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MICROBIOME AND BLADDER CANCER

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DECLARATION

This thesis has been composed by myself and has not been used in any previous application for a degree. Throughout the text I use both 'I' and 'We' interchangeably.

All the results presented here were obtained by myself, except for:

1) **Sequencing and diversity microbial metrics in urine and tissues** – done in collaboration with Dr. Roberto Ferrarese and the Microbiology Department, IRCCS Ospedale San Raffaele (data presented in Figure 19 and 21).

2) **Sequencing and microbiome analysis** – done in collaboration with Dr. Michela Riba and the Center for Omics Sciences, IRCCS Ospedale San Raffaele (data presented in Figure 24 and 25).

Part of the results presented in this thesis have been published in two publications:

- Pederzoli F. *et al.*, "Sex-specific Alterations in the Urinary and Tissue Microbiome in Therapy-naïve Urothelial Bladder Cancer Patients", European Urology Oncology 2020 (reference 452 in the present thesis);
- Pederzoli F. *et al.*, "Is There a Detrimental Effect of Antibiotic Therapy in Patients with Muscle-invasive Bladder Cancer Treated with Neoadjuvant Pembrolizumab?", European Urology 2021 (reference 457 in the present thesis).

All sources of information are acknowledged by means of reference.

ABSTRACT

The microbiome has gained increasing momentum in cancer research, as it has become clear that microorganisms residing within our body are involved in mediating the cellular and tissue metabolism in health and disease. In bladder cancer research, there are different microbial communities that may mediate cancer pathobiology and response to therapy: the gut microbiome, the urinary microbiome, the urothelium-bound microbiome. These bacterial communities may mediate the processes of carcinogenesis or recurrence, modify the response to local intravesical therapies or influence the activity of systemic anticancer protocols.

Based on these premises, my research project aimed to unveil the urinary and urothelium-bound microbiome in therapy-naïve bladder cancer patients, describing the differently enriched bacterial communities using a sex-based stratification. Compared to healthy controls, I found that the urine of men affected by bladder cancer were enriched in the order *Opitutales* and subordinate family *Opitutaceae*, together with the isolated class *Acidobacteria-6*, while in female patients I found enriched the genus *Klebsiella*. Notably, the bladder cancer tissue was enriched in the genus *Burkholderia* in both men and women, when compared to non-neoplastic, paired urothelium biopsies. Then, I also characterized the gut microbiome of bladder cancer patients undergoing neoadjuvant pembrolizumab to understand if the intestinal bacteria may influence the immune-mediated anticancer activity. In this set, I have reported that antibiotic therapy has a negative effect on immunotherapy efficacy. Second, the gut microbiome of patients not responding to neoadjuvant pembrolizumab was characterized by a higher abundance of *Ruminococcus bromii*, while patients who showed a response were enriched in the genus *Sutterella*. Lastly, I started the implementation of *in vivo* and *in vitro* systems to test the mechanistic role of the bacteria identified in human samples.

This thesis work reported innovative data on the role of different microbial communities (urinary/urothelium-bound/fecal) in bladder cancer and bladder cancer therapy, and provided novel *in vivo* and *in vitro* models to validate those finding and uncover the complex microbiome-host cells crosstalk in bladder cancer patients.

1. Table of Contents

2. Acronyms and abbreviations

5-FU: 5-fluorouracil

95% CI: 95% confidence interval

AJCC: American Joint Committee on Cancer

ALA: aminolaevulinic acid

APC: antigen-presenting cell

APOBEC: apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like

ASIR: age-standardized incidence rate

ATB: antibiotic

ATM: ATM serine/threonine kinase

AUS: atypia of unknown significance

B2M: beta-2-microglobulin

Ba/Sq: basal/squamous

BCa: bladder cancer

BCG: Bacille Calmette-Guerin

BMI: body mass index

BRCA: Breast related cancer antigens

CD: cluster of differentiation

CDC2: Cyclin dependent kinase 1

CDH1: cadherin 1

CDKN2A: Cyclin dependent kinase inhibitor 2A

CDT: cytolethal distending toxin

CIS: carcinoma in situ

CPS: combined positive score

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

CT scan: computed tomography scan

CTLA-4: cytotoxic T-lymphocyte antigen 4

CXCL13: C-X-C Motif Chemokine Ligand 13

CXCR2: C-X-C Motif Chemokine Receptor 2

CyTOF: mass cytometry

DALYs: disability-adjusted life-years

DC: dendritic cell

DCE MRI: dynamic contrast-enhanced magnetic resonance imaging

ddPCR: digital droplet polymerase chain reaction

DMEM: Dulbecco's Modified Eagle Medium

DNA: deoxyribonucleic acid

DWI: diffusion-weighted imaging

EAU: European Association of Urology

EBV: Epstein-Barr virus

ECOG PS: Eastern Cooperative Oncology Group performance status

EPIC: European Prospective Investigation into Cancer and Nutrition

EQUC: expanded quantitative urine culture

ER: estrogen receptor

ERBB2: extracellular domain human epidermal growth factor 2

ERK: extracellular signal-regulated kinase

EU: European Union

EZH2: enhancer of zeste homolog 2

FBS: fetal bovine serum

FDA: Food and Drug Administration

FFPE: formalin-fixed paraffin-embedded

FGFR3: fibroblast growth factor receptor 3

FISH: fluorescence in situ hybridization

FMT: fecal microbiome transplant

FOBT: fecal occult blood test

FOXC2: forkhead box C2

GATA2: GATA-binding factor 2

GATA3: GATA binding protein 3

GM-CSF: granulocyte-macrophage colony-stimulating factor

GSTM1: Glutathione S-Transferase Mu 1

GSTP1: Glutathione S-transferase P1

HAL: hexaminolevulinate

HOXA13: Homeobox A13

HR: hazard ratio

HRAS gene: Harvey Rat sarcoma virus gene

IARC: International Agency for Research on Cancer

ICOS: inducible T-cell costimulatory

IFN: interferon

IgA: immunoglobulin type A

IGFBP5: Insulin Like Growth Factor Binding Protein 5

IL: interleukin

IMRT: intensity-modulated radiotherapy

IQR: interquartile range

irAEs: immune related adverse events

ISUP: International Society of Urological Pathology

ITSM: immunoreceptor tyrosine-based switch motif

IVIS: in vivo imaging system

JAK1/2: Janus kinases 1/2

KDM6A: lysine demethylase 6A

KMT2D: lysine methyltransferase 2D

KRAS gene: Kirsten rat sarcoma virus gene

LDA: linear discriminant analysis

LEfSe: linear discriminant analysis effect size

LPS: lipopolysaccharide

LumNS: luminal nonspecified

LumP: luminal papillary

LumU: luminal unstable

MALT: mucosa-associated lymphoid tissue

MAPK: mitogen-activated protein kinase

MCR: multivariable Cox regression

MDK: Midkine

MDSC: myeloid- derived suppressor cell

MEK: alias for Mitogen-Activated Protein Kinase Kinase (MAP2K)

MHC: major histocompatibility complex

MIBC: muscle invasive bladder cancer

MIP-1β: Macrophage inflammatory protein 1β

MLH1: MutL Homolog 1

MLR: multivariable logistic regression

MMC: mitomycin C

MRI: magnetic resonance imaging

mRNA: messenger ribonucleic acid

MYD88: myeloid differentiation primary response 88

NAT2: N-Acetyltransferase 2

NBI: narrow band imaging

NCCN: National Comprehensive Cancer Network

NE: neuroendocrine

NGS: next-generation sequencing

NK: natural killer

NMIBC: non-muscle invasive bladder cancer

NMP22: nuclear matrix protein 22

Npl: neoplastic

OD: odd ratio

OTU: operational taxonomic unit

PBS: phosphate buffer solution

PCA: principal component analysis

PCR: polymerase chain reaction

PD-1: programmed cell death protein 1

PD-L1/2: programmed death-ligand 1/2

PDD: photodynamic diagnosis

PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha

PRR: pattern-recognition receptor

PTEN: phosphatase and tensin homolog

PUNLMP: papillary urothelial malignancy of low malignant potential

QIIME: Quantitative Insights into Microbial Ecology

RB1: RB transcriptional corepressor 1

RC: radical cystectomty

RECIST: Response Evaluation Criteria In Solid Tumors

RFS: relapse-free survival

ROC: receiver operating characteristic

rRNA: ribosomal RNA

RUNX3: Runt-related transcription factor 3

SCFAs: short-chain fatty acids

SEER: Surveillance, Epidemiology, and End Results

SHP-2: Src homology region 2 domain-containing phosphatase-2

SNAIL: Zinc finger protein SNAI1

Spp.: species

STAG2: stromal antigen 2

STK11/LKB1: serine/threonine kinase 11/liver kinase B1

TACC: transforming acidic coiled-coil containing protein

TAMC: tumour-associated myeloid cell

TCR: T cell receptor

TERT: telomerase reverse transcriptase

TGFβ: transforming growth factor beta

TLR: toll-like receptor

TMB: tumor mutational burden

TME: tumor microenvironment

TNF-α: tumor necrosis factor-α

TNM system: Tumor-Node-Metastasis system

TP53: Tumor Protein P53

TURB: transurethral resection of bladder tumors

UK: United Kingdom

US: ultrasound

USA: United States of America

VEGF: vascular endothelial growth factor

VHL: Von Hippel-Lindau

WHO: World Health Organization

YLDs: years of healthy life lost due to a disability

YLLs: years of life lost

ZEB1: Zinc Finger E-Box Binding Homeobox 1

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4. Introduction – Principles of anatomy of the urinary bladder

The urinary bladder is a hollow and elastic organ located in the human pelvis, with the main function of working as a reservoir for urine collection (**Figure 1**). The shape, position and anatomical relations with surrounding structures depend greatly on its filling volume; when empty, its shape resembles somehow a tetrahedron, with a fundus (base), a neck, an apex, a superior surface and two inferolateral surfaces.

4.1 Anatomic relations

The bladder fundus is triangular in shape and is related to the anterior vagina in women, and to the rectum in men. The bladder neck, which essentially corresponds to the internal urethral orifice, is located more inferiorly than the fundus, 3-4 cm behind the lower portion of the symphysis pubis. In men, the bladder neck sits on the base of the prostate, while in women it is related to the pelvic fascia. The anterior bladder surface is divided from the transversalis fascia by fat in the retropubic space of Retzius, while the superior bladder surface is bounded by lateral borders from the apex to the *ostia* of the ureters. In men, the superior surface is completely covered by the peritoneum and is related to the sigmoid colon and the most caudal ileal coils. In women, the superior surface is largely covered by the peritoneum, and it is related to the uterus. At birth, the bladder is located more cranially than during adulthood, as the true pelvis is shallower. Then, the bladder progressively moves downwards with growth, reaching the adult location at puberty.

4.2 Bladder interior

The vesical urothelial mucosa is only loosely attached to the detrusor muscle laying below for its vast majority, so that the mucosa could stretch when the bladder is filled, while it folds when it is empty. Instead, the mucosa is firmly attached to the underlying bladder wall over the trigone, immediately above and behind the internal urethral orifice, and it appears always smooth independently from the bladder's filling. At the trigone, the muscle bundles are organized into two layers, the superficial trigonal muscle and the deep trigonal detrusor muscle. The superficial trigonal muscle is a distinct structure, formed by small-diameter bundles that are in continuity with the musculature of the intramural ureters proximally. Moreover, the superficial trigone muscle fuses, at the level of the ure-

