult to accurately diagnose bladder cancer before taking a biopsy from tumor tissue, and this is one of the main problems related to the patient in determining the stage of the tumor, so many laboratory tests are followed to determine some blood parameters and inflammation degree with UTI (Armbruster *et al*.; 2018 ; Cui *et al*.; 2019). However, researchers are trying to reach a rapid diagnosis based on the type and

percentage of TLRs on lymphocytes in the blood because of their important role in tumor immunity, especially programmed cell death (PCD) according to TLRs signaling pathway (Ayala-Cuellar *et al.*, 2019 ; Moghadam and Nowroozi , 2019 ; Mokhtar *et al.*, 2020) . Programmed cell death is divided into apoptosis, autophagy, and programmed necrosis, is suggested to constitute cell death in a tumor condition to maintain host homeostasis via unique cellular mechanisms and diverse communication pathways (Ou *et al*., 2017).

Toll-Like Receptors activation in tumor cells may have both tumorigenic (positive regulation of cancer) and antitumor (negative regulation of cancer) effects (Ohadian and Nowroozi, 2019). TLR expression appears to be complicated, as it appears as part of innate antitumor immunity at times and as an oncogenic component at other times, resulting in up-regulation of the NF-B cascade and production of anti-apoptotic proteins that promote cancer cell proliferation and carcinogenesis. That's mean, they play a significant role not only in progression of tumour but also in treatment of cancer (Müller *et al*., 2017 ; Kashani *et al*,. 2020) .

Multiple immunological methods can be used to identify and estimate Toll-like receptors, but given the rapid advancement of technology in the current era, as well as the massive explosion in the invention of electronic devices and the discovery of various methods of laboratory tests, researchers have resorted to using the flow cytometry method to detect TLRs in order to obtain quick results and set targets. Flow cytometry is the most widely used immunological method for evaluating a wide range of characteristics in complex cell populations. Flow cytometry is a technique for analyzing the expression of a large number of variables in a single cell

at a rate of thousands of events per second (Bendall *et al*., 2012; Katherine, 2019).

A- Toll like receptor 2(TLR-2):

Toll-like receptors 2 (TLR2) was discovered molecularly characterized, and cloned for the first time in 1998, together with TLR1, TLR3, TLR4, and TLR5 (Rock *et al*., 1998), were granted a portion of the Nobel Prize in Medicine and Physiology last year (Beutler, 2009). TLR-2 belongs to the extracellular group based on its classification by location, its located on the surface of cells, plays a pivotal role in pathogenesis of different malignancies, among these is urinary bladder cancer (UBC) (Ohadian and Nowroozi, 2019). TLR2 has an important role in vertebrate immunity, according to more than a decade of study. This receptor is the first TLR to be discovered that can create functional heterodimers with more than two other TLRs. TLR2 interacts with several non-TLR molecules, allowing it to identify a wide variety of PAMPs (Zähringer *et al*., 2008).

Gram positive bacterial component (ligand) activates TLR2 to produce inflammatory responses. Only lipoproteins/lipopeptides (LPs) are "true" TLR2 ligands, according to a recent research, since they are detected at physiological quantities by this receptor (Hoppstädter *et al*., 2019). Immune cells, endothelial cells, and epithelial cells have all been found to express TLR2 . This might indicate an early bladder immune response prior to the onset of symptomatic UTIs, supporting TLR2's potential protective function against UTIs (Livia and Molly , 2020). It backs up what Hawn *et al*., said, about TLR signaling is associated with interactions in the tumor microenvironment between cancer cells, immune cells, and PAMPs in (2009).

A variety of methods have been developed to investigate the involvement of TLR2 in immune protection or infection pathogenesis. Ligand specific recognition and signaling through TLR2 occurs via heterodimerization with TLR1 or TLR6. On the cell surface, TLR2/TLR1 and TLR2/TLR6 heterodimers are considered to be pre-formed. TLR2 homodimerization was proposed in the absence of TLR1 and TLR6, however it has not been observed with current techniques (Jin *et al*., 2007).

There is ample evidence pointing to an important immunological role of TLR-2 in controlling infections caused by Gram-positive bacteria in general, but it is possible that another group of receptors may be involved in controlling Gr+ve bacteria when they are not secreted or activated. Thus , to enhance TLR2 responses, a variety of accessory molecules and co-receptors have recently been identified that concentrate microbial products on the cell surface or inside phagosomes (Jimenez - Dalmaroni *et al*., 2009 ; Oliveira - Nascimento *et al*., 2012).

B- Toll like receptor 4(TLR-4):

Toll-like receptors and their downstream components are expressed on bovine mammary epithelial cells (MECs), according to Strandberg *et al*. (2007) .TLR4 has a 608-residue extracellular domain and a 187-residue intracellular region that participates in intracellular signaling cascade (Medzhitov *et al*., 1997). In a variety of solid tumors, studies have linked increased TLR expression and malfunctioning immunity within the tumor microenvironment to cancer development and decreased patient survival (Mantovani *et al*., 2008). TLR2, TLR4, and TLR5 are the most efficient TLRs in preventing UTIs in the urinary system (Song and Abraham, 2007 ; Abraham and Miao, 2015) , allowing them to detect bacterial LPS

anywhere in the bladder milieu (Song *et al*., 2007) , While that TLR4 signaling pathway might cause bladder cancer to be suppressed or develop . TLR-MyD88 signaling and downstream NF-kB activation appear to be important mediating elements in this process (Li *et al*., 2017).

TLR-4 recognizes the lipopolysaccharide (LPS) exogenous molecules from Gram-negative bacteria as a target ligand, spatially type I and Pfimbriae,heat skock protein (HSPs) that are crucial in the elimination of *E.coli* from bladder epithelial cells. This mechanism is elicited by a TLR on macrophages that detects LPS and subsequently triggers a number of molecules in the inflammatory response, including macrophage recruitment and activation, as well as natural killer (NK) and dendritic cell activation (key agents in the presentation of antigen to T cells) In other words, the severity of UTIs affects the degree of TLR4 expression (Hawn *et al*., 2009 ; Zhang *et al*., 2012 ; Judith *et al* ., 2013). It has been shown that TLR4 transfection alone is insufficient for LPS recognition, and that physical interaction of TLR4 with myeloid differentiation 2 (MD2) on the cell surface is necessary for ligand-induced activation (Nagai *et al*., 2002).

In bladder cancer , TLR4 signaling was shown to be intact and capable of promoting the creation of soluble immune mediators that might aid the tumor's immunological defenses (Huang *et al*., 2005). Both innate and adaptive immune responses are controlled by toll-like receptor signaling in immune cells. TLR activation, on the other hand, can result in enhanced regulatory T-cell proliferation and suppressor function, which can encourage tumor growth (Liu *et al*., 2006 ; Kabelitz, 2007) . At the same time,tumor grade plays an important role in the reactivation of TLR2 and TLR4 were significantly lower in low-grade, non-muscle invasive and muscle invasive bladder carcinoma than normal, according to (Stopiglia *et*

al., 2015), and this may contribute to the high tumor relapse and progression rates (Al- biaty *et al.*, 2015). While some study revealed that significantly reduced TLR4 expression in more aggressive high-grade tumors could result from a loss of cell differentiation associated with cancer development (Krieg, 2008).

1.4.2 T-helper 17 cell (Th-17):

T-cells represent a specialized cellular immune response, which is activated by chemical agents known as Lymphokines, and the cellular immune system is regulated by T-helper cells (Th) of all kinds that develop different types of immune response depending on the type of stimulus (Chen and Kolls , 2017). Th17 cells were originally identified in 2005 as a novel subset of T helper lymphocytes , Th cells distinct from Th1 and Th2 (Harrington *et al*., 2005 ; Lin *et al*,. 2019). CD4+ T helper (Th) cells are an important component of adaptive immunity because they are required to control CD8+ T cell and B cell responses as well as to activate innate immune cells at inflammatory sites and in autoimmune disorders (Chugh *et al*., 2013; Guéry and Hugues, 2015) . Several studies have indicated the role of Th-17 in anti-inflammatory immunity of the urinary system and its relationship to tumor immunity, as well as the importance of understanding the relationship between T cells and pathogen-specific antibodies as immune markers, especially in bladder tumors, to achieve early detection of the tumor (Papotto *et al*., 2017 ; Llosa *et al*., 2019). Also the production of related cytokines and a critical transcription factor that aids in the formation of Th17 cells can be employ to determine the involvement of Th17 cells in bladder cancer, indicating a bladder tumor-induced local induction of this sub-group. However , the research in this field is still limited (Chou *et al*., 2015 ; Saleh and Elkord , 2020).

The important role of Th-17 in immunology lies in its plasticity feature and the ability to transdifferentiated into other types of helper T cells, Th1, Th2 or other subset allowing them to elicit qualitatively distinct responses depending on different tumor microenvironments position in urinary system carcinoma (Guéry and Hugues, 2015 ; Shahid and Bharadwa , 2019). Furthermore, as compared to healthy tissues, Th17 cells accumulate particularly in a variety of malignancies (esophageal carcinomas, breast, colon, and melanoma) (Bilska *et al*., 2020 ; Bai *et al*., 2022). In humans ,Th17 cells specific for Staphylococci sp. ,generated IL-17 and IL-10 after being restimulated, indicating that plasticity can allow Th17 cells to drive respons to different infections (Alessandra *et al*., 2019). Th-17, like other TLRs, has an effect on the human body. It works in two ways: either as an anti-tumor mechanism or as a tumor progression. Many connections between Th17 cell infiltration and prognosis in human cancer were adequately described in a few reviews (Chang , 2019 ; Marques *et al*., 2021).

IL-1, IL-6, and IL-23 are important cytokines secreted by cancer cells, tumor-derived fibroblasts, and antigen-presenting cells for Th17 development. Th17 cells may stimulate CD8+ T cells both directly and indirectly in the setting of tumors. Th17 cells were transferred in the tumors, promoting the recruitment of immune cells such as CD4+ T cells, CD8+ T cells, and DCs (Hu *et al*., 2018 ; Simoni *et al*., 2018). Th17 levels in tumors are linked to microenvironmental Th1 cells, cytotoxic CD8 T cells, and natural killer cells. Finally, several studies have discovered a Th-17 cell subset that serves as a regulator by suppressing the activity of other T cells (Wang *et al.*, 2017 ; Dai *et al*., 2018).

1.4.3 Cluster differentiation 14 (CD14) expression:

Various types of molecular markers appear on the surfaces of B-cells in the bladder epithelium that contribute to a non-specific cellular immune response; these markers are cluster of differentiation 14 (CD14) and their importance in assisting Toll-like receptors in identifying Gram-negative and Gram-positive bacteria, both directly or indirectly (Mousa *et al*., 2017 ; Mabrey *et al*., 2021). Bladder epithelial cells use TLR-4 and CD14 for LPS recognition and response to bacterial infection (**Figure 1-4**), as well as macrophages have been shown to be activated by peptididoglycan (PGN) and lipoteichoic acid in a CD14-dependent manner (Ge and Ding, 2020).

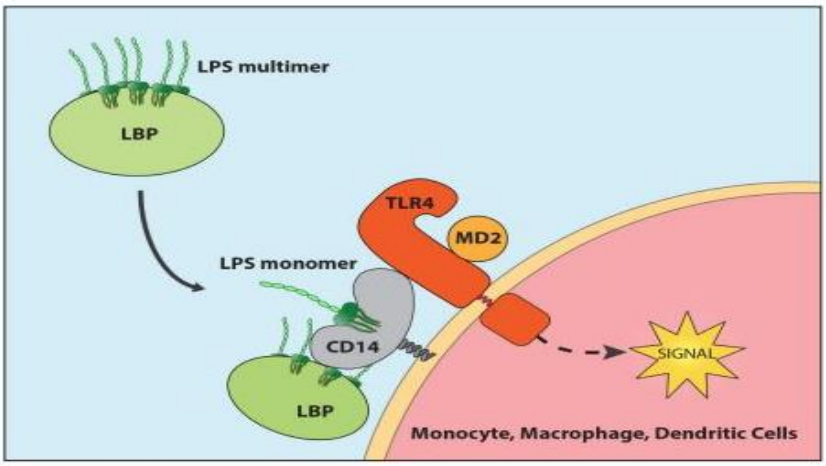


Figure 1.4: TLR-4 and CD14 interaction for LPS recognition in urinary tract infection. (Tan *et al* ., 2015).

To date, only a few studies have investigated CD14 expression in urothelial of the urinary bladder. CD14 an antigen was first characterized as a membrane-associated glycosyl-phosphatidylinositol (GPI)-linked glycoprotein that act as co-receptor and has been demonstrated to play a crucial role in Toll-like receptor signaling pathways (Ichise *et al*., 2020). In tumor microenvironment , to an increased cytokine production and

tumor development need CD14 high expression in bladder cancer according to(Ming *et al*., 2015 ; Doming *et al*., 2021), thus CD14 considered as favors receptor complex internalization. It presents on monocytes, macrophages, dendritic cells, and neutrophils (Zanoni *et al*., 2011 ; Ryu *et al*., 2017 ; Rodney , *et al*.,2019).The recruitment and polarization of monocytes and macrophages to acquire immune-suppressive features is directly driven by inflammatory mediators generated high CD14 by bladder cancer cells (BC). On the other hand , BC cells with low CD14 expression , have a greater relative proliferation, which is significantly enhanced when stimulated by CD14 high cell factors. To monitor the development of bladder tumors, can rely on the CD14 antigen, so need a immunohistochemistry (IHC) technique, IHC laboratory method that uses antibodies to check for certain antigens (CD14 markers) in bladder tissue (Lin and Prichard, 2015 ; Kim , *et al.*, 2016). In normal bladder tissue, the acute inflammatory response is activated upon recognition of exogenous factors which may be microbial or non-microbial in nature, or by endogenous factors resulting from cell or tissue damage (e.g. necrotic tissue) (Vera *et al*., 2018 ; Tamadonfar *et al* ., 2019 ; Crispen and Kusmartsev, 2020).