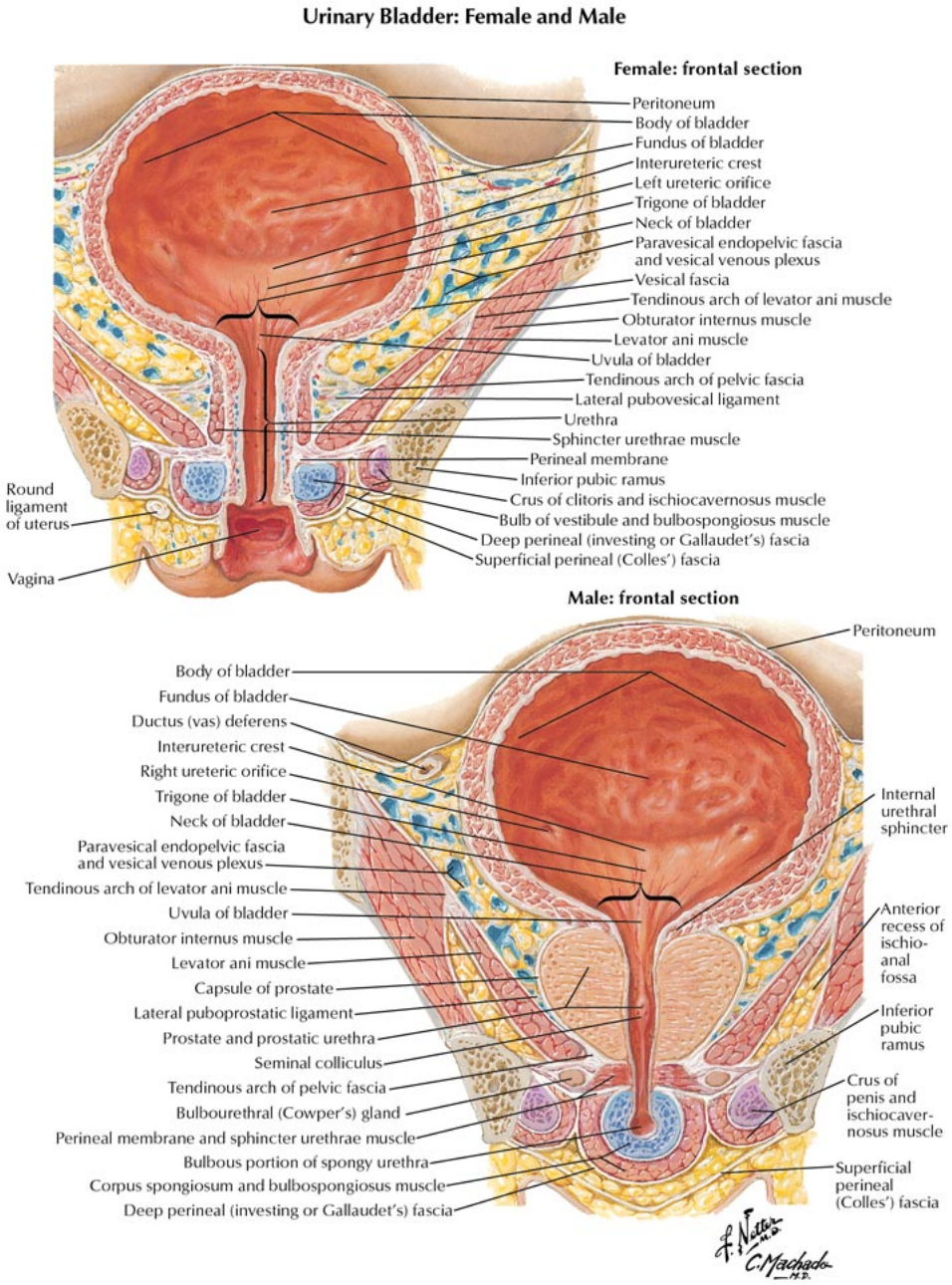


Figure 1 – Main anatomical relationships of the urinary bladder in the female and male pelvis. 2021. Used with permission of Elsevier. All rights reserved.

thral orifice, with the muscle layer of the proximal urethra. As far as the deep trigonal detrusor muscle is concerned, it is simply the posteroinferior portion of the proper detrusor muscle. At the posterolateral angles of the trigone, there are the ureteric *ostia*, which are ~2.5 cm apart in an empty bladder. Instead, the internal urethral orifice is located at the trigonal apex, the lowest part of the bladder.

The bladder neck has histologically and pharmacologically distinct smooth muscle fibers. In women, the bladder neck is formed by distinct, small-diameter, muscle fascicles that extend longitudinally or obliquely into the wall of the urethra. The female bladder neck is supported mostly by the pubovesical ligaments, the endopelvic fascia and the levator ani muscle, altogether contributing to the regulation of urinary continence and micturition. In men, the bladder neck is wrapped around by a round-shaped collar of smooth muscle fibers arranged in thin bundles and separated by a large connective tissue stroma, independently innervated by their own nerve fibers. The bladder neck constitutes what is generally called the "proximal sphincter mechanism" or "preprostatic sphincter". The preprostatic sphincter is richly innervated by sympathetic noradrenergic fibers, and it is almost totally devoid of parasympathetic cholinergic fibers. It is not involved in continence, but it allows antegrade ejaculation of the semen, preventing retrograde ejaculation into the bladder.

4.3 Vascular supply, lymphatic drainage, and innervation

The arterial blood supply to the bladder is mostly provided by the superior and inferior vesical arteries, deriving from the anterior trunk of the internal iliac artery (**Figure 2**). The superior vesical artery supplies the bladder fundus and, through some branches, the vas deferens and the ureter. The inferior vesical artery supplies the bladder base, lower ureter, and the prostate and seminal vesicles in men. In women, additional branches to the bladder derive from the uterine and vaginal arteries. The complex venous plexus draining the bladder lays on the inferolateral surfaces, eventually ending in the internal iliac veins. The lymphatic drainage, through the mucosal, intermuscular and serosal plexuses, mostly leads to the external iliac nodes (some drainage may go to the internal iliac and common iliac nodes, or to the nodes in the obturator fossa from the inferolateral surfaces of the bladder). The nerves supplying the bladder derive from the pelvic plexuses and consist of both sympathetic and parasympathetic fibers (**Figure 3**).

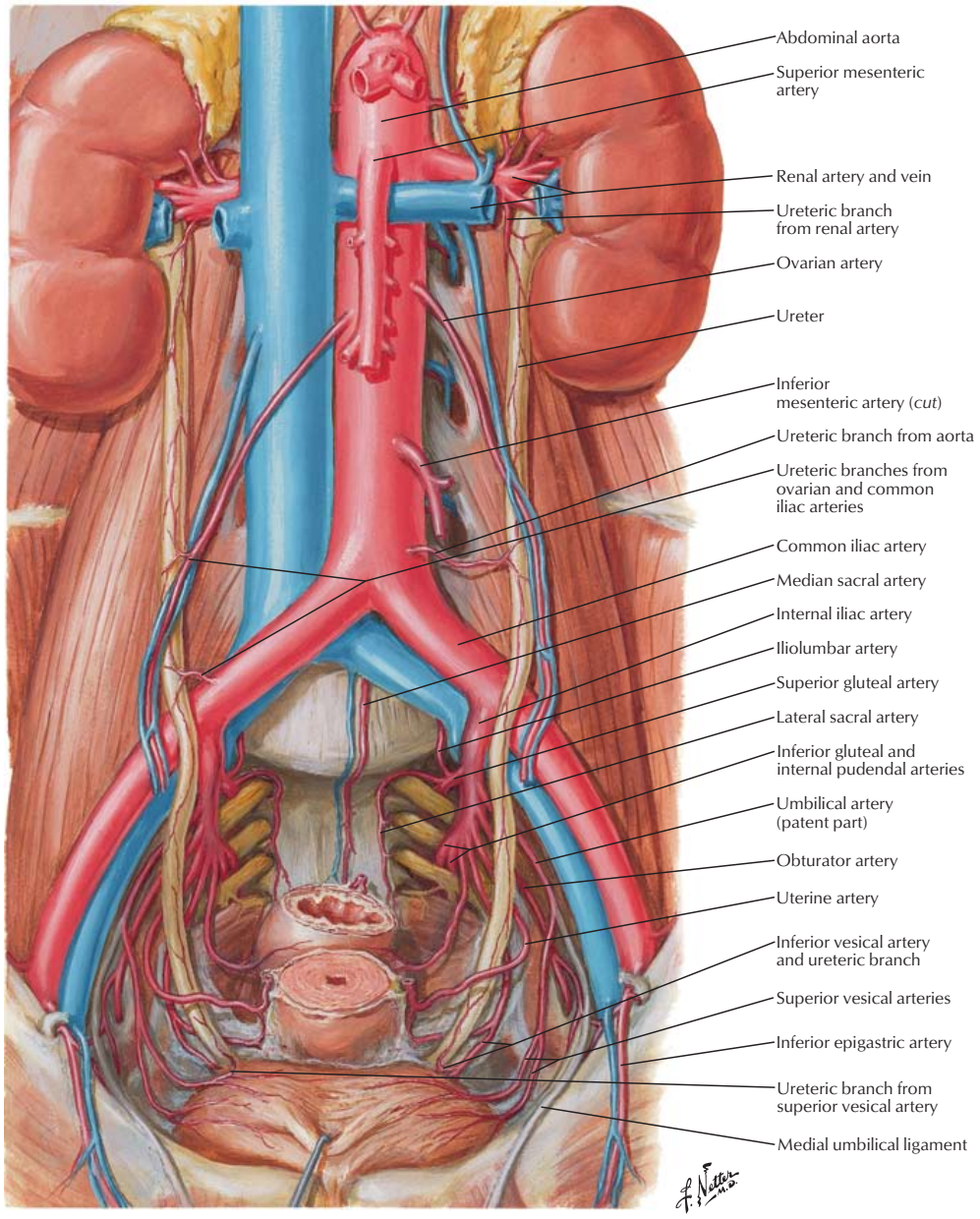


Figure 2 – Vascular anatomy of the female urinary bladder. 2021. Used with permission of Elsevier. All rights reserved.

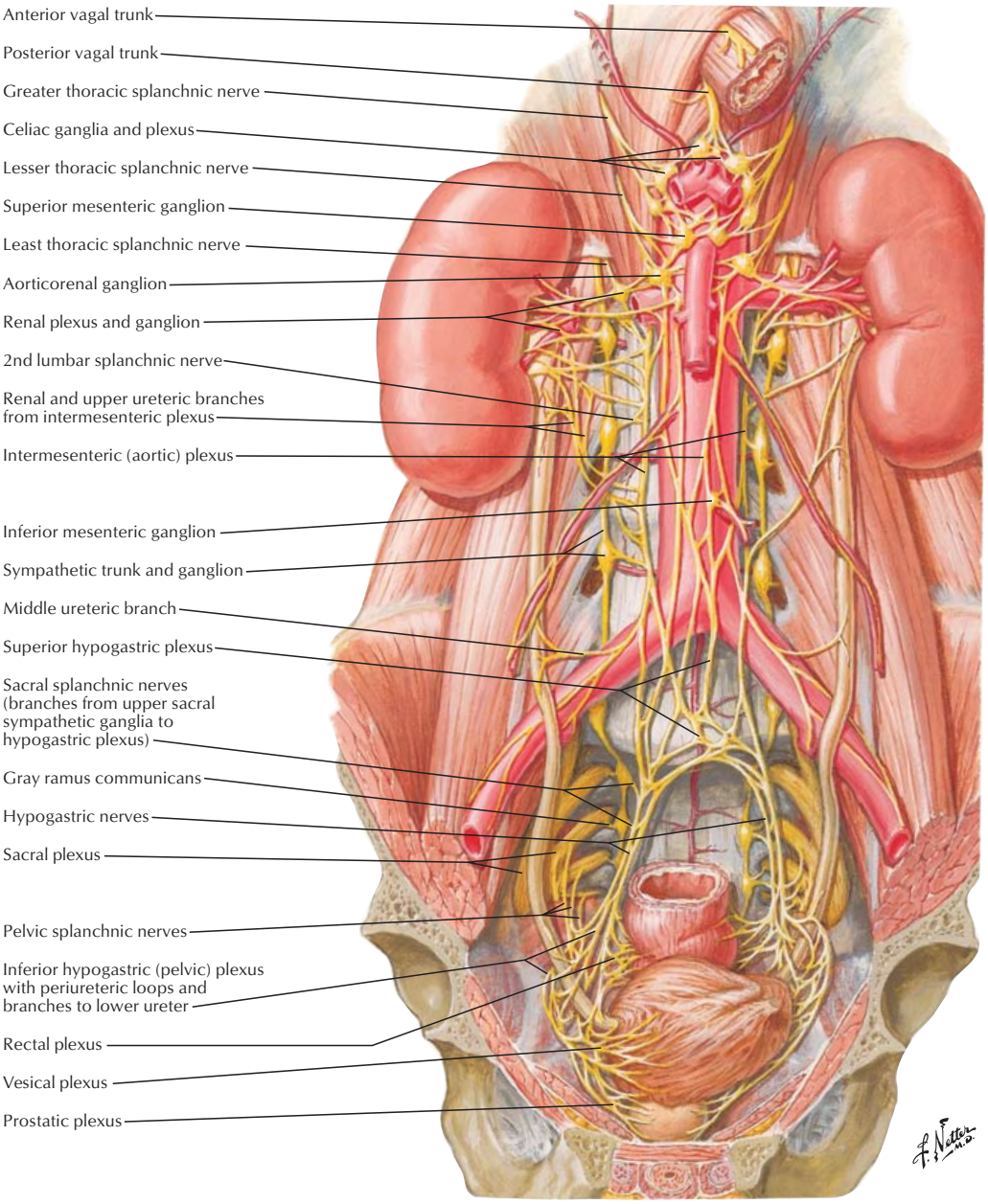


Figure 3 – Innervation of the male urinary bladder. 2021. Used with permission of Elsevier. All rights reserved.

5. Introduction – Epidemiology and pathology of bladder cancer

Bladder cancer represents a global threat, with around 500,000 new cases and 200,000 deaths worldwide each year[1,2]. Over the years, it has become clear that the general term "bladder cancer" encompasses a heterogenous spectrum of diseases, ranging from non-invasive but highly recurrent tumors managed mostly conservatively, to invasive malignancies requiring aggressive and multidisciplinary approaches. Altogether, the long-lasting and complex management of bladder cancer makes it the most expensive tumor to treat per patient over patients' lifetime ($129,000-$251,000 treatment cost/patient)[3]. In the European Union (EU), bladder cancer cost €4.9 billion in 2012, with health care accounting for €2.9 billion (59%)[4]. In other words, the EU spent €57 per 10 EU citizens for bladder cancer healthcare, with costs varying significantly between countries. Indeed, Bulgaria showed the lowest cost (€8 per 10 citizens), while Luxembourg the highest (€93 per 10 citizens).

In the following sections, I will provide an overview of the most important data about the epidemiology, pathology, and molecular pathophysiology of bladder cancer, setting the background for my work.

5.1 Epidemiology

In the course of a lifetime, the risk of being diagnosed with bladder cancer is about 1.1% in men and 0.27% in women[1]. While age-adjusted incidence decreased in both men and women in the 1990s and early 2000s, this decline has currently reached almost a plateau (**Figure 4**). Conversely, unadjusted incidence rates of bladder cancer grew by 31% between 2005 and 2015, an effect probably due to the increasing life expectancy and the derived increasing aging of the population[5]. In 2013, men living in developed countries had the highest age-standardized incidence rate (ASIR) of bladder cancer (23.6), much higher than the ASIR for women living in the same countries (4.8), and altogether higher than ASIRs in developing countries (5.3 for men, 1.4 for women)[6]. Other studies[5,7] confirmed geographic variations in bladder cancer incidence and mortality around the world, with 55% of all cases and 43% of all deaths occurring in 20% of the world population living in developed countries (identified as countries with a very high Human Development Index)[8]. However, this gap in incidence between high- and low-income countries has been narrowing over the years, potentially due to the increasing life expec-

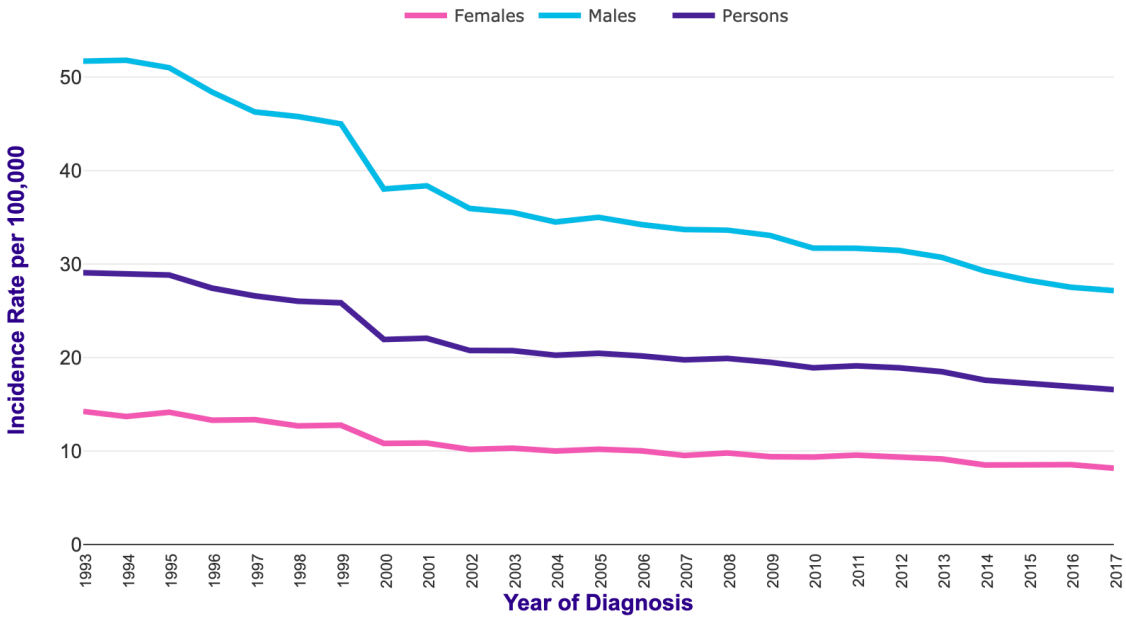


Figure 4 – European age-standardized incidence rates of bladder cancer (ICD-10 C67) in the United Kingdom from 1993 to 2017 (from Cancer Center UK: Bladder cancer incidence statistics, 2021; available online at https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/bladder-cancer/incidence#ref-2).

tancy for people living in these countries, thus increasing the overall aging of the population even in developing countries.

In 2015, bladder cancer was the cause of 3.4 million disability-adjusted life-years (DALYs; i.e., the primary metrics implemented by the World Health Organization to measure the burden from mortality and morbidity caused by a certain disease[9]), with 92% DALYs deriving from the years of life lost due to premature mortality (YLLs) and 8% DALYs from the years of healthy life lost due to disability (YLDs)[5]. Overall, 5-year bladder cancer relative survival rate in the United States is around 77%, but survival rates are largely influenced by disease stage. Using the Surveillance, Epidemiology, and End Results (SEER) database and its classification of disease stages (classification that does not follow the American Joint Committee on Cancer (AJCC) TNM system), the 5-year relative survival rate is 69% for localized disease, 37% for locally advanced tumors (i.e., tumors that extend through the bladder to the surrounding tissue or have spread to nearby lymph nodes or organs), and 6% for tumors metastatic to distant organs[10].

Regional variations in bladder cancer incidence and mortality can be appreciated even between European countries[7]. In men, the highest incidence rates were observed in Southern Europe countries, in particular Spain (age-standardized incidence rate, 36.7/100,000) and Italy (age-standardized incidence rate, 33.2/100,000). Moreover, male incidence rates have been increasing in Southern, Central, and Eastern Europe over the last 30 years, while they have been decreasing in Western and Northern Europe (except for the Baltic countries). Mortality rates were the highest in Eastern and Southern European countries (Spain, age-standardized mortality rate, 8.2/100,000) and in the Baltic area (Latvia, age-standardized mortality rate, 7.5/100,000). In general, mortality rates decreased over time in many European countries, with some exceptions in Southern (Slovenia, Croatia), Eastern (Bulgaria) and Baltic countries. Regional differences can also be appreciated for European women. Indeed, the highest incidence rates were recorded in Northern Europe (Denmark, age-standardized incidence rate, 8.4/100,000; Norway, age-standardized incidence rate, 6.4/100,000). Differently from men, incidence rates increased in women over the last 30 years in most European regions except for Northern Europe. Similar to the incidence, the highest mortality rate was registered in Denmark (age-standardized mortality rate, 2.3/100,000). Overall, mortality rates generally decreased all over Europe over the last 30 years.

Bladder cancer can be considered, in a simplified way, a disease of aging and of chronic exposure to external environmental *noxa*. Therefore, it comes with no surprise that its incidence and prevalence increase with age (average age of diagnosis in the USA: 73 years)[11]. Moreover, bladder cancer is more frequent in men than women, and its incidence is 3-4 higher in men vs. women. The potential importance of those sex-based differences will be discussed in detail in a following section. Caucasians are more likely to be diagnosed with bladder cancer than African-Americans, Native Americans or Hispanic Americans, but African-Americans are more likely to present with locally invasive tumors and with non-urothelial histologies compared to the other groups[12].

5.2 Etiology and risk factors

Like other body organs and tissues exposed to the external environment, the main risk factors are linked to exposure to external *noxa*.

Tobacco smoking (cigarette, pipe, cigar) represents the most important cause of bladder cancer, and smokers have a 2x-3x greater lifetime risk of developing bladder cancer than non-smokers[13–15]. Moreover, the incidence of bladder cancer is proportional to the duration of smoking and the number of daily cigarettes, and the risk of developing bladder cancer remains higher for former smokers for many years when compared to never-smokers[16,17]. However, quitting smoking decreases the relative risk of getting bladder cancer proportionally to the time spent from the last cigarette smoked: smokers who quitted from 1-3 years show a 2.6 relative risk of being diagnosed with bladder cancer, relative risk that decreases to 1.1 after 15 years from quitting smoking[18]. Active smoking at bladder cancer diagnosis increases the risk of local recurrence in patients with non-invasive tumors and of cancer-specific death in patients with invasive disease. While there are no univocal data about the potential cancer-specific survival benefit from quitting smoking after bladder cancer diagnosis, smoking cessation is of paramount importance to decrease the risk of concurrent onset of other smoking-related malignancies and diseases.

Occupational exposure to carcinogenic products is the second most important cause of bladder cancer, accounting for up to 20% of all cases[15]. The most dangerous substances are aromatic amines, used in the production of tobacco, dyes and rubber, and polycyclic aromatic hydrocarbons[19].

Obesity and metabolic syndrome are well-known risk factors for many oncological diseases, including bladder cancer[20,21]. In a large prospective study conducted in Europe, the Metabolic syndrome and Cancer project (Me-Can 2.0), metabolic aberrations, especially elevated blood pressure and triglycerides, were linked to an increased risk of bladder cancer among men, whereas high body mass index (BMI) was associated with decreased risk of developing bladder cancer[22]. Although the precise mechanisms are largely unknown, the pathobiological link between metabolic syndrome and bladder cancer probably resides in a combination of chronic inflammation, insulin resistance and hyperinsulinemia, and increased steroid hormone bioavailability.

Bladder schistosomiasis, an infectious disease caused by parasitic flatworms called schistosomes, remains a major cause of bladder cancer in developing countries. Indeed, schistosomiasis preventive measures were needed for at least 237,000,000 people worldwide in 2019[23]. Of the four species that infect humans, the *S. haematobium* is the only one linked to bladder carcinogenesis (especially squamous cell bladder cancer). The pathogenesis of schistosomiasis-induced bladder cancer is linked to the establishment of a chronic infection by *S. haematobium*, which is able to live in the venules of the bladder wall, laying its eggs and causing continuous inflammation, tissue fibrosis and a TH-2 type pro-inflammatory immune response[24]. With a pathobiological mechanism like schistosomiasis, chronic urinary tract infections have been associated with an increased susceptibility to bladder cancer[25,26]. Nevertheless, no perspective study has tested this hypothesis yet, and recent studies have not confirmed the association[27].

Several medical conditions can be linked to bladder carcinogenesis either directly, usually through a mechanism of chronic inflammation at the level of the urothelium causing keratinizing squamous metaplasia and then bladder cancer (e.g., bladder calculi), or indirectly, because of their treatment (e.g., oncological treatment with cyclophosphamide for another malignancy). For instance, a meta-analysis of case-control and cohort studies showed a positive association between urinary calculi and the development of bladder cancer[28]. Another known pathological condition that increases the risk of bladder carcinogenesis is bladder exstrophy, showing an incidence of bladder cancer (especially adenocarcinoma of the bladder) around 5% in the whole exstrophy population[29,30]. Some pharmaceutical drugs may promote bladder cancer development as a side effect. In this setting, a well-known example is represented by the chemotherapeutic agent

cyclophosphamide, which can cause bladder cancer through its mutagenic metabolite phosphoramide mustard[31]. Patients who received cyclophosphamide have a 4.5x risk of developing bladder cancer, and the risk appears to be dose-dependent, and with a peak between years 10-14 from cyclophosphamide administration[32,33]. Thiazolidinediones (pioglitazone and rosiglitazone) are oral hypoglycemic drugs used to treat type 2 diabetes mellitus, and they have been associated with an increased risk of bladder cancer, risk that seems to depend on the dose and the duration of treatment[34].

Radiation exposure to the pelvis is another recognized risk factor for bladder cancer. Higher rates of secondary bladder malignancies following pelvic external-beam radiotherapy to treat gynecological or prostate tumors have been reported[35,36]. The risk of bladder cancer development linked to modern radiation therapy techniques, such as intensity-modulated radiotherapy (IMRT), is still unknown.

Several dietary factors have been investigated as potential risk or protective factors towards bladder cancer development. However, any conclusion in this setting is still controversial. In the European Prospective Investigation into Cancer and Nutrition (EPIC) study, which aimed to examine the association between diet, lifestyle, environmental factors and cancer, no links between bladder cancer and fluid intake, red meat, vegetables, and fruit consumption were found. The only association found was an inverse association between dietary intakes of flavonols and lignans and the risk of bladder cancer, especially of aggressive disease[37].