Chapter Two

Materials and Methods

2. Materials and Methods

2.1 Laboratory materials and equipment :

2.1.1 Apparatuses and Tools:

The Apparatuses and tools used in this study (**Table 2-1**):

Table (2-1): Apparatuses and Tools

No.	**Apparatuses and Tools**	**Origin**
1.	Autoclave	Haramaya / Japan
2.	Automated Tissue Processor	Histo -Line- Italy
3.	Bunsen burner	China
4.	Centrifuge EBA 20	Hettich (Germany)
5.	Cotton swab	ATACO- Brand
6.	Cover slip	AFCO - Chaina
7.	Disposable syringes (5 mL)	Medical jet (Syria)
8.	Distillator	Bibby science (England)
9.	EDTA tube	AFCO (Jordan)
10.	Flask	.....
11.	Flowcytometer (BriCyte E6 / mindray)	China
12.	Gel tube	AFCO (Jordan)
13.	Hood	Labtech / Korea
14.	Incubator	Fisher scientific (Germany)
15.	Loop	LoopRhundon (England)
16.	Light microscope	Olympus- Japan
17.	Microtome blades	ERMA -Japan
18.	Micropipettes	Slamed (Germany)
19.	Multiple micropipettes	Watson Nexty (Japan)
20.	Para Film	BDH (England)
21.	pH- meter	Gallenkump (England)

22.	pH strip	
23.	Pipette tips (0.01 ml ,0.1 ml, 1ml)	China
24.	Plain test tube	Laiwu Yaohua - China
25.	Plastic pitridish	Sterilin (England)
26.	Plastic Test tubes 10ml.	AFCO (Jordan)
27.	Refrigerator	Concord- Ital
28.	Sensitive Balance	Sartorius (Germany)
29.	Swap media	AFCO (Jordan)
30.	Tips (different volumes)	China
31.	Transporter swabs	Girenier (China)
32.	UriSed mini(Urine microscopy Analyzer)	77 Elektronika Kft / Budapest
33.	Vortex (Electronic)	Kunkel /Germany
34.	Water path	Memmert - German
35.	Waxing Pen	Dako - Germany

2.1.2 Chemical Materials:

Chemicals and reagents that used in this study were listed in (**Table 2-2**):

Table (2-2): Chemical and Reagents

Chemicals	**Origin**
Distilled Water	Al Jazeera - Kuwait
Ethanol	Romil pure chemistry - United kingdom
Formalin CH_2O	BDH (England)
Gram's stain	Crescent (Saudi)
Hematoxylin Counter Stain	Bio SB- USA

Chemicals	Origin
Xylene	Romil pure chemistry- United kingdom

2.1.3 Immunological Kits:

The immunological kits used in this study are listed in **(Table 2-3).**

Table (2-3): Immunological Kits

Materials	Origin
Human TLR4 Marker Flowcytometry Kit	Bio SB- USA
Human Th17 Marker Flowcytometry Kit	
Human CD14 Marker IHC Kit	
Human TLR2 Marker ELIZA Kit	BT LAB
Vitek 2 System Kit	Biomerieux /France

2.1.4 Culture Media:

The culture media and purpose of use in this study are listed in (**Table 2-4**).

Table (2-4): Culture Media

Culture media	Purpose of Use	Origin
Chrom agar (CHROM) for UTI	UTI selective agar	Himedia (India)
Eosin Methylin Blue Media (EMB)	Isolation and identification UPEC	

Culture media	Purpose of Use	Origin
Nutrient agar (N.A)	Isolation and identification of members of Gram positive and negative bacteria	
Manitol salt agar (M.S.A)	Isolation and identification staphylococcus sp.	

2.2 Methods:

2.2.1 Solutions :

2.2.1.1 Neutral formalin solution (10%):

It was prepared by adding 10 ml of formaldehyde with a concentration of 40% to 30 ml of sterile distilled water, so that the final concentration of formalin was 10%. It was used to fixation the bladder samples as a general histological fixative for later histological examination.

2.2.1.2 Solutions for Immunohistochemical Staining Technique:

- **Immuno DNA washer solution:** it is a concentrated solution, when it utilized for Immunohistochemistry, should be diluted 1:10 with D.W.
- **20X Immuno DNA Retriever Citrate :** it is a concentrated solution diluted 1:20 with distilled water, it contains citrate buffer solution , detergents , stabilizers and non-sodium azide anti-microbial (Bio SB)
- **The 3, 3′-Diamino benzidine (DAB):** prepared through adding one drop of DAB chromogen after shaking into each 1mL of DAB substrate.

2.2.1.3 Phosphate buffer saline (PBS):

Prepare this solution according to the manufacturer's instructions by dissolving 9.86 gm of plate in 1000 milliliters of distilled water to obtain pH. 7.2

2.2.2 Stains :

2.2.2.1 Hematoxylin as a counter stain:

This dye was prepared by mixing 20 gm of potassium alum with 1 gm of Hematoxylin stain and 0.5 gm of mercuric oxide were dissolved in 200 ml of distilled water, then 10 ml of absolute ethyl alcohol was added to it, was used to stain tissue sections (Luna, 1968).

2.3 Study design :

This is a case coherst study. The study included 100 specimens of (70 blood , urine) and (30 tissue biopsy) from patients diagnosed with bladder cancer with infection and cystitis in equal number (50 case) , collecting under super version of specialized professional in private clinics (**Figure 2-1**).

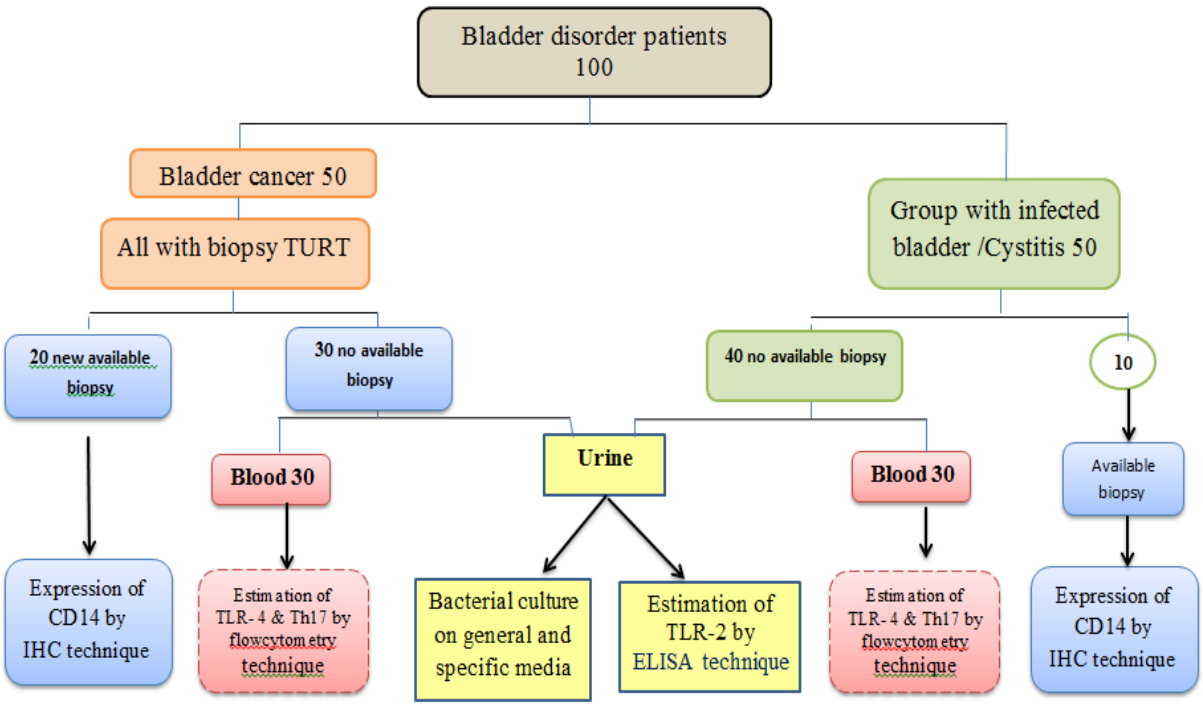


Figure (2.1): Scheme of study design

2.4 Sample collection:

This study was carried out on patients attended to Babylon oncology center and private laboratory in Babylon province, Iraq, during the period from March 2021 to March 2022. All patients were diagnosed and referred by urologist .

One hundred samples were collected from the patients, which varied between blood, urine and bladder biopsy from the patients were visiting the private laboratory , with information related to the patient recorded : name , sex , age and type of treatment . All samples were transferred shortly afterwards to the laboratory of college of Medicine, for bacteriological culture and conducting the rest of the tests.

2.4.1 Inclusion and Exclusion Criteria:

The included criteria of the patients group involved in this study included every patient who had recent histologically diagnosed with bladder tumor, and cystitis.

Excluded criteria , every suspected patient who had no urologic tumors , any patient with tumors other than bladder, and every patient with chronic health problem such as :Uncontrolled diabetes mellitus D.M, Autoimmune disease, Other malignancy with chemo and radio therapy and Pregnant women.

2.4.2 Ethical Approval:

Verbal agreement of all patients had been taken prior to the take any specimen. Moreover, the study design was approved by Research Ethical Committee in College of Medicine / Babylon University.

2.4.3 Blood samples:

Three ml of blood from (60) patients were put in a plastic tube containing an anticoagulant **,** and transported to the laboratory within 24h. for immunological tests by flowcytometry (Lewis *et al*., 2001).

2.4.4 Urine sample:

Five ml of midstream urine in test tube was collected from both groups : patients with bladder cancer (30) case and (40) case from cystitis group, and 3ml placed in a centrifuge at 3000 rpm speed for 5-10 minutes to wet smear of the precipitate was taken by swap media and calibrated loop technique was used for bacteriological culture (Vandepitte *et al*. 2003). While drop was taken for general urine examination (GUE) in UriSed mini (Urine microscopy Analyzer) (Barta *et al* , 2011).

2.4.5 Biopsy Samples:

Tumor tissue biopsies (bladder) have been obtained from the urological surgery unit after patient admission for transurethral resection (TUR) , partial secestomy . The biopsy was saved in plastic tube container with 10% neutral buffered formalin (NBF) before processing through automated tissue processor (Histo-Line. Italy) to create a formalin-fixed on slide, add Canada balsam paraffin-embedded (FFPE) blocks for immunohistochemical tests.

2.5 Bacteriological study:

2.5.1 General urine examination (GUE):

With the development of modern technical devices in laboratory diagnosis , the UriSed mini (Urine microscopy Analyzer) was used in

the parts of the GUE examination , leaving the traditional manual method . UriSed Technology is a unique system in the market for the automation of sediment analysis, making traditional manual microscopy automatic (Barta *et al* , 2011).

The test was carried out according to the instructions of the device by adding (175 µl) of urine sample , dispensed manually into the cuvette, the rest of the measurement procedure is fully automated : rotating the cuvette for a few seconds gently deposits produced elements onto a monolayer at the cuvette's bottom. The built-in digital camera captures 15 separate pictures of the sediment layer at various locations.

2.5.2 Preparation of culture media:

Selective media for identification of bacteria in urine of patients with bladder cancer and cystitis, such as (Nutrient agar, Manitol salt agar , Eosin Methylen Blue, MacConky agar and CHROM agar medium specific for UTI). All used culture media were prepared according to the manufacturers' instructions and sterilized with autoclave at 121°C for 15 minutes, at a pressure of 15 pounds/ ang, then poured into plates or tubes as required (Brown and Smith , 2017).

2.5.3 Urine culture:

To determine the presence of bacterial cells and other compounds, all specimens underwent a general urine examination. The calibrated loop technique was employed for quantitative culture and presumptive identification from each urine samples (Vandepitte *et al*., 2003). Separately, well-mixed urine samples (1µl) were inoculated on MacConkey agar, EMB agar, Manitol salt agar, and CHROMagar orientation plates. These plates were then incubated overnight at 37 °C in

aerobic bacteriological incubators. The identification on selective media , biochemical test, microscopically examination with Gram stain and validated by an automatic VITEK2 (MacFadden, 2000).

2.6 Immunological study:

2.6.1 Estimation of Toll like receptor 2 (TLR-2) in urine samples by ELISA technique :

A- Assay procedure:

A test was performed to measure the level of TLR-2 in the urine of cystitis and bladder cancer patients according to the manufacturer's instructions as follows:

1. All the components of the kit were leaved in the laboratory to reach room temperature and shake gently before use to ensure homogeneity.
2. Several concentrations of the standard solution were prepared by dissolving it in the dilution solution and making a series of dilutions, (0.000, 1.500, 3.000, 6.000, 12.000 and 24.000)ng/ml.
3. Group of reagents and solutions were performed at different times, some of them several hours before the start of work, and others came immediately according to the instructions of the manufacturer of the kit.
4. All the wells for the standard solution and urine of two group's cystitis and bladder cancer were labeled.
5. A (50) µl of standard solution was added to standard wells.
6. Fourty µl of urine was added to sample wells and then add (10 µl) anti-TLR-2 anti body to sample wells , then add (50 µl) streptavidin -HRP to sample wells and standard wells. Mix wells.

7. Then apply sealant to the plate. and incubate at 37^0C for 60 minutes.
8. After that , take off the sealer and 5times use wash buffer to wash the plate. Soak wells with (0.35ml) wash buffer for30seconds to 1 minute for each wash. Plate should be wiped with paper towels.
9. Fifty µl of substrate solution A was added to each well and then add (50 µl) substrate solution B to each well.
10. In this step, apply new sealant to the plate and incubate at 37^0C for ten minutes in the dark .
11. Fifty µl added from stop solution to each well.
12. Finally, read the optical density of each well immediately using microplate reader at a wavelength of 450 nm within 10 minutes after adding the stop solution .

B- Calculation of the ELISA Results :

The concentrations were calculated depending on the readings of optical density for each standard and samples by micro-plate reader. Also they can be calculated by Excel , through drawing a standard curve by plotting the meen OD values for each standard on the y-axis against the concentrations on the x -axis and draws a best fit curve through the points on the graph in Excel file (**Figure 2-2**).

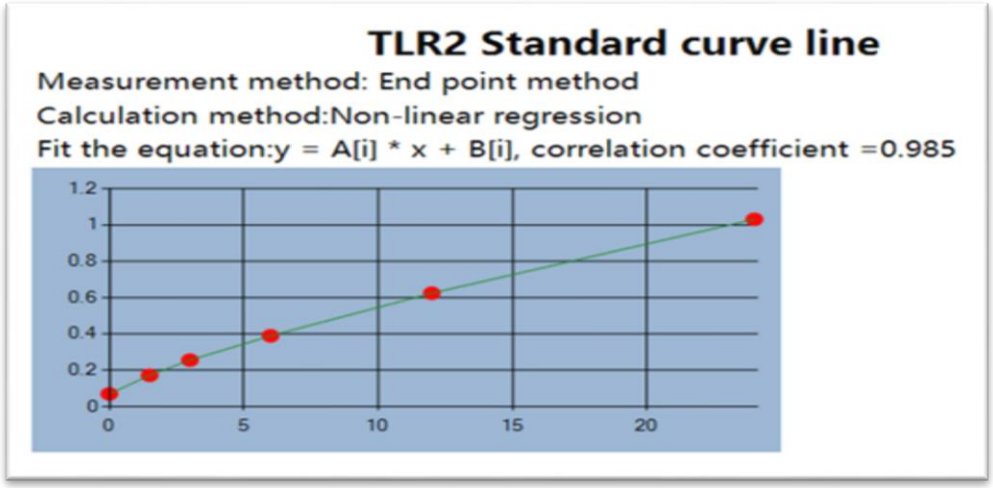


Figure (2-2) : TLR-2 standard curve

2.6.2 Estimation of Toll-like 4 receptors (TLR-4) in blood samples by flowcytometry technique:

Principle:

The basic principle of flow cytometry is the passage of cells in single file in front of a laser so they can be detected , counted and sorted. Cell components are fluorescently labeled and then excited by the laser to emit light at varying wavelengths (Bendall *et al*., 2012). Flow cytometry used to measure the cell surface levels of TLR4 at various time periods after the cells have been stimulated with LPS (Schappe *et al.*, 2018).