Although the main risk factors for the development of bladder cancer are environmental, there are some hereditary and genetic factors that put affected individuals at higher risk of bladder tumors. The most studied single genes in bladder carcinogenesis are involved in the catabolism of aromatic amines (GSTM1 and NAT2), thus having a great influence on the cancer susceptibility following exposure to external carcinogens[38,39]. Of interest, NAT2 is also involved in the metabolism and inactivation of nitrosamines and other toxic compounds present in tobacco smoke. In addition to single genes, genome-wide association studies conducted on bladder cancer patients have identified some susceptibility loci associated with bladder cancer risk, for instance on chromosome 8q24[40,41]. An increasing number of studies are investigating potential genetic susceptibility factors that may increase the risk of developing bladder cancer.

A population-based study of cancer risk conducted in relatives of bladder cancer patients found an increased risk for first- and second-degree relatives, even if there is not a clear Mendelian inheritance pattern[42]. In this kind of studies, given the importance of external risk factors, it is always important to weight in the contribution of a shared environmental exposure as a confounding factor over genetic predisposition[43,44]. Nevertheless, it is reasonable to think that familiar genetic predisposition may contribute to the increased risk of developing bladder cancer by magnifying the effect of external, environmental risk factors.

5.3 Bladder cancer histopathology

The wall of the urinary bladder (**Figure 5**) can be divided into 4 different layers (described from the innermost):

- urothelium: also known as transitional epithelium, it is composed by overlying layers of urothelial cells, which appear as 5-7 layers in contracted bladder. Urothelial cells in contact with the underlying basement membrane (basal layer) are cylindrical in shape and often show longitudinal nuclear grooves, while urothelial cells above (intermediate layer) are mostly cuboidal, with well-defined borders and glycogen-rich, amphophilic cytoplasm, regular and ovoid nuclei with small nucleoli and finely granular chromatin. On top of the urothelium, there is a single layer of specialized cells called umbrella cells, which are large and elliptical, with abundant eosinophilic cytoplasm and often prominent nucleoli. Of note, one umbrella cell covers multiple underlying urothelial cells.
- lamina propria: separated from the urothelium by a thin basement membrane, it is formed by loose to dense connective tissue, blood vessels, lymphatic vessels, and adipose tissue. It also contains "wisps of smooth muscle"[45] that form largely discontinuous muscular structures known as muscularis mucosa.
- muscularis propria: also known as detrusor muscle, it consists of three concentrical layers of thick muscular bundles: a longitudinal innermost, a circular intermediate and an outermost longitudinal. These layers can be clearly distinguished only around the bladder neck, while in most of the bladder they are greatly intertwined. To distinguish the muscularis mucosa from the muscularis propria in difficult cases, anti-smoothelin, a marker of terminally differentiated

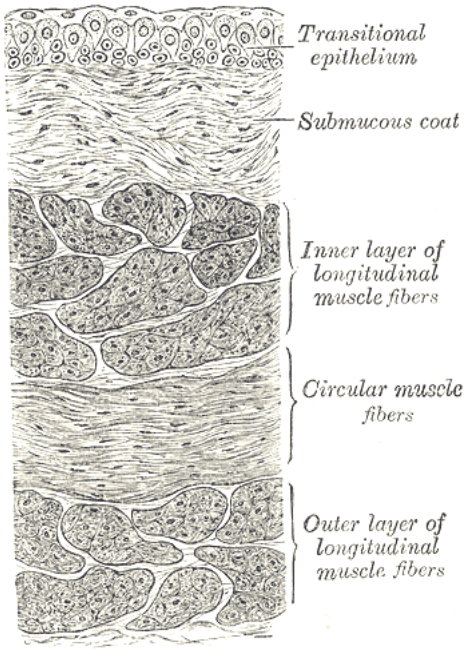


Figure 5 – Schematic representation of the histological appearance of a normal urinary bladder, in which it is possible to identify the urothelium or transitional epithelium, the submucosa and the three layers of muscularis propria. Public image available online at https://commons.wikimedia.org/wiki/File:Gray1141.png

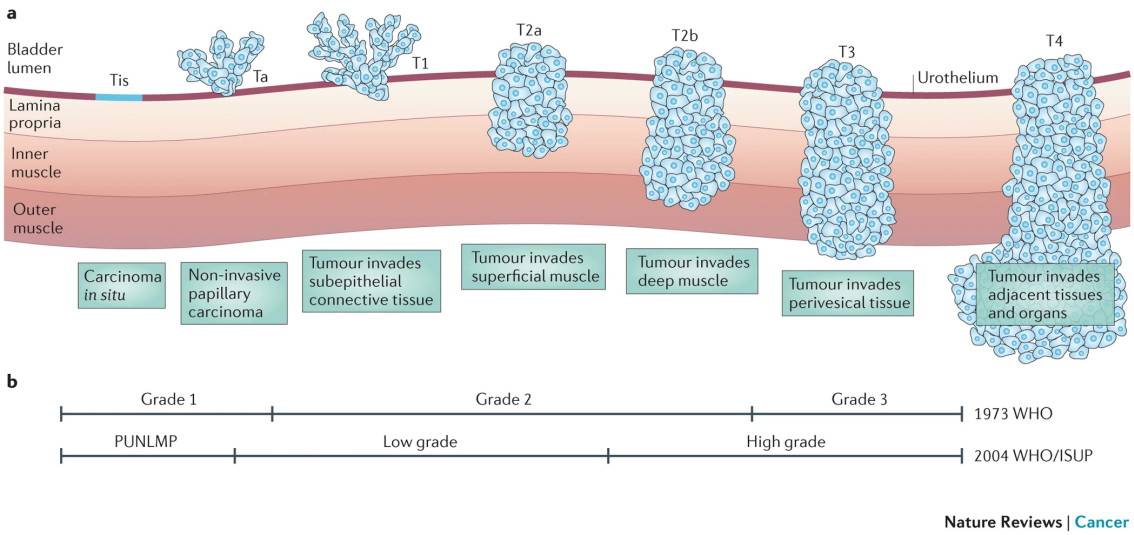


Figure 6 – Staging of bladder cancer according to the Tumour–Node–Metastasis (TNM) system is shown (**a**). Grading according to the 1973 World Health Organization (WHO) and 2004 WHO/International Society of Urological Pathology (ISUP) criteria is shown. The major difference is in the classification of papillary tumours, which are classified as grades 1, 2 and 3 in the older system and as papillary urothelial malignancy of low malignant potential (PUNLMP; equivalent to grade 1), low-grade papillary urothelial carcinoma or high-grade papillary urothelial carcinoma in the WHO/ISUP 2004 classification (**b**). (Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nature Reviews Cancer, "Molecular biology of bladder cancer: new insights into pathogenesis and clinical diversity" by Margaret A. Knowles *et al.*[46], ©2015 All rights reserved.)

smooth muscle, can be used (positive staining in the muscularis propria)[47].

- adventitia: also known as serosa, it is formed by a serosal layer outside the perivesical adipose tissue below the muscularis propria.

A clear appreciation of the different layers constituting the bladder wall is of fundamental importance to understand the pathologic tumor staging of bladder carcinoma, which is based on the extent of invasion deep into the layers of the bladder wall and surrounding organs (**Figure 6**). Indeed, the depth of invasion is one of the main features guiding the staging of bladder cancer based on the Tumor, Node, Metastasis (TNM) classification developed by the American Joint Committee on Cancer (AJCC).

5.3.1 Non-invasive urothelial lesions

Non-invasive urothelial lesions (**Figure 7**), either benign/premalignant or malignant, can be subdivided into flat or papillary (i.e., growing towards the bladder lumen) lesions:

→ dysplasia: in urothelial dysplasia, the normal thickness of the urothelium is maintained, as well as the general tissue organization, but the cytological appearance of urothelial cells is altered. The main cytological changes are loss of nuclear polarization, enlarged and darker nuclei, and loss of cytoplasmic clearing. The lamina propria is generally normal and urothelial cells are present. Although dysplasia is not a *bona fide* malignant lesion, it is suggestive of an unstable genomic environment that may favor recurrence or progression in patients with previous bladder cancer lesions. Indeed, up to 20% of patients with urothelial dysplasia will develop bladder cancer lesions, and in patients with a positive history for bladder cancer the presence of urothelial dysplasia is associated with development of carcinoma in situ (CIS) in ~60% of them[48–50].

→ urothelial hyperplasia: characterized by an increase in the number of urothelial layers, thus resulting in a thickening of the urothelium, without cytological changes. Flat hyperplasia may be a precursor lesion for low-grade papillary tumor, as it has been shown to harbor loss of heterozygosity at different sites (e.g., 3q22-q24, 5q22-q31, 10q26) and mutations in FGFR3[51,52].

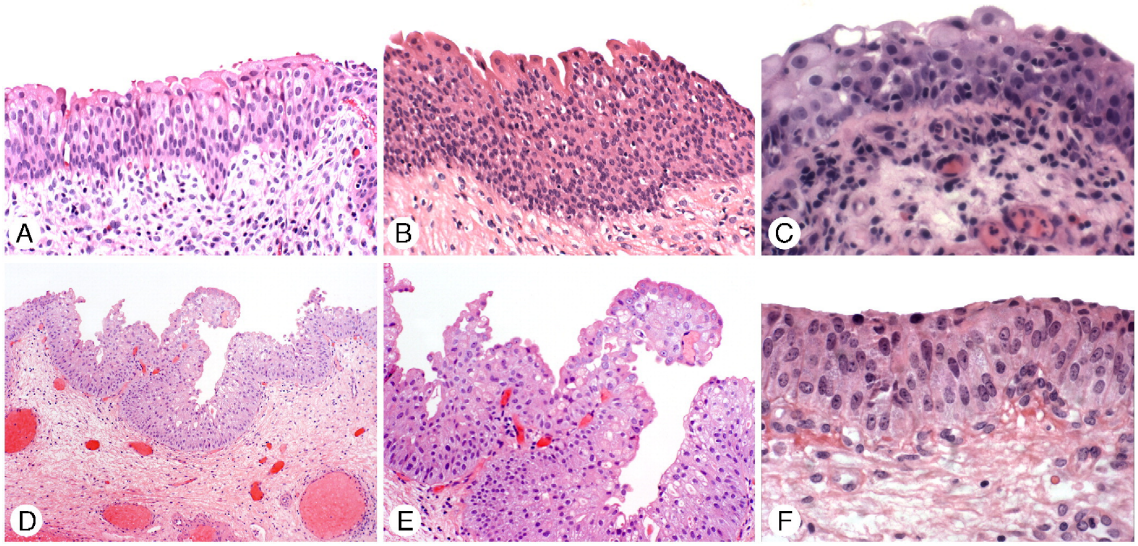


Figure 7 – Flat lesions of the urinary bladder. **A**. Normal urothelium. Note orderly maturation of the layers, with conserved polarity. **B**. Flat urothelial hyperplasia. Marked thickening of the urothelium is evident. Normal maturation from basal to superficial cells is maintained. Atypias and malignant mitoses are absent. **C**. Reactive, inflammatory urothelial atypia. Cells are uniformly enlarged. Some cells have prominent nucleoli. **D-E**. Papillary hyperplasia. There are folds of urothelium without fibrovascular cores, and cells maintain a benign appearance. **F**. Urothelial dysplasia. Several cytological changes appear, including loss of nuclear polarity, nuclear enlargement and nucleolar prominence. (Reprinted from Human Pathology, Kurt B. Hodges, "Urothelial dysplasia and other flat lesions of the urinary bladder: clinicopathologic and molecular features"[53], Copyright (2010), with permission from Elsevier)

→ reactive atypia: as the name suggests, this entity is characterized by cytologically abnormal urothelial cells in a context of generalized inflammation, but with a conserved tissue architecture. Patients with histologically diagnosed reactive atypia usually have a positive history for bladder inflammation (e.g., history of chronic urinary tract infections, recent endovescical chemotherapy, etc.). The areas of reactive atypia usually appear edematous and inflamed at the cystoscopy evaluation. Moreover, reactive atypia does not appear to be a premalignant lesion *per se*, even if it is often present together with dysplasia and/or CIS.

→ atypia of unknown significance (AUS): this term is used when it is not possible to discriminate whether the morphological changes in urothelial cells are reactive or dysplastic. AUS is generally linked to inflammatory signs in the lamina propria. AUS *per se* is not linked to a greater risk of bladder cancer development/recurrence, therefore its diagnosis is of limited clinical utility.

→ papillary urothelial neoplasm of low malignant potential (PUNLMP): PUNLMP is a papillary growth with minimal architectural abnormalities and minimal nuclear atypia[54]. Compared to papilloma, PUNLMP is thicker and with mildly larger and hyperchromatic nuclei. Mitotic figures are usually rare and, if present, are confined to the basal layer. About ¼ of patients with PUNLMP has a recurrence within 5 years from the first diagnosis, but recurrence is virtually always a PUNLMP or a low-grade lesion. Progression to invasive bladder cancer is very rare, and it is debated if that progression would be attributed to transformation of PUNLMP into an invasive and aggressive lesion or to the *ex novo* formation of an independent, invasive lesion[55,56]. At the molecular level, >50% of PUNLMP cases has alterations in TERT and/or FGFR3, in line with the molecular profile of low-grade lesions[57].

→ papilloma: the papilloma is a rare (around 1% of all urothelial lesions), exophytic bladder lesion, composed of thin papillary fronds covered by prominent umbrella cells. Papillomas occur mostly in patients <50 years of age and present as solitary lesions, with benign course (= no recurrence) if completely excised at first resection[58,59]. Macroscopically, it appears as a soft, pinkish, pedunculated urothelial growth with delicate papillary structures. Microscopically, it is formed

by discrete papillae with central fibrovascular cores, lined by normal urothelium in thickness and cytology, often with prominent umbrella cells. A recent genomic study using next-generation sequencing (NGS) techniques found that papilloma shows activating mutations in the MAPK/ERK pathway (e.g., KRAS and HRAS genes), have low tumor mutational burden (TMB) and no alterations in the apolipoprotein B mRNA editing catalytic polypeptide-like (APOBEC) gene mutational signature (mutated in the majority of invasive urothelial carcinomas)[60].

→ low grade papillary urothelial carcinoma: it is characterized by an exophytic growth in the shape of a papilla, with a central fibrovascular stalk, without invasion of the underlying lamina propria. Microscopically, the papillae do not show extensive fusing or branching and are lined by low-grade neoplastic urothelial cells. The overall histological architecture is mostly conserved, with some urothelial carcinoma cells showing loss of polarity and mild pleomorphism. Overall, cells are generally uniform in size, with occasional nuclear pleomorphism, nucleomegaly, or irregularities in the nuclear membrane. Mitoses may be present, but they are not prominent. Moreover, mitoses are confined to the lower layers of the urothelium. Molecularly, low-grade urothelial carcinomas usually have loss of chromosome 9 and mutations in FGFR3, TERT (50%), STAG2 (~40%), and PIK3CA (25%), among others[61–63]. Moreover, these lesions show epigenetic silencing of several tumor suppressor genes, including RUNX3, CDKN2A, MLH1, VHL, GATA2, GSTP1, and CDH1[64,65]. As far as the pathobiology of low-grade papillary lesions is concerned, it is thought that the malignant transformation of normal urothelial cells starts with the loss of chromosome 9, committing the cells to a hyperplastic proliferation. Then, further genetic alterations are gained (e.g., activating FGFR3 mutations), leading to the formation of a malignant, low-grade lesion[66]. While low-grade lesions rarely lead to disease progression and death (<5%), recurrence is common (>50% of cases) even after many years of negative follow-up.

→ high grade papillary urothelial carcinoma: it is characterized by an exophytic growth in the shape of a papilla with complex architecture (fusion of different branches, etc.), marked cytological and architectural atypias in the urothelium, but no deep invasion of the underlying layers[54]. Macroscopically,

these lesions appear as exophytic single or multiple vegetations, denser and more hyperemic compared to low grade lesions. Microscopically, the architecture of papillae and fibrovascular stalks appears complex, with fusions of the different branches. Urothelial cancer cells appear growing in a disordered, crowded way, with prominent nuclear pleomorphism visible at low power magnification. Nuclei are large, with prominent nucleoli and coarse chromatin. Mitoses are common and frequently atypical. Molecularly, the genomic alterations are similar to the ones described for low grade papillary lesions, with the difference that TERT is mutated in about ~80% of cases, and there are genetic or epigenetic alterations in TP53 gene or TP53 regulatory gene (e.g., CDKN2A, p16)[64,65].

→ carcinoma in situ: CIS is a flat lesion that does not invade the lamina propria by definition, and it is composed of cells with high cytologic grade. Presence of CIS generally confers a poor prognosis to patients. CIS may be classified as: primary, if isolated CIS is diagnosed in the absence of other tumors and of a history positive for bladder cancer; secondary, if CIS is diagnosed during the follow-up of a bladder cancer patient with no previous CIS lesions; concurrent, when CIS is diagnosed together with any other urothelial tumor in the bladder[67]. Macroscopically, CIS lesions appear as flat, grossly erythematous areas, with no clear masses. Microscopically, CIS appears as a flat lesion composed of urothelial cancer cells with large (5x that of lymphocytes, normal urothelium 2x), irregular, hyperchromatic nuclei, showing prominent nuclear pleomorphism and high nuclear/cytoplasm ratio[68,69]. Cells are not cohesive and do not form solid lesions, so that shedding of CIS cells in the urine is common. Molecularly, it seems that the carcinogenesis of CIS lesion is divergent and potentially independent from the one of concurrent, non-invasive and/or invasive urothelial carcinoma. CIS cells are generally monoclonal, aneuploid, and with more abnormal microsatellites than concurrent invasive carcinoma cells. Moreover, they show deletions of 9p21 or polysomy of chromosome 9[70,71]. However, due to the intrinsic difficulty of studying CIS, as it is difficult to isolate it from biological specimens, further studies are needed to precisely elucidate its pathobiology.

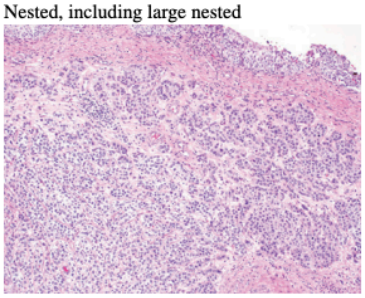
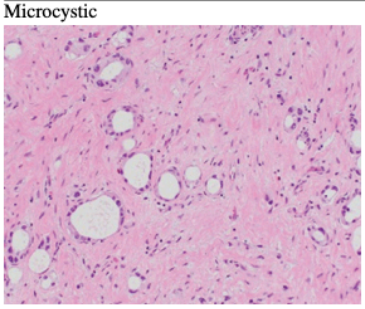
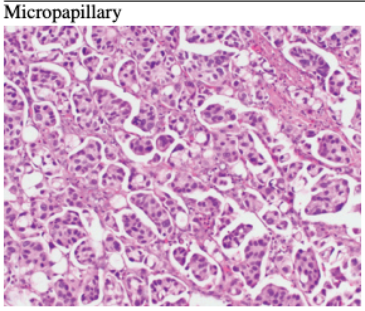
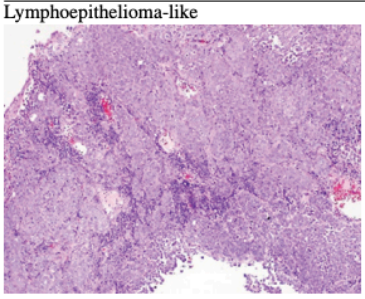
5.3.2 Invasive urothelial lesions

Invasive urothelial carcinoma lesions have penetrated through the basal lamina into the lamina propria or deeper. Based on the depth of invasion, they may be classified as non-muscle invasive bladder cancer (NMIBC), when the tumor does not break through the detrusor muscle, or muscle invasive bladder cancer (MIBC), when the tumor invades the detrusor muscle layer or deeper. NMIBC also includes by definition all the non-invasive malignant urothelial lesions described in section 5.3.1. Moreover, urothelial carcinoma can differentiate in several histological subtypes or variants[72] (better explained below). Depth of invasion into the bladder wall and presence/type of histological variant are the most important drivers of prognosis[73].

Macroscopically, the appearance of invasive carcinoma varies from a subtle bladder wall thickening to a clear exophytic/endophytic mass. Microscopically, neoplastic urothelial cells show high grade nuclear features and are arranged in irregular nests or single cells invading the layers of the bladder wall[74]. Molecularly, the most common genetic alterations are associated with cell cycle progression, (e.g., TP53, PIK3CA, RB1 and FGFR3)[75]. Urothelial carcinomas, especially invasive high grade tumors, have a tendency towards squamous or glandular differentiations[76]. Squamous differentiation is present when there are clear signs of keratinization and/or intercellular bridges between cancer cells. It is recognized in ~20% of invasive urothelial carcinomas, and it does not seem to carry prognostic significance. It is though that the squamous differentiation may somewhat decrease the efficacy of chemotherapy and radiotherapy, but the literature is not univocal[12,54,77]. On the other hand, glandular differentiation is less common (~5% of cases), and it is defined as the presence of true glandular spaces (tubular- or enteric-like) within the urothelial cancerous cells. Intracytoplasmic mucin *per se* does not indicate glandular differentiation. The glandular differentiation +/- mucin does not affect prognosis of urothelial carcinoma, and it seems to have no influence on chemotherapy and radiotherapy[12,54,77].

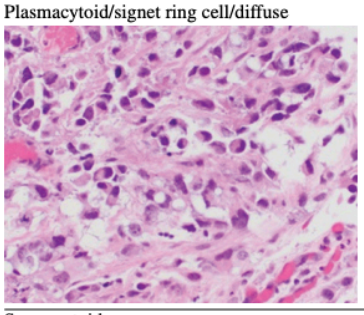
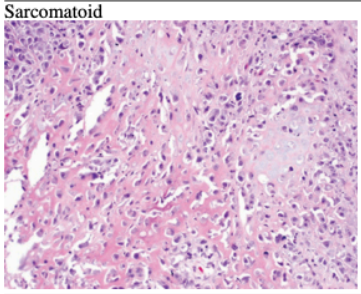
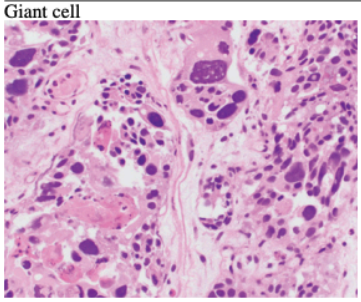
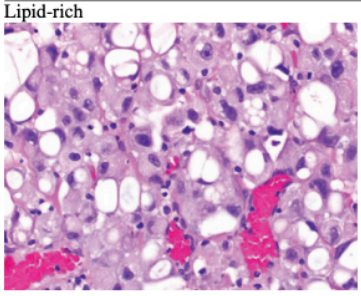
As it was previously mentioned, urothelial carcinoma has a tendency to variant histologies (**Figure 8**). The main variants are:

→ nested: nested urothelial carcinoma is an uncommon variant characterized by unusually bland, nested patterns of invasive urothelial cells. It shows a male predominance in the bladder, and it is reported in ~1% of all urothelial carcinoma cases. It generally presents with concurrent conventional urothelial carcinoma[54,78,79]. It is

WHO Recognized Urothelial Variant Histology	Diagnostic Features	Molecular Features	Clinical Features
Nested, including large nested 	Small or large nests of "low-grade" appearing urothelial cells with irregular infiltrating pattern	TERT promoter mutations; Luminal or urothelial-like molecular subtype	Often present at higher stage; however, stage for stage prognosis is similar to conventional urothelial carcinoma
Microcystic 	Round micro- or macrocysts with thin lining of low-grade urothelial cells, irregular infiltrating pattern; may be related to nested pattern	Unknown	Often presents at higher stage; behaves like conventional urothelial carcinoma
Micropapillary 	Nests of tumor without fibrovascular cores, multiple nests within single lacunar space, reverse polarity of nuclei	Downregulation of miRNA-296, activation of RUVBL1, overexpression of HER2; Luminal nonspecified molecular subtype	Aggressive disease; lymphovascular invasion. Stage for stage similar prognosis to conventional UC. Possible HER2 targeted therapy. No consensus on optimal treatment.
Lymphoepithelioma-like 	Undifferentiated syncytial growth of epithelial cells within dense mixed inflammatory cell infiltrate; EBV negative	Basal-like molecular subtype	Older data showing pure/predominant LELC may have better response to chemotherapy; no consensus on optimal treatment

(*Continued*)

(*Continued*)

WHO Recognized Urothelial Variant Histology	Diagnostic Features	Molecular Features	Clinical Features
Plasmacytoid/signet ring cell/diffuse 	Single discohesive cells infiltrating within myxoid background; morphologic overlap with plasma cells. Differential diagnosis includes plasmacytoma, melanoma, metastasis from GI or breast	Nonsense mutation in *CDH1*, e-cadherin loss by immunohistochemistry. Urothelial-like molecular subtype	Aggressive disease with frequent perivesical and peritoneal involvement; higher rates of recurrence when compared to conventional urothelial carcinoma
Sarcomatoid 	Biphasic malignant epithelioid and mesenchymal neoplasm; may have heterologous sarcomatous elements (osteosarcoma, chondrosarcoma)	Basal-squamous subtype	Aggressive, often presents with nodal or visceral metastatic disease. Cystectomy recommended. Heterologous elements may impart worse prognosis.
Giant cell 	Pleomorphic giant cells often admixed with poorly differentiated urothelial carcinoma; more common in renal pelvis	Unknown	Highly aggressive with uniformly poor outcomes
Lipid-rich 	Urothelial cells with numerous cytoplasmic vacuoles which indent the nucleus resulting in a "lipoblast-like" appearance	Unknown	Presents at higher stage with high mortality