Procedure

1- One hundred µl of anticoagulated (EDTA, ACD) whole blood was added to the bottom of a 12x75 mm polystyrene tube, along with the conjugated antibody, as directed by the manufacturers product insert. Vortex and incubate in the dark at room temperature for the time specified.

2- One hundred µl of Reagent A was added to each sample and vortex. Incubated for 10 minute at toom temperature in the dark.

3- One ml of reagent B was added to each sample and vortex. Incubated for 20 minute in the dark.

4- By centrifuge at (5500 rpm) for 5minutes. Pour off supernatant and resuspend in (1ml) of PBS.

5- After that, centrifuge at (5500 rpm) for 5minutes. Pour off

supernatant and resuspend in (600 µl) of PBS. Mix by micropipette and transfer to can tube , vortex .

6- The six step is analyze the sample on flow cytometer.

7- In the last step, read the result computerization after detect stain type for TLR-4 Allophycocyanin stain (APS).

8- Compared the results according to normal value (less than 20% is negative, more than 20% is positive) (**Appendix I**).

2.6.3 Estimation of T- helper 17 (Th-17) in blood samples by flowcytometry technique:

This test was carried out using the same method mentioned in the paragraph (**2.7.1**) with changing the type of stain, as a dye was used phycoerythrin stain (PE) for Th-17 instead Allophycocyanin stain (APS) for TLR-4 .

2.6.4 Estimation of CD14 expression in bladder tissue by immunohisto chemistry technique:

Immunohistochemistry is useful tool in morphologic diagnosis and is regarded as a necessary test for standard of care, includes markers that assist identify the origin of tumor cells as well as prognostic and therapeutic indicators (Rodney , *et al*.,2019) .

2.6.4.1 Sample Processing for the IHC Staining Technique:

- By microtome which its blades are standardized at 4 µm, each block of formalin fixed paraffin embedded tissue was sectioned into 4 µm thickness section (one section for each IHC marker), then the sectioned tissue was put in water bath (37) °C for

seconds that allow the tissue to be relaxed , and allow the paraffin to stick to the glass . Then ribbon was mounted on positive charge slide.

- After that , the mounted slides were incubated in oven (58-60) °C melting point, and then left overnight .
- The third step is de paraffinization; three containers filled with Xylene were prepared, and then mounted slides have been immersed on each container for 15 minutes .
- The fourth step is hydration ; four jars were prepared for the serial dilution of ethanol from the first (100%, 90%, 70%, and 50%), then deparaffinized slides were immersed on each jar for 5 minutes , sequentially .
- In the last step: Retrieval container with 20X Immuno DNA Retriever Citrate were put in water bath until reach 65 °C ,then the hydrated slides were put in , and follow the temperature by thermometer until reach 95°C, for 20 minute . After that , the water path was turned off and waited to reach the 40 °C, and then wash slides by wash buffer 3 time.

2.6.4.2 The Protocol of Immunohistochemical Staining :

Initially , the sections in slides were marked by a waxing pen by drawing a circle around the tissue to prevent the leakage of any added solution from one section peace to the other during procedure .

1. The 1st step of IHC staining procedure involved ; the specimens in slides were shielded with Peroxidase Blocker for 5-10 minutes , to block the untargeted antigens and neutralize endogenous peroxidase activity . After that , these slides have been washed by wash buffer three times (5 minutes), and dried by

using wiping tissue to remove all access washing buffer, so it wouldn't dilute the antibodies used or any other used reagents.

2. At the end of peroxidase blocker step , the tissue specimens in slides were covered with either of primary antibody (CD14), after 45 minutes, these slides have been washed by wash buffer three times (5 minutes) , and then the area around the tissue section was dried out using paper tissue.
3. The 3rd step was flooding the tissue specimen with poly detector Horse radish peroxidase (HRP) Label for 15 minutes , then washed three times by wash buffer and wiped by paper tissue .
4. After that, tissue specimens were flooded with DAB substrate-chromogen solution for 5 minutes, and washed three times by buffer.
5. The tissues/slides were immersed in Hematoxylin jar as counter stain for 1 minute and then washed with D.W.
6. Mounting and covered the tissue by cover slide then examined by light microscope.

2.6.4.3 Evaluation of CD14 expression:

Presence of brown colored reaction in the nucleus or cytoplasm was considered a positive seaction. The intensity of the immuno staining was determined by modified way through taking pictures for each section through digital camera connected to conventional light microscope (40X power) , and further image analysis was done with the Image J software (version 1.46r, National Institutes of Health , USA) for each picture to count CD14 on cells.

For evaluation of CD14 marker expression is detected using an immunohistochemistry (IHC) approach based on CD14-specific

antibodies, as directed by the manufacturer instructions. Positive expression was defined as staining of the cell cytoplasm. The following basic criteria were used to establish a semi-quantitative rating based on the intensity of positive cells: weak (≤ 25%); moderate (25-50%), and strong (≥ 50%) (Diana *et al*., 2016 ; Moon *et al*., 2018).

2.7 Statistical analyses :

Statistical analysis was performed by using statistical package of social science (SPSS) version 26. The data were expressed as (mean ± SD). Statistical comparison between groups were made using students Chi-square test and a P value of ≤0.05 was considered significant; while the differences between more than two groups were analyzed using an analysis of variance (one – way ANOVA). Also, Pearson correlation coefficients were calculated to check the relationship between the studied parameters (Niazi , 2004).

Chapter Three

Results and Discussion

3. Results and Discussion:

3.1 Patients:

The study population consists of 100 patients divided into two groups following clinical diagnosis by super version of specialized professional : bladder cancer (BC) (50) case and cystitis (50) case as a control group for comparison while analyzing the variables according to the study design in (**Figure 2-3**) and statistically studied for different variable, as well as immunological parameters as a marker, and influence in relationship with bladder cancer.

3.1.1 Distribution of bladder cancer patients according to sex:

The results of the current study showed a significant difference ($P<0.05$) in the percentage of the incidence of bladder cancer between the sexes, it was found that the highest percentage to bladder cancer was recorded in males by (34) 68% and (16) 32% for female (**Figure 3-1**).

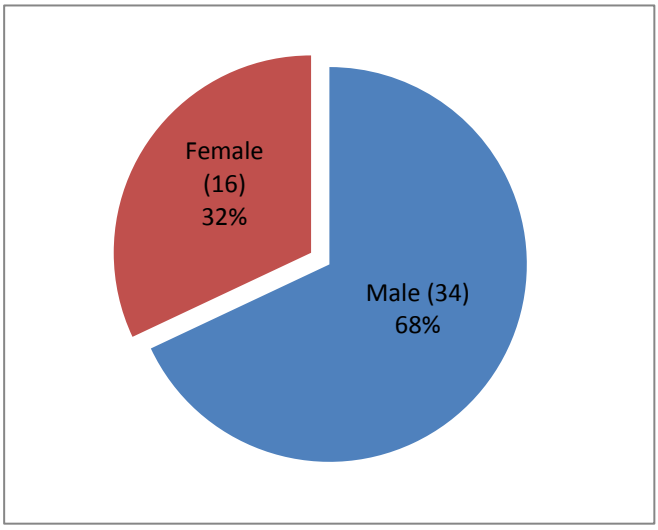


Figure (3-1): Distribution of bladder cancer according to the sex

These results were in consistent with local study in Iraq , with bladder cancer being the most prevalent cancer among Iraqi males, with a rate of 78% . In addition, bladder cancer was common in Iraqi cities, accounting for 13-15 % of all urinary system cancers (Abood *et al*., 2020).Howver, the present findings differed from those of an Egyptian study that found no significant differences in the incidence of bladder cancer (Kyritsi *et al*., 2018).

The statistical analysis of the current study revealed that there were significant differences for males compared to females, perhaps due to the male hormone factor (activity of the sex steroid hormone pathway), and the nature of working life (Alabdulkareem *et al***.,** 2017; Bray *et al*., 2018). Other variables that may predispose males to different behavior include environment, chemical exposure, carcinogenic exposures and other pollutants , as well as the use of alcohol,smoking and red meat (Etemadi *et al*., 2020; Abdolahinia *et al*., 2021**)**.

3.1.2 Distribution of bladder cancer according to age group:

In the current study, age was ranged from (40-87) years. The highest percentage (25) 50% was found in the age group (56-71) years, followed by the age group (40-55) with (15) 30%. The lowest incidence rate in the age group (72-87) was (10) 20% (**Figure 3-2**).

These finding were consistent with other studies (Hassanipour *et al*., 2019 ; Siegel *et al*, 2019) , indicating that bladder diseases are more prevalent in the age group more than 55 years about (80-90%) and prevalence among females than males depending on many factors. (Kalyan *et al*., 2020).

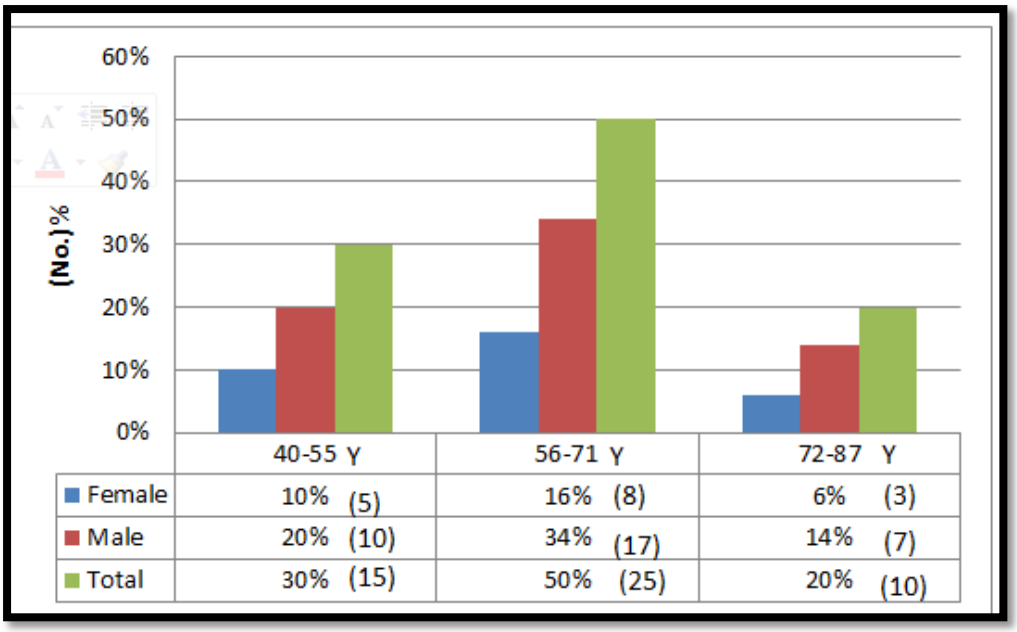


Figure (3-2): Distribution of bladder cancer according to the age group

The rise in the incidence rate in the age group (56-71) year may be attributed to the functional and physiological characteristics of the bladder tissue itself at this stage of life, and therefore age is one of the key risk factors in bladder cancer incidence that is not well known. However, according to certain research was undertaken to analyze the geographical disparities in the impact of exposure to chemical weapons and depleted uranium over numerous wars on the histological characteristics of bladder cancer (Hussein *et al*., 2017).

The study was accordance with many previous studies, which found that the high incidence of infection in the age group (65-70), and it's possible that this is due to the disease's course, which takes decades after exposure to mutagens to overcome some immune defense pathways and culminate in carcinogenesis in this age group, so the disease is rare in children and young people (Cormio *et al*.,2018 ; Saginala *et al* ., 2020).

3.2 Bacteriological study:

3.2.1 Bacterial isolation and identification:

Figure (**3-3**) showed that the urine specimens that gave a positive culture were (7) 23.4% for bladder cancer patients from (30) case, while (23) 76.6% negative result compare to cystitis specimens (28) 70% and (12) 30% respectively from (40) case. All this result from (70) case (**Figure 3-3**).

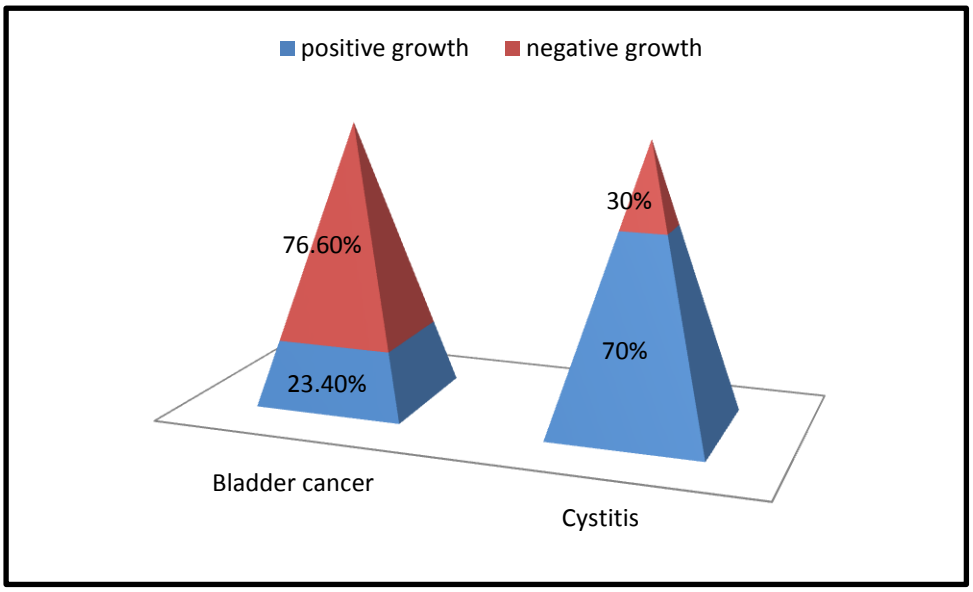


Figure (3-3): Isolation rate of bacterial growth in urine of patients with bladder cancer and cystitis

According to one theory, the diversity of bacterial colonization inside the bladder has a role in the absence of clinical symptoms, which adds to the pathogenicity of some isolate colonizing more dangerously to induce cells to proliferate irregularly (Urbaniak *et al*., 2016 ; Xingxing *et al* ., 2021).The present study showed the bacteriological examination for patients urine supported the diagnosis and explain inflammation state in bladder.