(*Continued*)

(*Continued*)

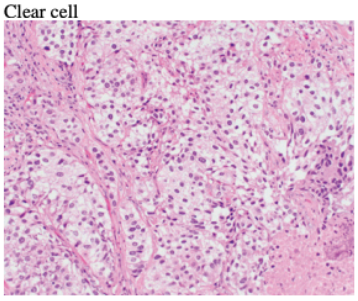
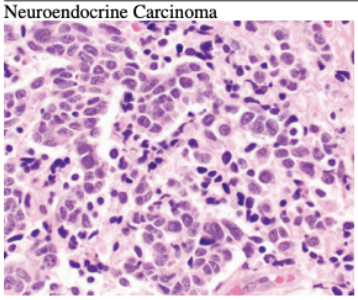
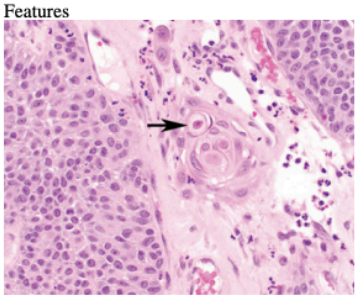
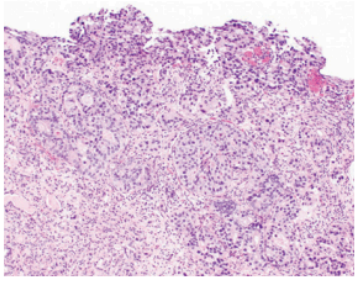
Clear cell 	Voluminous, clear glycogen-rich cytoplasm; differential diagnosis includes clear cell adenocarcinoma	Unknown	Rare variant, prognosis uncertain. Radical surgery with adjuvant chemotherapy suggested
Neuroendocrine Carcinoma 	Small cell carcinoma demonstrates high nuclear to cytoplasmic ratio with nuclear molding, frequent mitotic figures and necrosis	Neuroendocrine-like group, TP53 and RB1 mutations	Aggressive disease often presenting with metastatic disease; platinum-based chemotherapy with etoposide; atezolizumab in trials
Urothelial Carcinoma with Squamous Features 	Keratinizing cells (present at arrow) or intracellular bridges consistent with squamous derivation. Must be admixed with conventional urothelial carcinoma	Basal-squamous subtype; high PD-L1 expression	Worse prognosis than conventional urothelial carcinoma without squamous features. Increasing amounts of squamous features may drive behavior
Urothelial Carcinoma with Glandular Features 	Intestinal type glands with mucinous secretions or extracellular mucin containing malignant cells. Must be admixed with conventional urothelial carcinoma.	Unknown	Worse prognosis than conventional urothelial carcinoma. Increasing amounts of glandular features may drive behavior. Fluoropyrimidine-based chemotherapy often used for advanced disease. No clear role for neoadjuvant chemotherapy

Figure 8 – Main variant histologies of urothelial bladder cancer and their main diagnostic, molecular and clinical features. Adapted from Alderson *et al.*, "Histologic Variants of Urothelial Carcinoma: Morphology, Molecular Features and Clinical Implications", Bladder Cancer 2020[76] (the original article is distributed under the terms of the Creative Commons Attribution Non-Commercial License, CC BY-NC 4.0).

important to distinguish this variant from benign, florid von Brunn nests, which represents its main differential diagnosis. While von Brunn nests are relatively round, evenly distributed and with uniform borders, nested urothelial carcinoma shows anastomosing, irregularly sized nests lacking cytoarchitectural orientation, with irregular projections into deep bladder wall layers[80–82]. The cytological appearance of nested urothelial cells is bland and not particularly remarkable. Differently from other variants, nested variant does not seem to negatively impact prognosis, with outcomes comparable to conventional urothelial carcinoma of matched stage[83].

→ microcystic: microcystic urothelial carcinoma is a very rare variant that was firstly described in bladder in 1991[84]. Microscopically, it is characterized by urothelial carcinoma cells with micro/macro cysts (ranging from micrometers up to 1-2 millimeters in diameter), which may contain necrotic material or pink pale secretions, and are lined by single to multilayered cuboidal or flattened cells with bland cytologic atypias[80,81]. The microcystic variant often coexists with the nested variant and with conventional urothelial carcinoma (~40% of cases), and it is recommended that the microcystic component should represent at least 25% of the tumor volume to define the lesion as microcystic variant[85]. Due to its rarity and the limited reports, its prognostic significance has not been established, although it does not seem to differ from stage-matched conventional urothelial carcinoma[80,81].

→lymphoepithelioma-like: it is a rare entity (~1% of cases) of bladder cancer, particularly diagnosed in elderly men (mean age 70 years)[54]. These tumors are so-called because they resemble lymphoepitheliomas of the nasopharynx but, unlike them, lymphoepithelioma-like urothelial carcinoma is not associated with an Epstein-Barr virus (EBV) infection[86–89]. Microscopically, it appears as sheets of undifferentiated, round or spindle (pleomorphic) cells arranged in sheets, nests, or individual cells, admixed with an abundant, almost obscuring, mixed inflammatory infiltrate (B and T lymphocytes, histiocytes, eosinophils and plasma cells)[90,91]. Concurrent CIS is rather common on the urothelial surface. The main differential diagnoses to exclude are malignant lymphoma, poorly differentiated urothelial carcinoma with lymphoid stroma, and poorly differentiated squamous cell carcinoma. The clinical behavior of lymphoepithelioma-like variant, when admixed with conventional urothelial carcinoma, seems to be similar to the

one of conventional urothelial carcinoma, but lymphoepithelioma-like lesions seem to respond better to chemotherapy and immunotherapy[90,92,93].

→ micropapillary: this rare (~2% of cases) variant has a male predominance, it usually presents with advanced bladder tumors and has an aggressive clinical behavior[94–96]. Microscopically, it is composed of high grade malignant urothelial cells arranged in tight, small, infiltrating clusters and nests within lacunae/empty spaces. Lymphovascular invasion is virtually always present. The surface of the lesions appears lined by slender, delicate papillary and villiform processes. Cytologically, nuclei are peripherally oriented, with marked and high grade atypias and intracytoplasmic vacuoles[97]. The percentage of micropapillary variant present in the tumor volume can greatly vary, and the remaining of the tumor areas is usually occupied by conventional urothelial carcinoma. It is recommended to report the percentage of micropapillary variant as it is an important prognostic factor[80,98,99]. NGS of micropapillary bladder lesions revealed a high frequency of activating alterations in the extracellular domain human epidermal growth factor 2 (ERBB2) gene, raising the possibility of targeted treatment using ERBB2 inhibitors[100].

→ plasmacytoid: as the name suggests, plasmacytoid variant resembles a plasma cell infiltrate within a loose, myxoid stroma. Microscopically, it is composed of sheets of discohesive, high grade cells with eosinophilic cytoplasm, eccentrically placed round nuclei and inconspicuous nucleoli. Plasmacytoid variant cells are positive for the plasma cell marker syndecan-1 (CD138), but they are also positive for urothelial carcinoma markers, like GATA binding protein 3 (GATA3)[80]. NGS studies found that truncating alterations in the CDH1 gene occur in 84% of plasmacytoid cases and are specific to this urothelial carcinoma variant[101], partially explaining the aggressive behavior and generally poor prognosis associated with this entity[102,103].

→ poorly differentiated: it is a very rare variant of high-grade urothelial carcinoma, mostly diagnosed in elderly men, showing an aggressive behavior and poor outcomes. Microscopically, it appears as a mixture of mononuclear malignant epithelial cells (characterized by abundant cytoplasm, vesicular nuclei, mild atypia and variable mitotic index) and multinucleated osteoclast-like reactive giant cells (characterized by light cytological atypia, no mitoses and rare phagocytic activity)[54,104].

→ sarcomatoid: this extremely rare (<1%) variant of urothelial bladder cancer is characterized by the concomitant presence of epithelial and mesenchymal components (biphasic neoplasia). It has a male predominance, and it has been associated with previous exposure to radiotherapy or intravescical cyclophosphamide[79]. Microscopically, it appears as sarcomatoid areas (mostly high-grade spindle cell) admixed with conventional high-grade urothelial carcinoma. Heterologous elements that may be sometimes present include osteosarcoma, chondrosarcoma, rhabdomyosarcoma, leiomyosarcoma, liposarcoma and angiosarcoma[81]. It is typical an overexpression of epithelial-mesenchymal transition markers, including vimentin, FOXC2, SNAIL and ZEB1[81]. NGS studies support a monoclonal origin from a common ancestor clone of both the epithelial and mesenchymal components in sarcomatoid urothelial carcinomas[105,106]. These tumors have a poor prognosis, are usually diagnosed at advanced stages, are chemotherapy-resistant and associated with a worse survival when compared to stage-matched conventional urothelial carcinoma[79].

5.4 Bladder cancer molecular pathology

The traditional and well-established classifications of bladder cancer are based on the appearance of the tumor lesion (flat vs. papillary) and the depth of invasion (non-muscle invasive vs. muscle invasive). However, it has become clear that under the label "urothelial carcinoma of the bladder" there is a wide spectrum of different, molecularly distinct entities that we are just starting to appreciate. The increasing availability of NGS data obtained from human bladder cancer specimens has given the possibility to describe molecular-based classifications of bladder tumors and define therapeutic subgroups of tumors with common genetic alterations, thus potentially increasing the chances of success of targeted therapies.

5.4.1 Molecular subtypes of bladder cancer

The notion that differential expression of mRNAs could be exploited to define molecularly classes of tumors associated with different clinical behaviors and response to treatment dates back 20-25 years ago, when pioneering work in breast cancer led to the definition of molecular subtypes of breast tumors[107]. From those studies, multiple research teams around the world joined the effort to study the mRNA expression profile

of the malignancy of interest, including urothelial bladder carcinoma. The first studies conducted using tissue microarrays showed that unsupervised clustering was able to define molecular subclasses of bladder tumors that correlate with traditional staging and grading[108–110]. For instance, Dyrskjot and colleagues designed a 32-gene molecular classifier able to distinguish between different tumor stages, and provided the possibility to discriminate between recurring and non-recurring pTa tumors[109]. In the following years, several groups in Europe and North America performed independent studies to identify discrete molecular subtypes of muscle invasive bladder cancer[75,111–113]. With the aim to reconcile the similar but independent classifiers developed and to provide a shared classification that may help in the stratification of patients' prognosis and appropriate therapy, an international working committee was created to establish a consensus molecular subtyping classification[114] (**Figure 9**). Overall, six classes were defined: luminal papillary, luminal non-specified, luminal unstable, stroma-rich, basal/squamous and neuroendocrine-like. Basal tumors are more likely to be diagnosed at advanced stages (muscle-invasive or metastatic) and are more common among women – a possible factor explaining the worse prognosis of bladder cancer in affected women compared to affected men. Nevertheless, patients with basal tumors seem to benefit the most, compared other molecular classes, from perioperative traditional chemotherapy regimens or novel immunotherapy protocols[111,115,116]. On the other hand, luminal tumors have a better clinical course and are often downstaged following neoadjuvant chemotherapy, but they do not show such a great benefit from perioperative treatments (in terms of prognosis and survival) as basal tumors do[111,114–117]. However, further studies are needed to better elucidate the differences in prognosis and response to therapies (including potential targeted therapies) between the different subtypes (papillary, unstable, non-specified) of luminal tumors. Stroma-rich tumors are mainly characterized by stromal infiltration, with overexpression of several stroma-related gene signatures, like the smooth muscle, endothelial, fibroblast, and myofibroblast signatures[114]. In terms of survival, stroma-rich tumors seems to have a fair prognosis, comparable with the luminal papillary subclass[114]. The clinically aggressive neuroendocrine subtype is quite rare (~3% of cases), but it has become of particular interest because it may have exceptional responses to immunotherapy protocols (potentially due to low transforming growth factor beta (TGFβ)