The surge in negative bladder cancer outcomes might imply that many of the medicines and antibiotics used to strengthen patients' immune systems are working. (Lene *et al*, 2020).

The great difference in the appearance and absence of bacterial growth between cystitis and bladder cancer is due to the role of bacteria that cause inflammation and their superiority over the rest of the microorganisms (Cai *et al*., 2020). Despite the low percentage of bacterial culture for the bladder cancer group, this suggested that the urine of patients acts as an enrichment medium as a result of the tumor's molecular changes (Wong *et al*., 2018). The present study's findings contrasted with those of other study at the time, which suggested that the high number of bacteria in duel with bladder cancer was due to a suitable tissue environment that allowed bacteria to evade immune regulation by inhibit anti-tumor activity when compared to normal tissues (Laliani *et al*., 2020; Xingxing *et al*., 2021). Tumor mass act as forging body to risk of infection.

The isolation and diagnosis of bacterial species in this study were based on the culture characteristics, particularly on CHROM agar medium and microscopic examination, as well as diagnosis using the VITEK2 system (**Appendix II**) (MacFaddin, 2000).

CHROM agar media contains enzymatic chromogenic substrates, which combine with certain enzymes secreted by the types of bacteria when they grow on this medium, which leads to different colors depending on the bacteria species, this test is useful in the laboratory diagnosis of bacteria (Murray *et al*.,2005).

Figure (**3-4**) showed the bacterial isolates in the studied groups differed between pure cultures of Gram-positive and Gram-negative bacteria and

both culture. The results indicated the apperance of uropathogenic *E.coli* (UPEC) and *Staphylococcus saprophyticus*, in significantly different proportions in patients with bladder cancer compared to cystitis. Both isolates of *S. saprophyticus* as pure culture in two groups bladder cancer and cystitis, appear to have a high percentage of cystitis. By (4) 11.8 % in bladder cancer patients group and (6) 14 % to cystitis patients group, whereas UPEC accounted for (1) 4.6 % in bladder cancer patients group and (14) 35.1 % to cystitis patients group. A similar finding was reported in previous study (Guoqin *et al*., 2019) who had shown the same result (35%) about the cause of infection by *E.coli*.

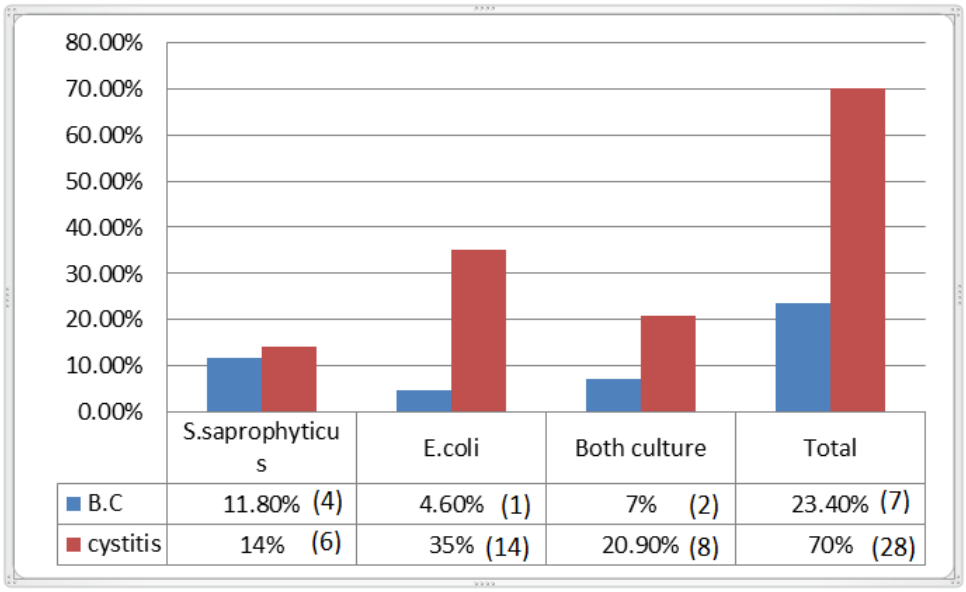


Figure (3 - 4): Percentage of bacterial species isolated from patients with bladder cancer and cystitis

On the other hand, another study conducted by Wu, *et al* (2018) who showed that another type of Gram negative bacteria was more abundant isolated from urine of patients with bladder cancer such as *Enterobacter*.

Bacterial infection of the urinary bladder is the most common cause of acute cystitis. Prevalence of *Staphylococcus saprophyticus* in study as

pure culture reached to (11.8%) with bladder cancer compared with cystitis group about (14%). Results were consistent with the recent study (Lawal *et al*., 2021) who revealed that *S. Saprophyticus* producing UTI in humans belonged to two primary clonal lineages (G and S), which arose in food/production (Noor *et al*., 2013; Karla *et al*., 2020). Also, the reason for the high rate *S. saprophyticus* (11.8%) may be due to the bacteria's adaptation to live in the urinary tract environment and its tolerance to unfavorable environmental conditions. In addition to having ability to inhibit other bacteria's development via generating the urease enzyme, haemagglutination, and adhesion to human uroepithelial cells, are among the virulence characteristics (Agwal *et al*.,2020). However *E.coli* bacteria demonstrated the greatest incidence of all samples (35) that give positive results in the current investigation, followed by *S.saprophyticus* with (15) 42.5%, (6) 16.9% , respectively (**Figure 3-5**).

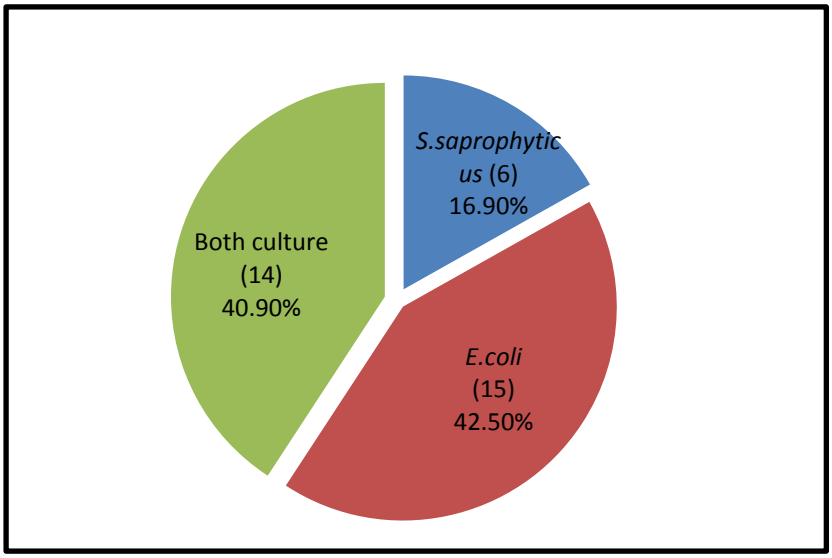


Figure (3-5): Percentage of bacterial growth in study groups (both cystitis and bladder cancer)

E.coli bacteria can induce pathogenicity in host cells by a variety of mechanisms, including direct integration of host DNA, production of

reactive oxidation types in some strains, and effect on the (Wnt/ β -catenin) pathway which conceder regulated key cellular functions and its manipulation to encourage cell proliferation in many cancer (Gagnaire *et al.*, 2017).

The intricate involvement of virulence factors and intervening toxic secretions in enhancing pathogenicity may explain the presence of (14) 40.9% of the both culture. The microbiome can potentially generate chronic inflammation, which can promote tumor growth, or elicit immunosuppressive responses, which can impede cancer immunosurvival. Bacterial metabolism of host-derived metabolites, dietary components, or xenobiotics can produce toxic chemicals that can induce cancer even in remote parts of the body (Viljemka *et al*., 2017).

Bacteriological study reveals the ability of *E.coli* bacteria to adapt in the environment of the urinary tract through the results of bacterial culture of urine, while another study revealed the role of mechanical forces for bladder tissues in addition to the high regulation of the immune system to reduce microbial attack depending on the person's lifestyle (Ueda *et al*., 2020). From the result, factors that predispose the urothelium to a state of prolonged bacterial infection appear to enhance the risk of bladder cancer.

All data from bacterial cultures were statistically analyzed and grouped into four groups (No growth, *E.coli* , *S. saprophyticua*, and Both culture) in order to be reliant upon and compressed with an immunological study.

3.3 Immunological study:

3.3.1 Estimation of immuno-markers (TLR-2, TLR-4 and Th17) in study population:

The descriptive data of the immunological study groups were shown in table (**3-1**), the table showed an increase in immunomarkers value (TLR-2, TLR-4 and Th-17) of bladder cancer patients compared with cystitis group.

Table (3-1): Evaluation of immunological markers in bladder disorder by ELIZA and flowcytometry technique

Bladder disease	Marker	Sample	Mean ± S.D	N
Bladder cancer	TLR-2 ng/ml	Urine	5.85217 ± 1.8627	30
	TLR-4 %	Blood	3.649 ± 1.8267	30
	Th-17 %	Blood	33.697 ± 16.3788	30
Cystitis	TLR-2 ng/ml	Urine	2.99734 ± 1.1494	30
	TLR-4 %	Blood	2.777 ± 1.5529	30
	Th-17 %	Blood	29.497 ± 14.4778	30
S.D: Std. Deviation				

The results of the current study showed that Th-17 percentage was higher in bladder cancer patients group than cystitis in (33.697%) , (29.497%) respectively (**Table 3-1**). This finding refer to the fact of Th17 levels correlate positively with microenvironmental Th1 cells, cytotoxic CD8 T cells and natural killer cells as a tumor-associated marker (Curiel *et al* ., 2004 ; Kryczek. *et al*. , 2009).

When evaluating the expression profile of immune markers and by comparing the results of current study between bladder tissues, found that the variance of expression of TLRs in general was unregulated in the case of bladder cancer compared to cystitis. These results were consistent with (Lu *et al*., 2021).

Urine is arguably the most unusual setting for studying tumor markers, because the urine is an environment that only a few organs are exposed to, detecting markers in urine samples frequently yields better specificity than those discovered in serum samples. Thus, in current study evaluated TLR-2 as highly sensitive and specific receptor for gram positive bacteria (Livia and Molly , 2021).

In order to acquire insight into the changing bladder tumor microenvironment, the current clinical trials in bladder cancer often incorporate the collection of blood samples. Regarding to understanding immunological statue in tumor microenvironment to bladder, must study the immune surveillance in the patient's blood . TLRs expression in tumor statue has tumorigenic effects (positive regulation of cancer). TLRs expression with cancer cells can upregulate the NF-κB signaling cascade, the production of pro-inflammatory cytokines, and the production of anti-apoptotic proteins, all of which contribute to tumor growth and cancer cell immune evasion and proliferation. Furthermore, additional immune cells are recruited to boost immunity in the tumor's microenvironment (Ohadian and Nawroozi, 2019). Multiple immunosuppressive cell type and inflammatory mediators are mobilized into bladder cancer in response to bacterial infection to controlling on state or not.

3.3.2 Estimation of Toll- like Receptors-2 (TLR-2) in urine by Enzyme - Linked Immunabsorbent Assay (ELIZA) technique:

3.3.2.1 TLR-2 concentration according to age group:

The current findings indicated that there was a significant rise ($P \leq 0.05$) in the concentration of TLR-2 and for all age groups of patients (5.55, 7.696 and 2.675) ng/ml with bladder cancer compared to cystitis,

and the highest levels appeared at the age group of (56-71)year of bladder cancer patients in table (**3-2**).

The immune response in some diseases especially cancer has been well characterized (Ayala *et al*,2019) , the mechanisms involved in reactivation in humans are still unclear. Knowledge of the anti-tumor and anti-inflammation immune response is important. Present studies pointed that the concentration of TLR-2 in urine is a result of systemic overproduction because of dendritic cells , neutrophils and regulatory T cells (T regs) activation by bacteria contacts with TLR-2 receptor, which plays an essential function as a biomarker in the innate immune response, according to (Joseph *et al*, 2019).

Table (3-2): Level of TLR-2 in patients with bladder disorders

Age group	**Concentration of TLR-2 ng/ml Mean ± S.D**	
	Bladder disorders group	
	Cystitis	Bladder cancer
40-55 y	4.352 ± 0.245	5.55 ± 0.284*
56-71 y	2.577 ± 0.088	7.696 ± 0.235*
72-87 y	1.374 ± 0.104	2.675 ± 0.161*
*L.S.D under (P<0.05) = 0.362 S.D: Std. Deviation		

The present study revealed a significant increasing in the concentration of TLR-2 for all age groups of patients with bladder cancer compared to cystitis and the highest concentration of them for the age group (56-71) years (7.696 ± 0.235 ng/ml) . The immune response accompanying tumors includes both humeral and cellular types in an integrated manner, and therefore it is noted that both innate and adaptive immunity are involved

to limit tumor development. Immune cells can be harmed by cancer and are frequently shown to be dysregulated in patients (Patras *et al*., 2019; Hoppstadter *et al*, 2019).

3.3.2.2 Association of TLR-2 with bacterial culture:

The concentration of TLR-2 was measured statistically according to bacterial culture results as shown in table (**3-3**). There was very varied in TLR-2 concentration in bladder cancer compared to cystitis as a result to number of cases that appear in current study. Statistical analysis showed that TLR-2 concentration (2.68467 ±.182717 ng/ml) was significant 'affected (P≤0.05) in B.C patients with *S. saprophyticus* bacteria as compared with others bacterial isolates , while in cystitis showed non-significant effect (P≤0.05) of bacterial culture on TLR-2 concentration.

Table (3-3): Comparison of TLR-2 concentration in patients according to bacterial culture

Bladder disease	**Bacterial culture**	**N**	**Concentration of TLR-2 ng/ml Mean ± S.D**	**P *value* (p≤0.05)**
Bladder cancer	No growth	23	6.11422 ±1.698308	.009*
	E.coli	2	6.28700 ±1.265721	
	S. saprophyticus	3	2.68467 ±.182717*	
	Both culture	2	7.15500 ± .234759	
	Total	30	5.85217 ± 1.862784	
Cystitis	No growth	9	2.66678 ± 1.107464	.629 NS
	E.coli	11	3.33845 ± 1.117237	
	S. saprophyticus	4	2.75700 ± .346462	
	Both culture	6	2.98800 ± 1.550737	
	Total	30	2.99734 ± 1.149459	
* The mean difference is significant at the 0.05 level by one way ANOVA S.D: Std. Deviation. NS: Non-significant difference				

Regarding to TLRs low concentrations significantly with *S.saprophyticus* in bladder cancer may indicate a delay in early identification of bacteria and activation of signaling pathways, in addition to size of sample (Greten and Grivennikov , 2019). Study evidence pointing to an important immunological role of TLR-2 in controlling infections caused by Gram-positive bacteria in general, but it is possible that another group of receptors may be involved in controlling Gr^{+ve} bacteria when they are not secreted or activated (Ohadian and Nowroozi, 2019).

To the best of knowledge may be, this the first study in Iraq that evaluating TLR-2 concentration in patients urine with bladder cancer and explain their association with bacterial culture results.

3.3.3 Estimation of Toll- like Receptors-4 (TLR-4) by flowcytometry technique:

In this study, TLR- 4 was examined as innate immunity of bladder cancer patients and compared to cystitis. In figure (**3-6**), flowcytometry technique analysis has been used to evaluate TLR-4 percentage as an innate immune response and their role in tumor progressing with bacterial infection conditions.

Levels of TLR-4 were increased in bladder cancer patients than cystitis about (3.649 ± 1.8267 %) and (2.777 ± 1.5529 %), respectively (**Table 3-1**). This indicates that patients in B.C were suffering from multi TLR-4 signaling pathway may prevent or delay the development of bladder cancer (Li *et al*., 2017).

Malignant cells and immune cells both express TLR- 4 . From another perspective, the increase in the level of TLR-4 could explain how immune

cells and other toll like receptors might overcome bacterial colonization (Bajic *et al*., 2019 ; McCall *et al*., 2020).

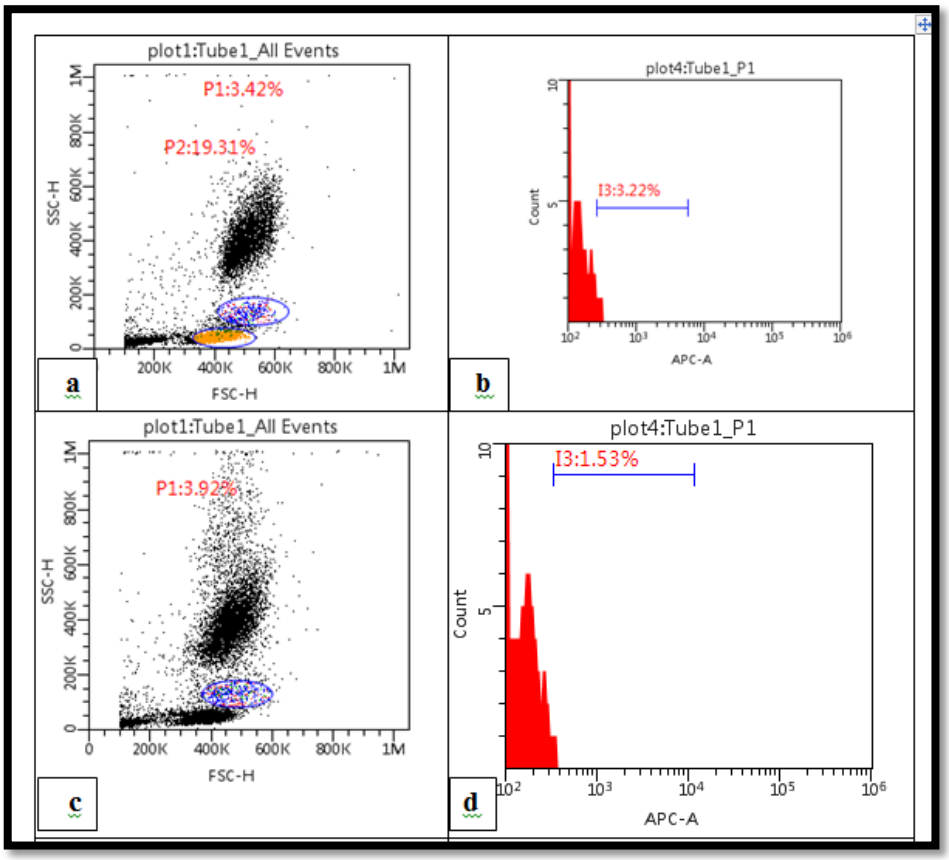


Figure (3-6): Diagrams showing a forward scatter height (FSC-H) and side scatter height (SSC-H) the TLR-4 markers according to patients groups that use the flowcytometry technique. (a,b) : anti-TLR-4 to TLR-4 percentage in cystitis group. (c,d) : anti-TLR-4 to TLR-4 percentage in bladder cancer group**.**

3.3.3.1 Level of TLR-4 according to age group:

In the study analyses, TLR-4 level was examined in blood of bladder cancer patients , and there was non-significant difference (P≤0.05) between age group with the highest level at the age group (40-55y) (4.444 ± 1.6241%) . While detecting a significant difference (P≤0.05) in all age group to cystitis patients, with high level at age (72-87y) (4.4401.7925%) , as showed below in (**Table 3-4**).

Table (3-4): Level of TLR-4 in patients with bladder disorders

Groups	Age group	N	TLR-4 % Mean ± S.D	95% Confidence Interval for Mean		P *value* (p≤0.05)
				Lower Bound	Upper Bound	
Bladder cancer	40-55y	9	4.444 ± 1.6241	3.196	5.693	.305^{NS}
	56-71y	15	3.325± 2.0149	2.210	4.441	
	72-87y	6	3.267 ±1.4624	1.732	4.801	
	Total	30	3.649 ±1.8267	2.967	4.331	
Cystitis	40-55y	9	3.267 ±1.3360 ^{a}	2.240	4.294	.002*
	56-71y	16	1.981±1.0547^{*b}	1.322	2.438	
	72-87y	5	4.440±1.7925 ^{*a}	2.376	5.991	
	Total	30	2.777±1.5529	2.179	3.334	

NS: Non-significant difference
* significant difference ($p \leq 0.05$) by one way-ANOVA, different letters in same column different significantly

There was no risk factor evaluated when it came to age variance and TLR-4 levels with bladder cancer. In cystitis, on the other hand, age was an effect element that might contribute to that older patients with cystitis have increasing immune cell expression in their receptors , which may important immunosurveillance in those patients (Ahn *et al*., 2017).

It's also possible that the high TLR-4 levels in old age group are due to their frequent exposure to carcinogens or environmental pollutants during the Iraq wars, which may increase inflammation and immunesupressor in the bladder and throughout the body, causing a permanent genetic mutation or encouraging the creation of an appropriate cancerous environment when a bladder infection occurs . This explanation agree with (Saginala *et al*., 2020).

3.3.3.2 Distribution of TLR-4 According to Bacterial Culture:

The lavel of TLR-4 was measured statistically according to bacterial culture results as shown in table (**3-5**). There was very variation in TLR-4 lavel in bladder cancer compared to cystitis in the current study. Statistical analysis showed that TLR-4 level were non-significantly affected (P≤0.05) in BC patients . While in cystitis showed significant effect (P≤0.05) of bacterial culture on TLR-4 level, *S. saprophyticus* as (1.900 ± 0.1000 %) pure culture and both culture (4.400 ± 2.2450 %) respectively (**Table 3-5**).

Table (3-5): Comparison level of TLR-4 in patients according to bacterial culture

Bladder disease	Bacterial culture	N	TLR-4 % Mean ± S.D	P *value* (p≤0.05)
Bladder cancer	No growth	23	3.664 ± 2.0337	.813 NS
	E.coli	2	4.000 ± .5657	
	S. saprophyticus	3	4.067 ± .1528	
	Both culture	2	2.500 ± 1.4142	
	Total	30	3.649 ± 1.8267	
Cystitis	No growth	9	2.778 ± .8555	.004*
	E.coli	12	2.433 ± 1.0030	
	S. saprophyticus	3	1.900 ± .1000 *	
	Both culture	6	4.400 ± 2.2450 *	
	Total	30	2.777 ± 1.5529	

* The mean difference is significant at the 0.05 level by one way ANOVA
S.D: Std. Deviation. NS: Non-significant difference

TLR-4, which is found on the cell surface, recognizes the structural components of bacteria with the highest affinity, table (**3-5**) showed no significant link between TLR-4 and bacterial growth in bladder cancer group. On the other hand, the highest level was found with pure cultures of

Gram-negative and Gram-positive bacteria (4.000 ± .5657%) and (4.067 ± .1528%) respectively in bladder cancer patients . The non-significance of the raised TLR-4 level with *E.coli* also suggests that the LPS concentration within the bladder may not be similar to that which appears upon bacterial culture of the urine and thus induce an innate immune response to the uroepithelium. Additionally to the vital role of TLR-4 in progressing the inflammation condition to cancerous state (Lu *et al*., 2018; Yeh *et al*., 2019). In other side, there was a significant association between TLR-4 and bacterial culture in cystitis group. Pathogen sensing by TLR-4 induces the production of various soluble factors which are secreted by antigen presenting cell such as (NF-κB , TNF-α , IL-1,and others) (Al-biaty *et al*., 2015).

The present results were comparable with other studies have pointed to bacteria and their products, particularly lipopolysaccharide, lipotechoic-acid and exotoxin, as one of the most important factors causing an increase in the secretion of a number of Toll-like receptors during infection, such as TLR-4, TLR-3, and TLR-2, to speed up both types of immune response innate and specific immunity (Merrill *et al*., 2016 ; Ueda *et al*., 2020).

3.3.4 Estimation of T-helper 17 (Th-17) by flowcytometry technique:

In this study, T-helper 17 was examined as adaptive immunity of bladder cancer patients and compared to cystitis. In figure (**3-7**), flowcytometry technique analysis has been used to evaluate Th-17 percentage as an specific immune response by T-lymphocyte cell and their role in tumor progressing with bacterial infection conditions.

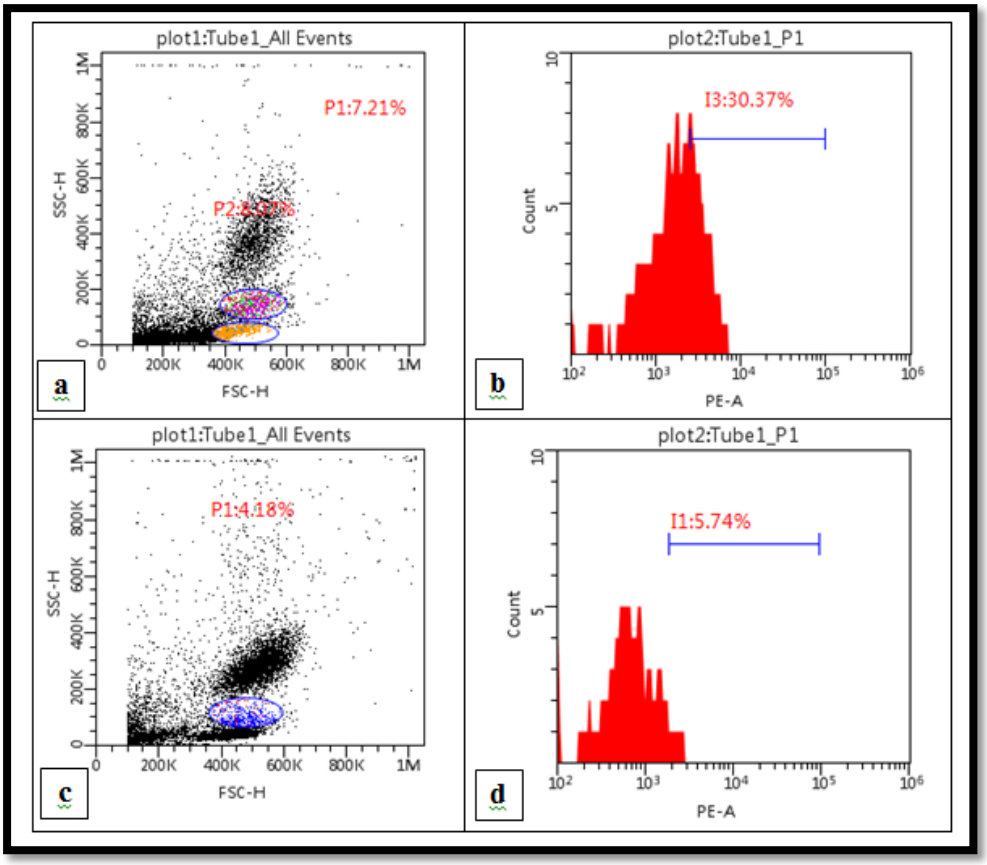


Figure (3-7): Diagrams showing a forward scatter height (FSC-H) and side scatter height (SSC-H) the Th17 markers according to patients groups that use the flowcytometry technique. (a,b) : anti-Th17 to Th17 percentage in cystitis group. (c,d) : anti-TLR-4 to TLR-4 percentage in bladder cancer group.

Despite the fact that Th17 cells make up a small population within the tumor microenvironment (Curiel *et al* ., 2004 ; Kryczek. *et al*. , 2009), Th17 was detected in table (**3-1**) proportionally higher numbers in bladder cancer patients comparison with cystitis about (33.697 ± 16.3788%) and (29.497 ± 14.4778%) respectively.

3.3.4.1 Level of Th-17 according to age group:

The current findings showed variance in Th-17 in all age groups without a significant difference (P≤0.05) in patients with bladder cancer and cystitis. However, table (**3-6**) showed that a significant increase (38.360 ± 4.6859 %) in bladder cancer occur in people aged (56-71 y) , while in

cystitis (33.433 ± 5.5494 %) peaking in people aged (40-55 y). The result was consistent with a previous report (Al-Humairi *et al*., 2019).

Table (3-6): Level of Th17 in patients with bladder disorders

Groups	Age group	N	Th-17 % Mean ± S.E	95% Confidence Interval for Mean		P *value* (p≤0.05)
				Lower Bound	Upper Bound	
Bladder cancer	40-55 y	9	33.967 ± 4.5734	23.420	44.513	.104 NS
	56-71 y	15	38.360 ± 4.6859	28.310	48.410	
	72-87 y	6	21.633 ± 3.9264	11.540	31.726	
	Total	30	33.697 ± 2.9903	27.581	39.813	
Cystitis	40-55 y	9	33.433 ± 5.5494	20.636	46.230	.630 NS
	56-71 y	16	27.525 ± 3.2127	20.677	34.373	
	72-87 y	5	28.720 ± 7.6795	7.398	50.042	
	Total	30	29.497 ± 2.6433	24.091	34.903	

NS: Non-significant difference by one way-ANOVA
S.E: Std. error.

According to the present findings, patients' age in bladder disease was not risk factor that affects their Th-17 levels. This result depends on the substance capacity to increase inflammatory mediator production, and increase from glucose transporter to enrich tumor microenvironment and more induce cell division (Zhao *et al*., 2019; Sullivan *et al*., 2019). Another study looked at the concentration of IL-17, which is a key cytokine secretion , to determine if Th-17-induced inflammatory mediators engage myeloid cells to promote cancer growth (Veldhoen, 2017) . It highlighted how neutrophils, myloid cells, and other immune cells might infiltrate a tumor's microenvironment by T-helper cell 17 to create favorable conditions for irragulatory cell development (Veglia *et al*., 2018).