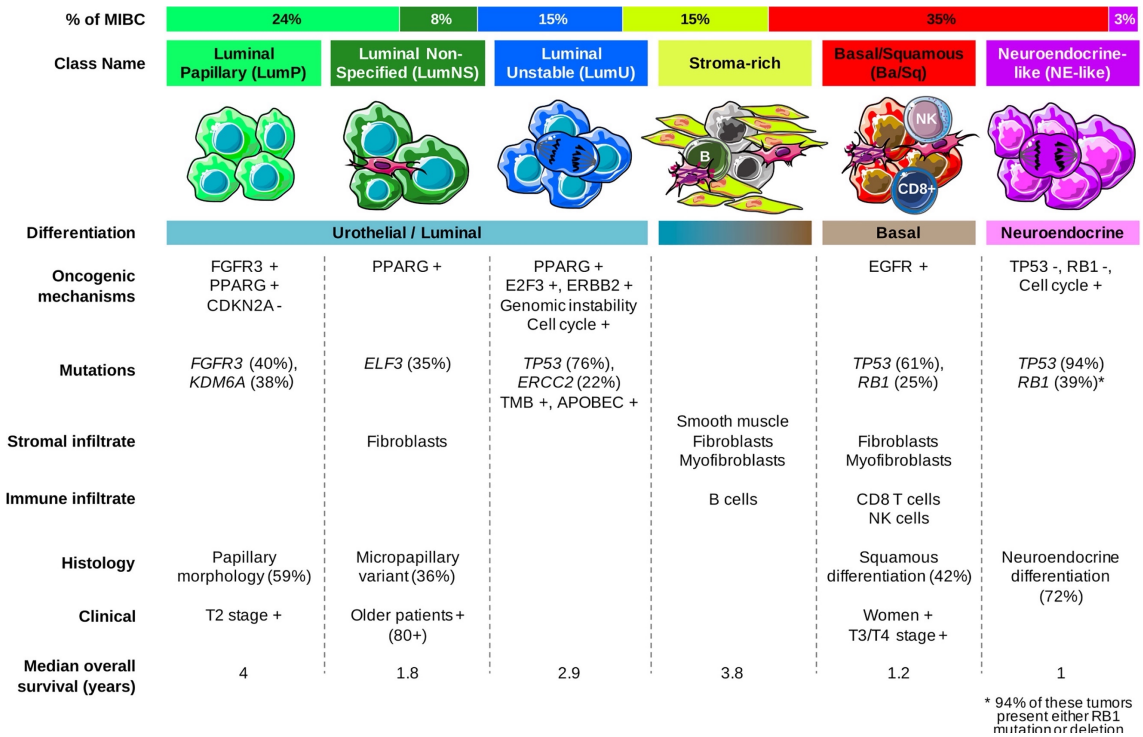


Figure 9 – Consensus molecular subtyping of muscle invasive bladder carcinoma. Ba/Sq = basal/squamous; LumNS = luminal nonspecified; LumP = luminal papillary; LumU = luminal unstable; MIBC = muscle-invasive bladder cancer; NE = neuroendocrine; NK = natural killer. Reproduced from Kamoun *et al.*, "A Consensus Molecular Classification of Muscle-invasive Bladder Cancer", European Urology 2020[114] (the original article is distributed under the terms of the Creative Commons CC-BY-NC-ND license).

expression and high tumor mutational burden), though this finding needs to be validated in future studies[114,118,119].

As previously mentioned, the studies used for the consensus molecular classification mostly, if not almost exclusively, collected and sequenced advanced, invasive tumors, and the potential translation of these (or similar) classes to NMIBC has been questioned. In the setting of NMIBC, the UROMOL study has reported expression mRNA profiles of >400 cases, and unsupervised clustering led to the definition of three major classes[120]. Based on molecular and clinical features, UROMOL tumor class 1 and class 2 displayed luminal-like characteristics, but with different levels of aggressiveness, while UROMOL tumor class 3 showed basal-like characteristics (probably early basal-like phenotype rather than true MIBC basal phenotype)[120,121].

Of note, UROMOL tumor class 2 showed the worst clinical outcomes and expressed a CIS-like gene signature, potentially explaining the shortest progression-free survival. Nevertheless, the reliability of these classes to stratify NMIBC based on the likelihood to benefit from endovesical therapy with Bacille Calmette-Guerin (BCG) or other therapies (including novel immunotherapy protocols for BCG-refractory or BCG-naïve NMIBC) has not been clearly demonstrated yet. At this point, it is worth remembering that bladder cancer often presents as a multifocal disease, and these concomitant lesions may be the expression of the same ancestor clone or may be multiclonal. Therefore, it may happen that different lesions within the same bladder may be classified within different molecular subtypes, thus making molecular-based stratification difficult to be clinically applied. Nevertheless, future studies exploiting single-cell sequencing, patient-derived organoids and clonal lineage tracing would shed more light on these topics[122].

5.4.2 Molecular alterations and response to therapy

As it was briefly described in the above section about risk factors, the main mechanism leading to bladder cancer is thought to be field cancerization through the exposure to environmental *noxa*, which cause the first, preneoplastic mutations, eventually leading to *bona fide* tumor development. This hypothesis has been recently confirmed by whole organ mapping studies, in which several normal-appearing areas biopsied in non-cancerous bladders and in areas far from tumor lesions in cancerous bladders were seque-

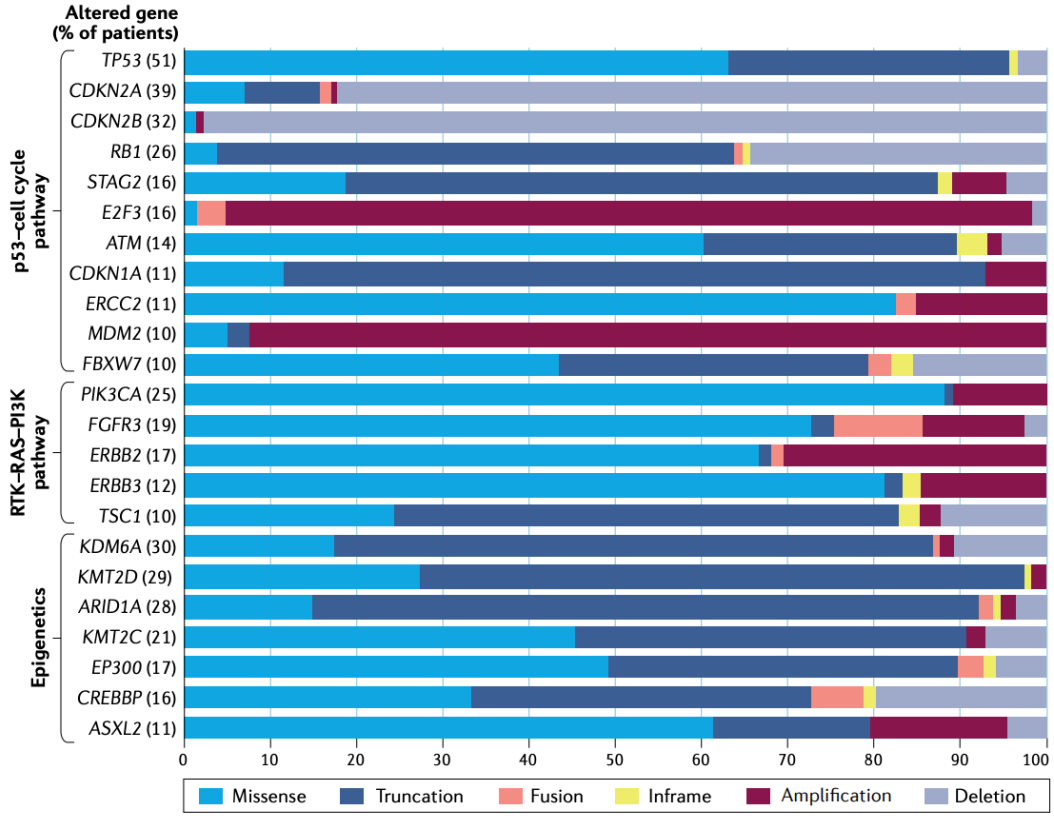


Figure 10 – Major genomic alterations in human bladder cancer. (Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nature Reviews Cancer, "Advances in bladder cancer biology and therapy" by Linda Tran *et al.*[122], © 2020 All rights reserved.).

nced[123,124]. Cells in those areas harbor genomic alterations (**Figure 10**) involving inactivation of chromatin-modifying enzymes (e.g., KMT2D and KDM6A), but they do not show the traditional alterations found in bladder cancer (e.g., TERT, FGFR3, TP53, etc.). These findings have a twofold importance. Firstly, they suggest that urine liquid biopsy tests for the early diagnosis of urothelial carcinoma should not assess alterations in chromatin-modifying enzymes, as they are also common in non-neoplastic tissues, while traditional mutations associated with bladder cancer should be preferred and may be more specific. On the other hand, it is tempting to think that blocking pharmacologically the effects of the inactivating alterations in chromatin-modifying enzymes may be a strategy to prevent bladder cancer development either in high-risk subjects or to consolidate recurrence-free status in previously treated patients (e.g., in patients with frequently recurring low grade, pTa lesions).

Alterations in the FGFR3 gene are quite common across the whole spectrum of urothelial carcinoma lesions. For instance, activating alterations in FGFR3 causing ligand-independent activation are very common in low grade, non-invasive pTa tumors, leading to a constitutive activation of the Ras signaling pathway in cancer cells[46,121]. Moreover, ~20% of invasive tumors have FGFR3 alterations (including FGFR3-TACC fusions)[117,125]. Therefore, it is not surprising that FGFR3 inhibitors (like erdafitinib) have been tested in clinical trials, also with very promising results (e.g., 40% confirmed responses in unresectable, locally advanced or metastatic bladder cancer patients enrolled in the BLC2001 trial[126]). Ongoing trials (e.g., THOR-2) are also testing FGFR3 targeting in NMIBC[127].

The mainstay of chemotherapy for urothelial carcinoma is represented by cisplatin, an alkylating antineoplastic drug that interfere with DNA replication causing DNA damage and the death of replicating cells (especially, in theory, the fast-replicating tumor cells). Therefore, cells with alterations in the DNA repair pathway (e.g., alterations in BRCA1/2, RB1, ATM, etc.) are more sensitive to cisplatin-based chemotherapy. However, available studies have led to contrasting results about an increased benefit from cisplatin-based chemotherapy for patients with urothelial carcinoma harboring mutations in DNA repair genes[128–131]. Perspective trials (e.g., NCT03609216) are ongoing to test if these patients may have effective, lasting, and complete responses that may avoid surgery, and novel

biomarkers are needed to monitor response and identify early signs of tumor resistance and/or progression.

Alterations in the DNA repair pathway are also associated with an increased benefit from immunotherapy, probably because the higher genomic instability leads to higher TMB values, thus higher immunotherapy efficacy[132]. However, it is still to be understood if this benefit is only evident in "hot" tumors, in which the immune system has already been recruited to provide immune-mediated tumor clearance, or also in "cold" tumors, in which the immune system does not promptly counteract tumor growth and spread.

5.5 Sexual dimorphism in bladder cancer pathobiology

As previously mentioned, there are clear differences between the two sexes in the pathobiology and oncological outcomes of urothelial carcinoma (**Figure 11**)[133–135]. Moreover, it is important to note that studies in the context of immune checkpoint inhibition showed a worse response in female compared to men[136]. For the purposes of this dissertation, I will briefly cover the link between the sexual dimorphism, different hormonal regulation and the immune system in bladder urothelial carcinoma, while the potential genetic bases for these sex-based differences will not be detailed.

Steroid sex hormones constitute one of the major factors contributing to the sexual dimorphism in urothelial bladder cancer. Indeed, estrogens seem to play a protective role against bladder carcinogenesis by means of the estrogen receptor (ER) 1 pathway in bladder cells[137–139]. Supporting this observation, castration in male animal models decreases the risk to develop bladder cancer, while oophorectomy in female animal models increases it[140,141]. Conversely, androgens seem to have a negative effect, as supported by preclinical[140,142] and clinical[143] studies. ERβ expression has been associated with recurrence, progression and bladder-specific mortality in MIBC patients, and loss of ERα was linked to more invasive and higher grade lesions[144].

Another critical factor in bladder urothelial carcinoma pathobiology is represented by the sex-specific differences in the immune system[145]. Those differences can be attributed to genetic and epigenetic factors, as many genes and regulatory RNAs involved in the immune response are located on the X chromosome[146,147]. Different proportions of circulating immune cells that may mediate the anticancer immunity in urothelial carcinoma have been reported in men and women, and at different ages. For instance,

concentrations of circulating monocytes are higher in young men than young women, but their proportions increase in women after menopause[148]. Moreover, plasmacytoid dendritic cells, another important player in the IFN-mediated anticancer response, show sex-based differences, with higher concentrations and activation levels in female[149]. Another factor that may affect the local concentrations of immune cells at the level of the urothelial mucosa is the different prevalence of urinary tract infections between the two sexes. While in physiological states the concentration of immune cells does not differ much between the two sexes, in case of infections important differences appear[150,151]. In a preclinical model of urinary tract infection by uropathogenic *E. coli*, female mice mounted a strong immune response against the infection, including increased pro-inflammatory cytokine expression (e.g., IL-17), and increased γδ T cells and group 3 innate lymphoid cells in the urothelial mucosa[151]. Conversely, male and testosterone-treated female mice remained chronically infected for up to a month, with only small increases in cytokine production or local immune infiltrates. Currently it is not known if those differences in the acute immune response during an infection would also occur in the setting of tumor growth, potentially influence anticancer therapy.