3.3.4.2 Th-17 distribution According to Bacterial Culture:

According to the current data (**Table 3-7**), which compare Th-17 levels in bladder disease patients based on bacterial culture, there was non-significant difference in bladder cancer but a significant difference in cystitis was found (P≤ 0.05).

Table (3-7): Comparison level of Th17 in patients according to bacterial culture

Bladder disease	Bacterial culture	N	Th-17 % Mean ± S.E	P *value* (p≤0.05)
Bladder cancer	No growth	23	31.535 ± 3.6134	.275 NS
	E.coli	2	46.200 ± 2.6000	
	S. saprophyticus	3	30.333 ± .4910	
	Both culture	2	51.100 ± 7.0000	
	Total	30	33.697 ± 2.9903	
Cystitis	No growth	9	18.400 ± 1.6449	.004 *
	E.coli	12	30.375 ± 4.1779 *	
	S. saprophyticus	3	29.667 ± .2603	
	Both culture	6	44.300 ± 6.1833 *	
	Total	30	29.497 ± 2.6433	

* The mean difference is significant at the 0.05 level by one way ANOVA
S.E: Std. error. NS: Non-significant difference

To explain the results of our current study in terms of the absence of significant differences for the level of Th-17 in the blood with the results of bacterial culture in patients with bladder cancer compared to the presence of a significant difference for cystitis that may be due to the heterogeneity of the sample size in general, but the significance can be explained by factors of bacterial virulence and toxins that the massive proliferation of T cells, especially Th-1 and Th-17, causes the secretion of an exaggerated amount of cytokines , which can switch the immune

system from one that is positive to one that is negative, leading to tumor immunosuppression (Dutta *et al*., 2020 ; Kaymak *et al*., 2021).

The results of the tables (**3-3, 3-5, 3-7**) show a significant increase in immune marker rates and concentrations with both cultures , as a result of the association of additional bacterial factors associated with colonization with the regulation of metabolic pathways and the interaction between them so that bacteria can be sensitive to biological signals within the bladder environment in order to be able to stimulate immune responses (Maria *et al*., 2017).

3.3.5 Correlation between immuno-markers (TLR-2, TLR-4 and Th17) in study population:

To answer about question mark on focus on toll like receptors in our study, the correlation between Toll like receptors 2,4 and T-helper cell 17 value of patients with bladder cancer and cystitis has been calculated and analysis statistically under ($P \leq 0.05$) in this study (**Table 3-8**).

Statistical analysis showed a significant ($P \leq 0.05$) positive correlation between levels of Th-17 and TLR-2 in BC patients group, as well as, there is non-significant ($P > 0.05$) negative correlation with TLR-4 in same group. While , there was non-significant ($P \leq 0.05$) invers correlation between levels of Th-17 and TLR-2 in cystitis patients group with present significant correlation with TLR-4.

The interaction between the results of immune tests for patients with bladder cancer and cystitis by observing the concentrations of immune receptors shows an important way to reach the type of immune response that occurs and the cells involved in it specifically, although the bacterial types present in the urinary tract, especially the bladder express the occurrence of different immune responses (Li *et al*., 2020).

However, this study came to investigate the relationship between what happens inside the bladder cells and what takes place between the components of the immune system by tracing the cellular pathway with the participation of some immune pathways, considering BC well recognized as an immunogenic and immunoresponsive tumor.

Table (3-8): Pearson Correlations between immunological markers in study population

Correlations					
Bladder disease			**TLR-2**	**TLR-4**	**Th-17**
Bladder cancer	**TLR-2**	Pearson Correlation	1	-.010-	.405*
		Sig. (2-tailed)		.959	.027
		N	30	30	30
	TLR-4	Pearson Correlation	-.010-	1	-.066-
		Sig. (2-tailed)	.959		.730
		N	30	30	30
	Th-17	Pearson Correlation	.405*	-.066-	1
		Sig. (2-tailed)	.027	.730	
		N	30	30	30
Cystitis	**TLR-2**	Pearson Correlation	1	-.008-	.238
		Sig. (2-tailed)		.966	.215
		N	30	30	30
	TLR-4	Pearson Correlation	-.008-	1	.443*
		Sig. (2-tailed)	.966		.014
		N	30	30	30
	Th-17	Pearson Correlation	.238	.443*	1
		Sig. (2-tailed)	.215	.014	
		N	30	30	30
*. Correlation is significant at the 0.05 level (2-tailed).					

As a result, various markers are being created to better study these cells and establish their physiological characteristics that indicate their proclivity to operate as cancerous cells. In other means, all receptors on immune cells or their secretion cannot be work alone in any pathway unless contributed as a team and form immune complex to act as anti-tumor or immunesupressor depended on natural of infection or immune

defect giving precedence to the toll like receptors sequentially (Tongtawee *et al*., 2019 ; Crispen and Kusmartsev , 2020) .

3.3.6 Correlation of immuno-markers (TLR-2, TLR-4 and Th17) to bacterial culture:

Table (**3-9**) summarizes the correlation between TLR-2, TLR-4 and Th17 with bacterial culture in bladder cancer to cystitis. Correlation analysis showed nonsignificant correlation ($p \leq 0.01$) between three immunomarkers with bacterial culture in bladder cancer cases . Although , finde inverse correlation with TLR-2 and TLR-4 about (-.216- and -.081-) respectively. The correlation was explained in below table (3-9), that pointed to present highly significant correlation ($p \leq 0.01$) to Th17 with bacterial culture in cystitis .

Table (3-9): Correlation of TLR-2, TLR-4 and Th-17 to bacterial culture in study population

Correlation					
Bladder disease			**TLR-2**	**TLR-4**	**Th-17**
Bladder cancer	**Bacterial culture result**	Correlation Coefficient	-.216-	-.081-	.269
		Sig. (2-tailed)	.251	.670	.151
		N	30	30	30
Cystitis	**Bacterial culture result**	Correlation Coefficient	.156	.040	.639**
		Sig. (2-tailed)	.419	.833	.000
		N	30	30	30
**. Correlation is significant at the 0.01 level (2-tailed).					

Despite there was big variation in our results about TLRs concentration and their correlation with bacterial culture in study population, but can be explained as a result of bacterial infection and irritation stimulating transcription factor kappa- B (NF-κB) activation as a

result of their interaction with TLR2, 4/(CD14) Cluster of Differentiation antigen receptor complexes, leading to suppression of apoptosis and reduced activation of Tumor protein53(p53) and its responsive genes (Gudkov *et al*., 2011).

Table (**3-9**), shows the highly significant correlation of Th17 with bacterial culture, it is noted that there are several immune responses to reduce the bacterial burden, and if one of them is fail, the body switches to an alternative pathway that involves the activation of helper T cells, particularly Th-17, to mediate activation of the adaptive immune response through the secretion of a group of cytokines and the stimulation of B cells to produce certain antibodies and control to bacterial invasion (Dutta *et al*., 2020).

3.3.7 Expression of CD14 by Immunohistochemistry (IHC) technique in study group:

The sample population consisted of 30 cases (biopsy), divided into three groups, cystitis and bladder cancer (low grade and high grade), each include 10 cases.

3.3.7.1 Distribution of CD14 immuno-expression in study group according to sex:

Study was conducted on 30 patients of two sexes, with (7) 70 % of men in cystitis and high grade bladder cancer (H.G) samples and (8) 80 % of males in low grade bladder cancer (L.G) samples (**Figure 3-8**).

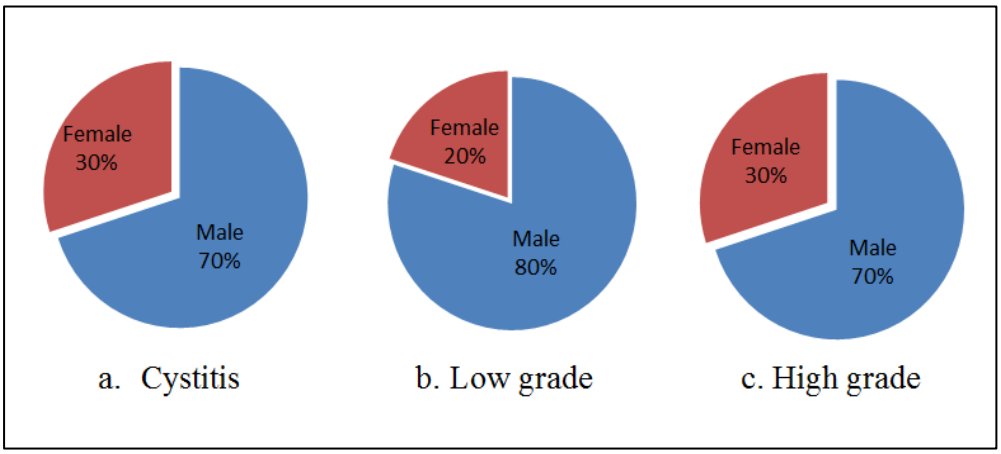


Figure (3-8) : Distribution of study samples CD14 according to sex

The current study agrees with others have found that males are more likely than females to develop cystitis and bladder cancer of both sorts (low and high grade). The rising risk of cystitis and bladder cancer in males, as opposed to women, has been correlated to their physiological status and lifestyle, additional to cigarette use being more prevalent in men (Tyagi *et al*., 2018 ; Afshar *et al*., 2018).

3.3.7.2 Distribution of study sample with CD14 expression according to age group:

The study also revealed that bladder disease was classified into several age groups, ranging from 40 to 87 years old, with varied percentages. The highest frequent appear in age group [40-55 y] to cystitis, while frequency of patients with bladder cancer group (L.G/ H.G) appear in age group [56-71 y] (**Figure 3-9**).

According to the present study, bladder cancer was more prevalent in people over 55 year , whereas cystitis was more common in people under 55 year, which was consistent with personal history and might be linked to other urinary system disorders or treatment (Kalyan *et al*., 2020) .

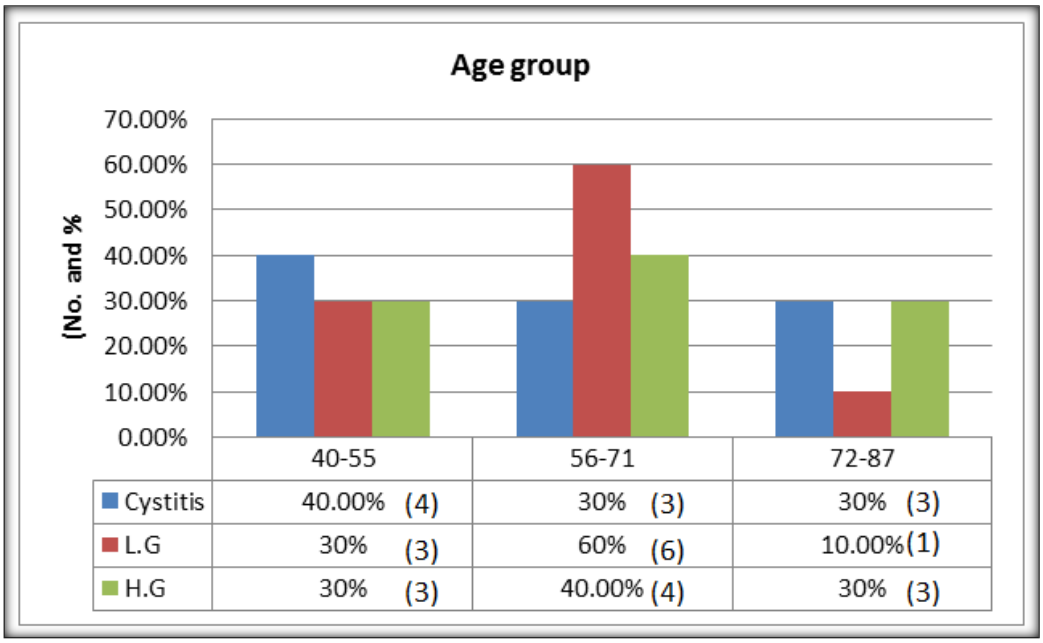


Figure (3-9) : Expression of CD14 by IHCaccording to age group

3.3.7.3 Expression of CD14 in bladder cancer and cystitis patients:

Inflammatory diseases of the bladder are among the vital problems that should be taken care of, especially bladder cancer, which requires careful diagnosis and follow-up by focusing on the patient's immune system to prevent the inflammatory condition (cystitis) from progressing to malignant status (Bray *et al*., 2018). Figure (**3-10**) showed a positive anti-CD14 response by uptake IHC stain in a bladder biopsy revealed CD14 expression, as seen in the pointed brown region of the image, compared to non-staining cells (negative CD14 expression).

Additionally, the positive CD14 expression was appeared in varying, with cystitis accounting for around 30% of the positive CD14 expression, while L.G and H.G account for about 70% and 20% positive CD14 expression, respectively (**Figure 3-11**).

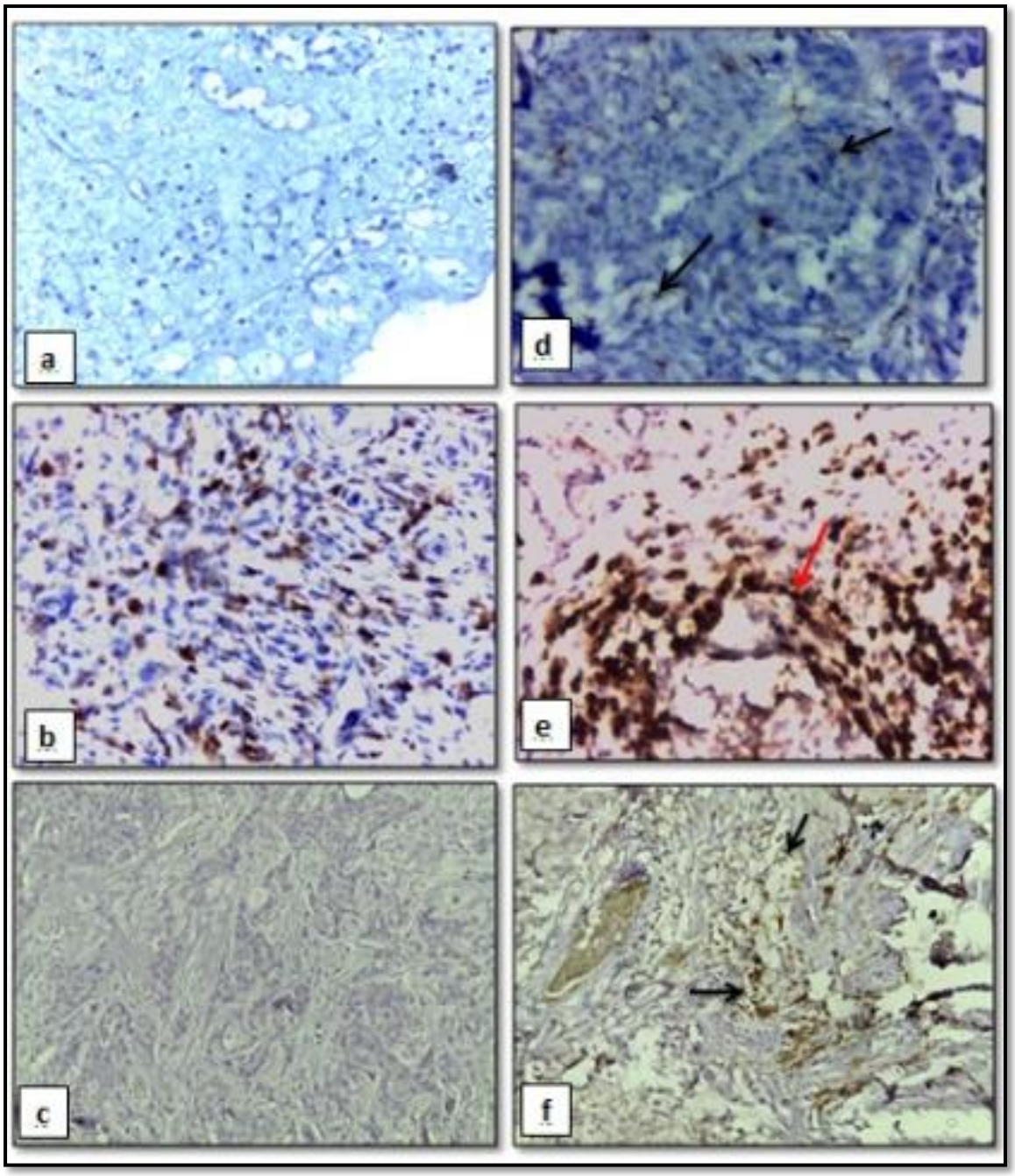


Figure (3-10) : Photomicrograph . (a,b,c) show negative result for CD14 expression in biopsy of cystitis, Low grade and high grade bladder cancer, respectively . (d,e,f) show positive CD14expression as pointed

cytoplasmic brown stain for three cases of bladders biopsy (Immunohistochemicalstaining for CD14 x 400).

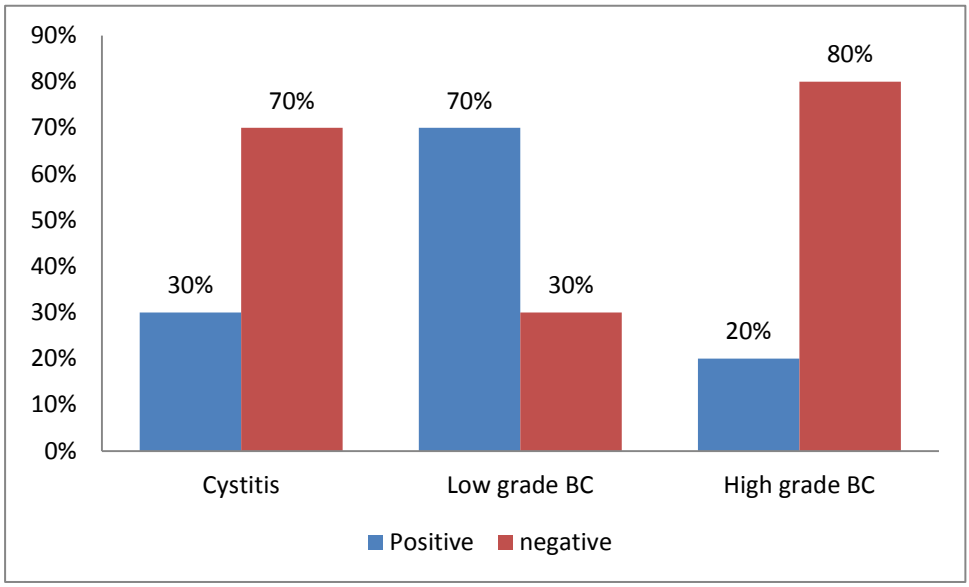


Figure (3-11) : Percentage of CD14 expression in cystitis and bladder cancer biopsy

By IHC technique for detection of CD14 expression showed different expression between current study groups. It can be explained by the role of the opposite side of immune activity, which occurs as a result of the formation of immune complexes from CD14 antigens/ receptors on the surfaces of urothelial cells, which works to suppress apoptosis by reducing the vitality of the oncoprotein p53, leading to the genetic activation of oncogenic cytokines (TNF-α, IL-1,IL-2,IL-6,IL8 and IL-10) and all these chain stimuli, it suppresses immunity and encourages tumor cells to grow uncontrollably (Al-biaty *et al*., 2015).

On the other hand, expression of CD14 , reduced in cystitis samples, despite the fact that it is a co-receptor for bacterial component. This might be due to the capacity of antibiotics to regulate inflammation before immunological control and chemotherapy, which inhibits immunity (Rodney *et al*., 2019) . The majority of high grade bladder cancer showed

lowest percentage (20%) for CD14 expression, this was supported by the fact that CD14 high cancer cells produce greater amounts of multiple inflammatory mediators and exhibit accelerated tumor development, resulting in bigger tumors, as compared to low CD14 expression in bladder cancer (Cheah *et al*., 2015).

Regarding to intensity of CD14 expression, Table (**3-10**) and (**Figure 3-12**) revealed that all bladder biopsy groups showed a high significant difference in CD14 expression (0.001 under $p \leq 0.05$), although there was no significant variation in the intensity of positive CD14 immunostaining. The present findings were consistent with other studies which showed non significant difference in CD14 expression when measured by IHC and other immune technique in different type of bladder cancer (Mousa *et al*., 2017).

Table (3-10) : Intensity of CD14 expression in study groups

Bladder diseases	**CD14 expression**		**Intensity of CD14 expression**			**Total**
	Result	No.	weak	moderate	strong	
Cystitis	positive	3	1	1	1	10
	Negative	7				
Low grade of bladder cancer	positive	7	2	2	3	10
	Negative	3				
High grade of bladder cancer	positive	2	2	0	0	10
	Negative	8				
P *value* ($p \leq 0.05$)	0.001 (highly significant)		0.1 (non-significant)			30

The intensity of positive CD14 expression in the current study was appeared non- significant difference , which was dependent on the amount of CD14 receptors present on cell surfaces, and hence may be deceptive due to immunostain, antibody, and chemical reagent inefficiency (Stanimir *et al*., 2012). Cheah *et al*., (2015), showed that CD14+ bladder cancer cells regulate tumor-promoting inflammation and drive tumor cell proliferation, both of which contribute to tumor development. According to scientific

evidence, the body's immune system protect itself by making inflammation flow through the bladder and out through the urine, but if it stays there for too long, it might develop into cancer (Mai *et al*., 2019).

3.3.7.4 Correlation of CD14 expression in bladder cancer (L.G/ H.G) to cystitis:

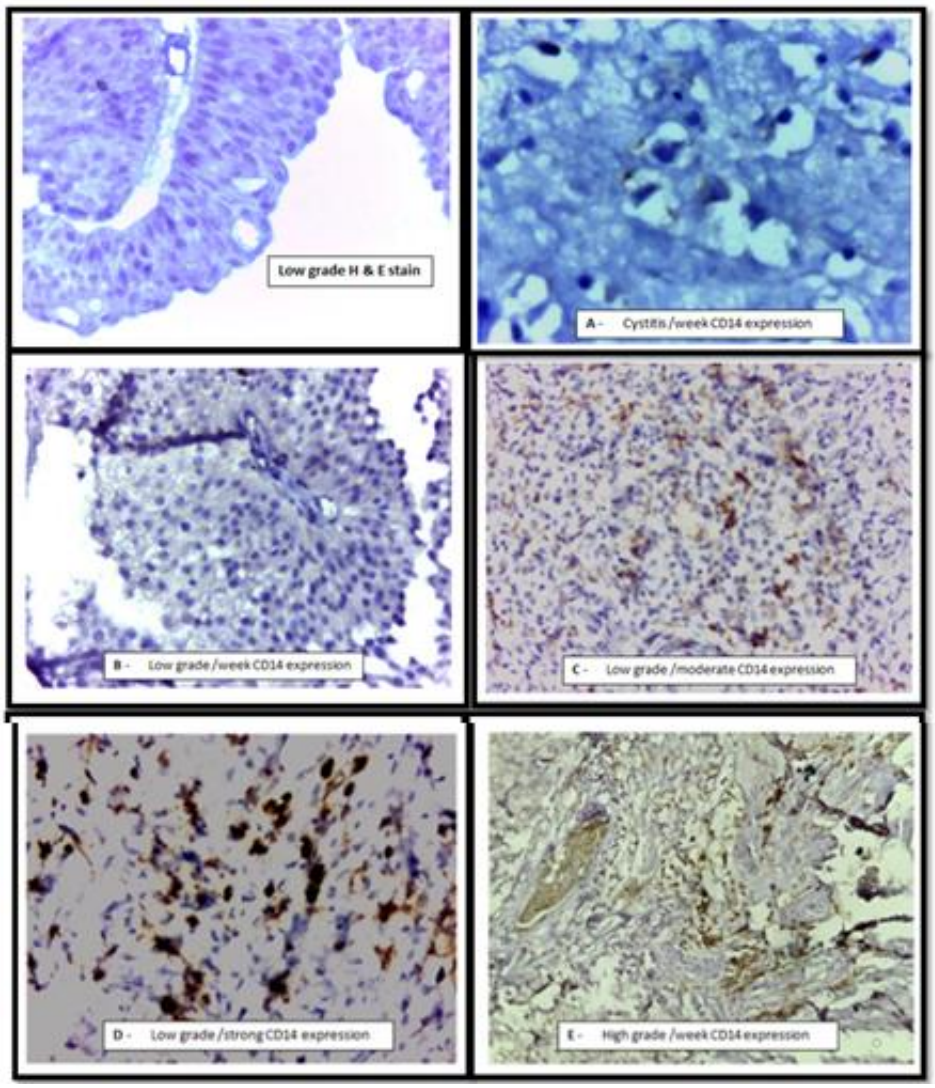
The current study, focused on the important correlation between bladder cancer (L.G / H.G) and cystitis correlations in terms of CD14 expression, demonstrating that there was a significant correlation between low grade bladder cancer and cystitis with inverse significant correlation under ($p \leq 0.05$). While there was a non-significant correlation between high grade bladder cancer and cystitis with inverse correlation. (**Table 3-11**).

Table (3-11): Correlations of CD14 expression between bladder cancer (L.G / H.G) to cystitis

Correlations				
		cystitis/ CD14	L.G of BC /CD14	H.G of BC/ CD14
cystitis/CD14	Pearson Correlation	1	-.048-	-.327-
	Sig. (2-tailed)		.896	.356
	N	10	10	10
L.G of BC /CD14	Pearson Correlation	*-.048-	1	-.218-
	Sig. (2-tailed)	.896		.545
	N	10	10	10
H.G of BC/ CD14	Pearson Correlation	-.327-	-.218-	1
	Sig. (2-tailed)	.356	.545	
	N	10	10	10
* Significant correlation with an inverse relationship				

To explain the fact of the degree of immune response in cystitis instances should be connected with the CD14 expression, which acts as a

first step in reducing inflammation progressing to carcinoma and increasing cytokines and toll-like receptor secretion (Mousa *et al*., 2017). Moreover, the inverse correlation of CD14 expression between pathological bladder cases indicates the degree and strength of the patient's bladder tissue defense cells, which then work to redirect the type of immune response through the secretion of growth factors and specialized cytokines, which suppressor or strengthen anti-tumor function and control the inflammatory state (Greten *et al*., 2019).



Figur (3-12) : Photomicrograph for bladder tissue with H&E stain. (A,B,C,D,E) show intensity of CD14 expression in bladder cancer type and cystitis. (A,B,E): week expression in cystitis, L.G and H.G. (D): strong expression in L.G

In response to bacterial invasion, the bladder tissue has a defensive mechanism that makes blood vessels more permeable. This attracts immune cells, such as macrophages, D.Cs, and other lymphocytes, which then start to express specific toll like receptors and cluster differentials on their surfaces to engulf the agent. During this process, certain bacteria enter the bladder's deep layers and spread their pathogenicity by altering immune pathways, which developed an inflammatory condition into cancer (Livia and Molly , 2020).

Finally, the results of the current study showed conclusive evidence for the participation of CD14 as a co-receptor with toll like receptors to synergize and achieve the immune pathway depending on the type of bacteria causing infection when compared with the immune markers included in present study in terms of significance. Regarding to the role of CD14 in secretion of interleukins with or without a bacterial stimulus such as LPS and thus affecting the bladder as a protumorigenic factor with contributed TLRs to trigger the NF-kB signaling cascade , pro-inflammatory cytokine and anti-appoptotic in tumor growth and cancer cell proliferation have been achieve (Moghadam and Nowroozi , 2019).

Conclusions and Recommendations

Conclusion:

1. There is high relationship between bacterial infection and induce or a compound with bladder cancer .
2. Innate immunity, which is represented by TLR-4, TLR-2, and CD14, as well as a specialized response with elevated blood Th17 level, were both appeared in the current study as immune responses to bladder cancer with bacterial infection.
3. The immune system exhibits both anti-tumor and anti-immune suppressor behavior, depending on the severity of bacterial pathogenesis.
4. CD14 expression in bladder tissues and correlated its intensity in relations with Low grade bladder cancer which indicate to the beginning of local immune response**.**

Recommendations:

1. Infiltrating immune cells in relation to bladder tumor prognosis could be investigated in future studies.
2. All TLRs type should be considered in bladder cancer study especially to diagram high or low risk tumor.
3. Genetic manipulation for immunological pathway in activation of anti-tumor effect in case of infection.
4. TLR-2 very important in current study so that, larger group was study could be included, its relationship to other microorganism in patient with bladder cancer.
5. Bacterial infection with *Staphylococcus saprophyticus* and its correlation with tumor could be studied furthermore and its relation to free radiation.

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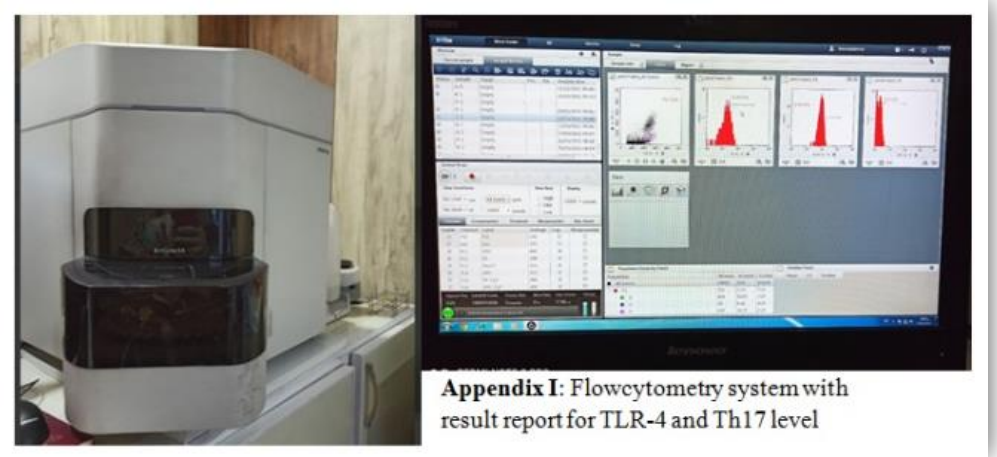
Appendix



Appendix I: Flowcytometry system with result report for TLR-4 and Th17 level

Appendix II: A/ VTEK2 report for *E.coli*

bioMérieux Customer:
CDT System #:

Laboratory Report

Printed March 13, 2021 09:32
Printed by: System

Patient Name: 64, Huyam
Isolate: 71020201-1 (Approved)

Patient ID: 71020201

Card Type: GN Bar Code: 24111432035418000 Testing Instrument: 00001B1B47B6 (19528)
Setup Technologist: Laboratory Administrator(Labadmin)

Bionumber: 0405610554526610
Organism Quantity: Selected Organism: Escherichia coli

Comments:	

Identification Information	Card: GN	Lot Number: 2411143203	Expires: Jan 12, 2021 12:00 CST
	Completed:	Status: Final	Analysis Time: 4.83 hours
Organism Origin	VITEK 2		
Selected Organism	98% Probability Escherichia coli Bionumber: 0405610554526610		Confidence: Excellent identification
SRF Organism			
Analysis Organisms and Tests to Separate:			
Analysis Messages:			
Contraindicating Typical Biopattern(s)			

Biochemical Details																			
2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	+
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	-	37	MNT	-	39	5KG	+
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	(-)
46	GlyA	-	47	ODC	+	48	LDC	+	53	IHISa	-	56	CMT	+	57	BGUR	+
58	O129R	+	59	GGAA	-	61	IMLTa	-	62	ELLM	-	64	ILATa	-			

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline:
AES Parameter Set Name:

Therapeutic Interpretation Guideline:
AES Parameter Last Modified:

Appendix II: B/ VTEK2 report for *S.saprophyticus*

bioMérieux Customer:
CST System #:

Laboratory Report

Printed March 13, 2021 09:05
Printed by: System

Patient Name: 60, Huyam
Isolate: 151220206-1 (Approved)

Patient ID: 151220206

Card Type: GP Bar Code: 2421207103137420 Testing Instrument: 00001B1B47B6 (19528)
Setup Technologist: Laboratory Administrator(Labadmin)

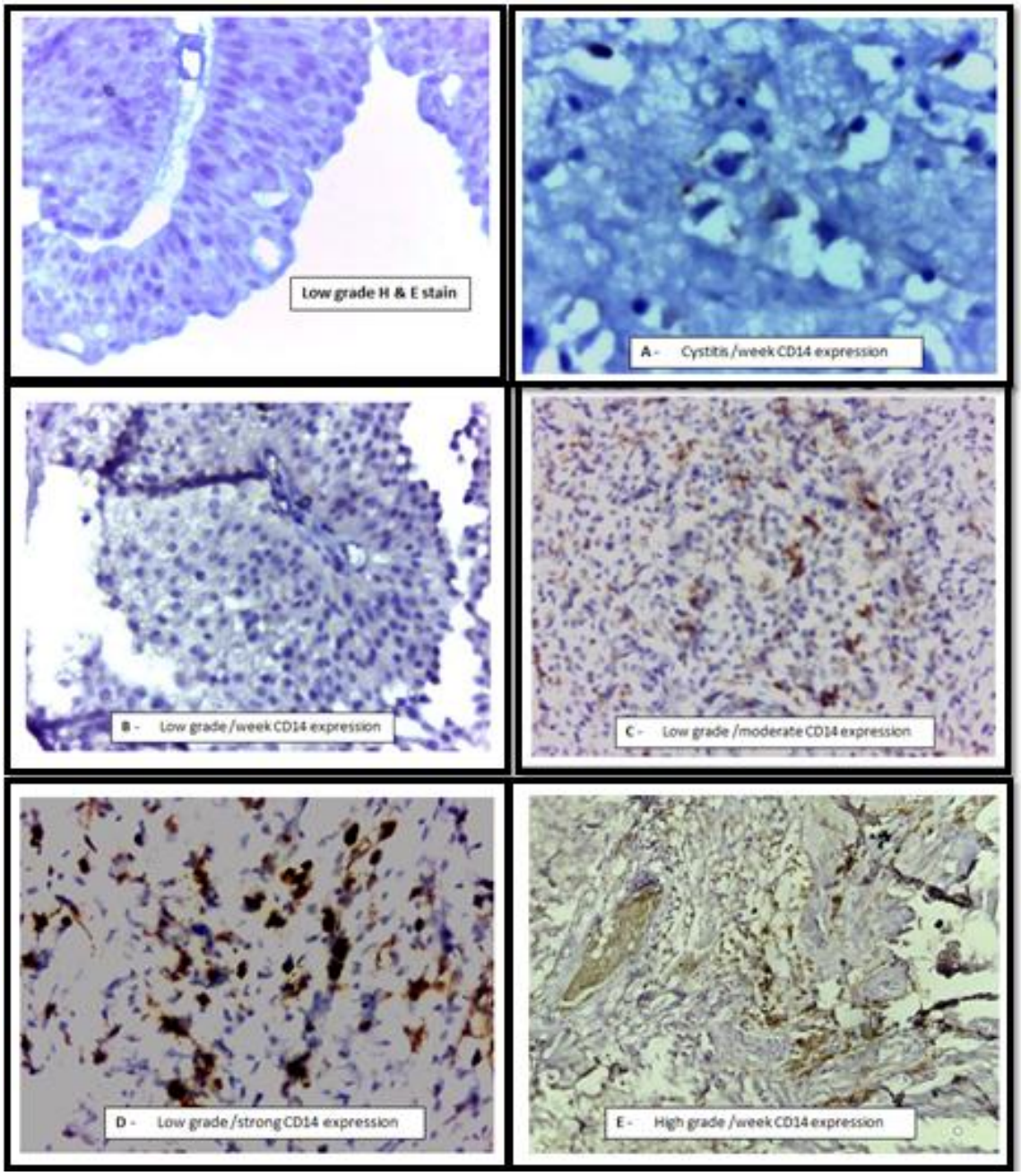
Bionumber: 0100000132702 31
Organism Quantity: Selected Organism: Staphylooooous saprophyticus

Comments:	

Identification Information	Card: GP	Lot Number: 2421207103	Expires: Mar 17, 2021 13:00 CDT
	Completed	Status: Final	Analysis Time: 4.83 hours
Organism Origin	VITEK 2		
Selected Organism	99% Probability Staphylooooous saprophyticus Bionumber: 0100000132702 31		Confidence: Excellent identification
SRF Organism			
Analysis Organisms and Tests to Separate:			
Analysis Messages:			
Contraindicating Typical Biopattern(s)			

Biochemical Details

2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	-	11	AGLU	-
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	-
20	LeuA	-	23	ProA	-	24	BGURr	-	25	AGAL	-	26	PyrA	-	27	BGUR	-
28	AlaA	-	29	TyrA	-	30	dSOR	-	31	URE	+	32	POLYB	-	37	dGAL	-
38	dRIB	+	39	ILATk	+	42	LAC	-	44	NAG	-	45	dMAL	+	46	BACI	-
47	NOVO	+	50	NC6.5	+	52	dMAN	+	53	dMNE	-	54	MBdG	-	56	PUL	-
57	dRAF	-	58	O129R	+	59	SAL	-	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															



Appendix III : Photomicrograph for bladder tissue with H&E stain. (A,B,C,D,E) show intensity of CD14 expression in bladder cancer type and cystitis

الخلاصة :

أجريت الدراسة الحالية للكشف عن البكتريا المصاحبة للمصابين بسرطان المثانة وتحديد الحالة المناعية من خلال الكشف عن التعبير للمعايير المناعية (TLR-2, TLR-4, Th17, CD14) وتقدير تراكيزها بالنسبة للمصابين مقارنة مع مرضى التهاب المثانة (مجموعة السيطرة) Cystitis. تضمنت الدراسة الحالية جمع ١٠٠ عينة (٧٠دم ، ادرار و ٣٠خزعة نسيجية) من المرضى الذين شخصت حالتهم بأنهم مصابين بسرطان المثانة والتهاب المثانة بواقع (٥٠) عينة لكل مجموعة من قبل الاطباء الاختصاص في العيادات الخاصة للفترة من آذار ٢٠٢١ ولغاية آذار ٢٠٢٢ .

وجدت الإصابة بسرطان المثانة لدى الجنسين إلا إن نسبتها لدى الذكور كانت أعلى من الاناث (٣٤) ٦٨% و(١٦) ٣٢% على التوالي ، وشملت الإصابة جميع الفئات العمرية الا إن الفئة (٥٦–٧١) عاماً هي الأكثر إصابة .

اجريت الدراسة البكتيرية على (٧٠) عينة ادرار (٣٠) حالة من المصابين بسرطان المثانة و (٤٠) حالة من الاشخصاص الذين يعانون من التهاب المثانة. نسبة العينات التي أعطت نتيجة موجبة للزرع البكتيري للمصابين بسرطان المثانة (٧) ٢٣.٤٠% في حين بلغت العينات التي لم تظهر نمو(٢٣) ٧٦.٦٠% ، مقارنة بنتائج المصابين بالتهاب المثانة كانت النسبة الموجبة للزرع البكتيري هي الأعلى بحدود(٢٨) ٧٠% . اذ تم عزل وتشخيص نوعين من البكتريا هي بكتريا اشريشيا القولون *E.coli* واحد اجناس بكتريا المكورات العنقودية *Staphylococcus saprophyticus* بنسب مختلفة لمجاميع الدراسة ، اذ كانت اعلى نسبة لبكتريا S. *saprophyticus* لمجموعة المصابين بسرطان المثانة (٤) ١١.٨% في حين كانت نسبة بكتريا *E.coli* اعلى لدى المصابين بالتهاب المثانة (١٤) ٣٥.١٠%. وقد كشفت الدراسة عن وجود حالات مزارع مفردة وثنائية (كلا الجنسين *E.coli, S. saprophyticus*) بنسب متباينة من اصل (٣٥) عينة اعطنت نتيجة موجبة للزرع البكتيري ، أذ وجدت نسبة المزرعة مفردة للانواع *E.coli* و *S. saprophyticus* (١٥) ٤٢.٥٠% ، (٦) ١٦.٩٠% على التوالي في حين سجلت المزرعة الثنائية نسبة ٤٠.٩٠% (١٤)

. واعتمادا عليها صنفت نتائج الزرع البكتيري الى اربعة مجاميع (عدم ظهور نمو ، مزرعة مفردة لل UPEC ، مزرعة مفردة للبكتريا المكورات العنقودية و مجموعة المزارع الثنائية) واعتمدت في الدراسة الحالية للمقارنة مع تراكيز المعايير المناعية (TLR-2, TLR-4, Th17).

بعد الكشف عن الاختبارات المناعية في ادرار و دم المصابين لمرضى لسرطان المثانة لوحظ هنالك تأثيراً معنوياً في زيادة تركيز المستقبل المناعي TLR-2 وعدم وجود فروق معنوية مع كلا من TLR-4, Th17 مقارنة بالمصابين بالتهاب المثانة التي اعطت نتائج عكسية بالنسبة للفروق المعنويه ولمختلف الفئات العمرية ، و تم تقدير تراكيز TLR-2, TLR-4, Th17 وعلاقتها بنتائج الزرع البكتيري للمصابين فبينت النتائج وجود فروق معنوية لتراكيز TLR-2 لدى المصابين بسرطان المثانة مع البكتريا الموجبة لصبغة غرام مقارنة بالتهاب المثانة على عكس مستويات TLR-4 التي اظهرت فروق معنوية لكلا من البكتريا الموجبة لصبغة غرام والمزارع المختلطة لدى المصابين بالتهاب المثانة . وكذلك لوحظ ارتفاع مستوى Th17 بفارق معنوي مع مزارع البكتريا السالبة لصبغة غرام والمزارع الخليطة في مجموعة المصابين بالتهاب المثانة.

تضمنت الدراسة أيضاً المناعة النسيجية من خلال تقدير مستوى التعبير ل CD14 لخزعات نسيج المثانة (٣٠) عينة من المصابين بسرطان المثانة بنوعيه عالي الدرجة وقليل الدرجة (L.G / H.G) مقارنة بنماذج التهاب المثانة بواقع (١٠) عينات لكل مجموعة. كان تعبير CD14 المناعي اعلى لفئة L.G بحدود (٧) ٧٠% في الوقت الذي كانت اقل نسبة للتعبير لدى فئة عالي الدرجة H.G (٢) ٢٠%. كما تم تحديد كثافة التعبير المناعي من خلال ايجاد النسبة المئوية لعدد الخلايا التي اخذت الصبغة وتصنيفها الى : ضعيف (اقل من ٢٥%) ، متوسط (٢٥-٥٠%) و قوي (اكثرمن ٥٠%)، اضافة الى تحديد نوع الارتباط للتعبير CD14 بين نوعي سرطان المثانة و التهاب المثانة اذ تبين وجود ارتباط معنوي ذو علاقة عكسية بين التهاب المثانة وسرطان المثانة للنوع قليل الدرجة .

وزارة التعليم العالي والبحث العلمي
جـــــــــــامعة بـــــــــابل
كـــــــــــلية الطب

التعبير المناعي لبعض السايتوكينات المرتبطة بالأصابة البكتيرية لدى مرضى سرطان المثانة

أطروحـــــــــــة

مقدمة الى مجلس كلية الطب/ جامعة بابل وهي جزء من متطلبات نيل درجة دكتوراه فلسفة في العلوم / الأحياء المجهرية الطبية

من قِبل

زينة شاكرخليل ابراهيم الهندي

بكـــــالوريوس /احياء مجهرية/ كلية العلوم للبنات/جامعة بابل (٢٠٠٦)

ماجـــــستير/ احياء مجهرية / كلية العلوم للبنات/جامعة بابل (٢٠١٢)

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