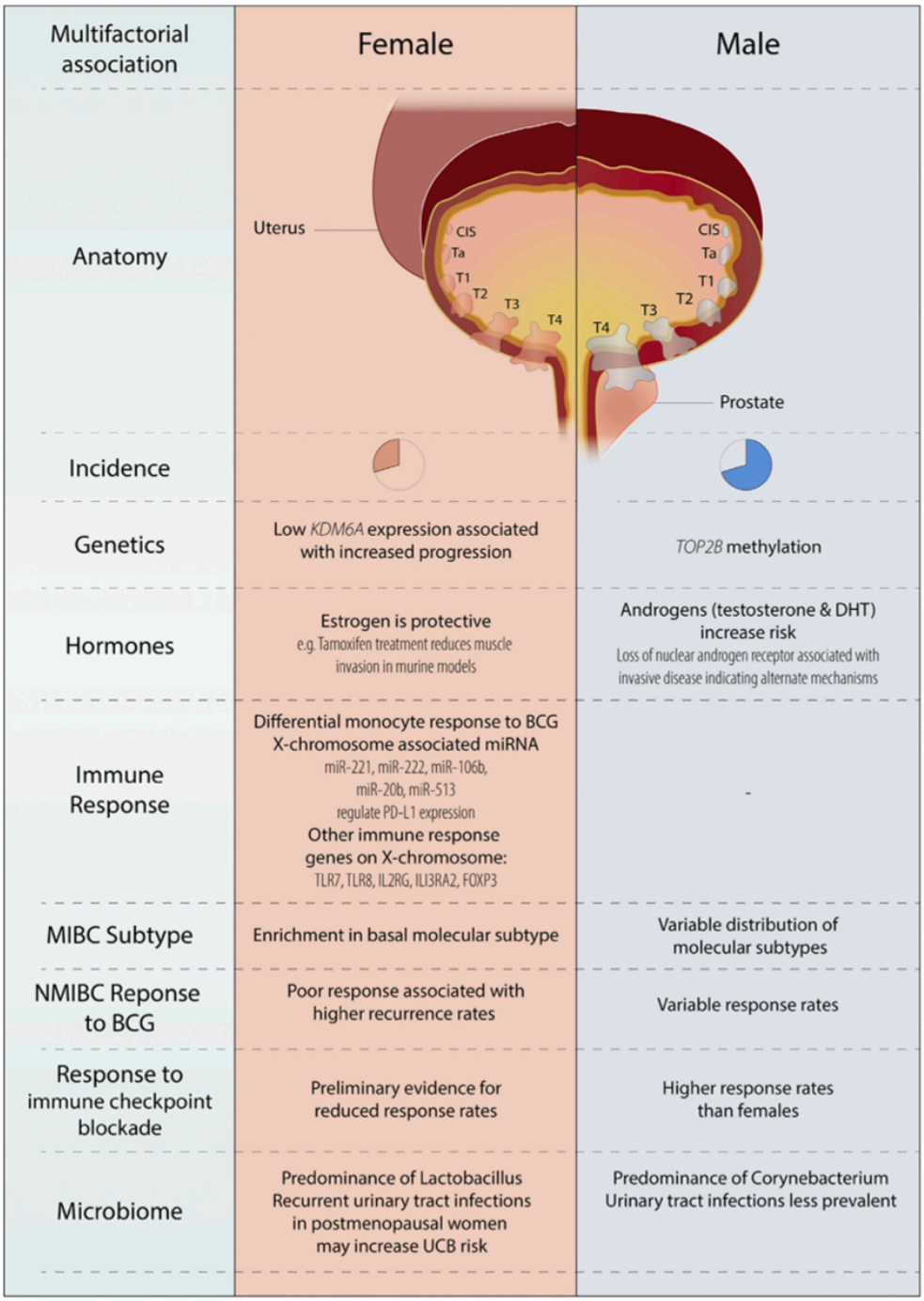


Figure 11 – Overview of the key sex-related differences associated with bladder urothelial carcinoma pathobiology. (Reprinted from European Urology Oncology, Koti *et al.*[152], "Sex Differences in Bladder Cancer Immunobiology and Outcomes: A Collaborative Review with Implications for Treatment", ©2020, with permission from Elsevier.).

6. Introduction – diagnosis and therapeutic management of the urothelial carcinoma of the bladder

Bladder cancer is rarely an incidental finding, but it is mostly diagnosed following the appearance of presenting symptoms and signs.

6.1 Diagnostic evaluation of bladder urothelial carcinoma

Among presenting symptoms and signs of bladder cancer, the most common presenting complain is painless, gross hematuria (in up to 85% of cases), defined as the presence of red blood cells in the urine, which appears of a bright, red color[153,154]. Therefore, any episode of gross hematuria, even if isolated, must be thoroughly evaluated to exclude the presence of bladder cancer. A complete diagnostic evaluation following gross hematuria includes medical history assessment and physical evaluation (which should include rectal and vaginal bimanual palpation), urinalysis and urine culture tests, cystoscopy, and upper tract imaging (preferentially a multi-phase contrast-enhanced computed tomography (CT) scan, including delayed phase images). It is also reasonable to ask for a urine cytology test. Other presenting symptoms and clinical signs include non-visible hematuria (virtually present in all bladder cancer patients), urgency, dysuria, increased frequency (irritative symptoms are particularly common if CIS is present), and, in more advanced tumors, pelvic pain and symptoms related to urinary tract obstruction.

6.1.1 Cystoscopy and endoscopy-based techniques

The gold standard diagnostic procedure for urothelial bladder cancer is still represented by cystoscopy +/- eventual biopsy of suspicious areas for histological analysis. Flexible, office-based cystoscopy is the option of choice in the suspect of bladder cancer, followed then by rigid cystoscopy and transurethral resection of bladder tumors (TURB) in case of suspicious areas. Nowadays, standard white light cystoscopy is the first choice for bladder evaluation, although its low sensitivity for small tumors and flat lesions as CIS[15]. Fluorescent-based cystoscopy (also known as photodynamic diagnosis, PDD) using photoactive porphyrins (hexaminolevulinate, HAL; 5-aminolaevulinic acid, ALA), which preferentially accumulate in neoplastic tissue, increases the detection of tumor lesions invisible to traditional white light cystoscopy. In a systematic review and meta-analysis, PDD demonstrated higher sensitivity than white light cystoscopy at both the patient-level

(92% vs. 71%) and biopsy-level (93% vs. 65%)[155]. It is worth mentioning that a potential drawback of PDD is the lower specificity when compared to white light cystoscopy, mostly because inflamed areas in the urothelium can accumulate the photoactive porphyrins similarly to the neoplastic tissue[155,156]. Moreover, a systematic review and meta-analysis of 14 trials with nearly 3,000 patients found that PDD decreased risk of tumor recurrence compared to the white light technique, but it did not decrease the progression and mortality rates[157].

Another endoscopy-based technique developed with the aim to increase bladder tumor detection is narrow band imaging (NBI), which uses a light of narrow bandwidth (blue, 415 nm; green, 540 nm) to enhance the contrast between the urothelial mucosa and the microvasculature. Some studies, mostly performed at single-institution or on a limited cohort of patients, have shown an improved cancer detection using NBI +/- resection over traditional white light cystoscopy +/- resection[158–160]. However, in a multicenter randomized clinical trial comparing NBI + resection vs. traditional white light cystoscopy + resection, both techniques showed similar results in the overall 12-month recurrence rate, even if NBI+resection significantly reduced recurrence in low-risk disease patients (pTa/low grade, < 30 mm size, no CIS)[161].

Other endoscopy-based techniques that have been preliminary tested in bladder cancer include confocal laser micro-endoscopy and the Storz professional image enhancement system (IMAGE1 S)[162,163]. However, future studies are needed to assess their utility and cost-effectiveness for the diagnosis of bladder cancer.

6.1.2 Urine cytology and other urine-based assays

Cytological examination of exfoliated urothelial cells collected from voided urine for the diagnosis of bladder cancer was invented by George Papanicolaou in the 1940s. Nowadays, urinary cytology has high sensitivity (84%) in high-grade urothelial tumors, low sensitivity (16%) in low grade lesions, and a more variable sensitivity (28–100%) in CIS[164,165]. Providing estimates for urinary cytology has always been a complex task because of the great operator-dependency of the cytological interpretation[166,167]. To solve the issue, a standardized reporting system (the Paris Reporting System for urinary cytology) has been introduced in 2016[168]. The Paris system has been validated in several

retrospective studies and it is strongly recommended to be adopted by every laboratories[169,170].

To reduce the number of invasive and costly cystoscopies patients must undergo, especially in NMIBC, several urine-based assays have been developed and studied for implementation in the diagnostic work-up for bladder cancer[171]. However, none of these assays has been confidently introduced in routine practice or medical societies' guidelines.

Fluorescence in situ hybridization (FISH) probes have been designed to target specific chromosomal alterations in bladder cancer cell (e.g., the commercial kit "UroVysion" targets aneuploidy of chromosomes 3, 7 and 17, together with 9p21 loss), to be used on exfoliated urinary cells. FISH sensitivity and specificity are overall 63% and 87%, respectively[172]. However, FISH-based techniques are not able to confidently detect low-grade urothelial carcinoma, and there is no consensus about the cost-effectiveness of this method over conventional urinary cytology, or about the most appropriate setting (diagnosis vs. follow-up) for its implementation.

Another urinary cell-based assay to be used in conjunction with cytology is the so-called ImmunoCyt assay. It is based on the use of three fluorescent-labeled antibodies targeting 3 bladder cancer antigens (2 mucins and a particular form of carcinoembryonic antigen) commonly found on exfoliated cancer cells. A metanalysis of 14 studies found an overall 78% sensitivity and 78% specificity[172]. Similarly to conventional cytology, the test is operator-dependent and suffers from high interobserver variability, though adequate training may help improving reproducibility[173,174].

Another cell-based assay is the so-called CxBladder test, which detects the presence of five mRNA (HOXA13, CDC2, CXCR2, MDK, IGFBP5) fragments[175,176]. Few studies have been conducted so far using the CxBladder assays, and its sensitivity and specificity seem to be ~80%[176,177].

Two Food and Drug Administration (FDA)-approved urine-based assays test for the presence of nuclear matrix protein 22 (NMP22), enriched in malignant urothelial cancer cells. In a metanalysis of 19 studies using the laboratory-based NMP22 quantitative assay, pooled estimates for sensitivity and specificity were 69% and 77%, respectively[172].

Although the great interest and intense research in the field of urinary biomarkers of urothelial carcinoma, nowadays there is no consensus about their routine use and

implementation. Moreover, well-designed, perspective, multi-institutional studies are needed to perform a head-to-head comparison of all those assays in different clinical settings (e.g., screening, follow-up, etc.).

6.1.3 Imaging

In general, traditional imaging techniques play a major role in advanced and late-stage disease, while several challenges have limited their use in early-stage lesions. Indeed, it is not easy to differentiate the different layers that compose the bladder wall; therefore, it is difficult by imaging to exactly measure the depth of invasion of bladder lesions, which is among the most important prognostic factors. Moreover, intravesical therapies and TURBs cause modifications/scars in the bladder wall that makes evaluation even more complex. Nevertheless, there is great interest to improve the actual imaging techniques and to develop novel ones, with the final aim to decrease the number of invasive and unpleasant cystoscopies and transurethral resections.

Ultrasound (US) can be used as a quick adjunct during an office consultation for bladder cancer and to preliminarily assess a patient presenting with hematuria, as it can provide a rapid overview of the urinary tract and highlight potential renal masses, hydronephrosis, or intraluminal bladder masses. However, its utility in upper tract malignancies, especially to evaluate the mid-lower portion of the ureters, is limited. Bladder cancer has generally a hypoechoic appearance (even if concomitant fibrosis, calcifications, or hemorrhages may change its appearance), in the shape of a papillary lesion with endoluminal projections or a plaque-like thickening of the bladder wall. Color Doppler US may sometimes highlight intralesional vascularization if the lesion is big enough. Although imaging of a filled and distended bladder with a high-frequency US probe can macroscopically highlight the different layers of the bladder wall (from inside out, a hyperechoic inner mucosa/submucosa, a hypoechoic muscularis propria and a hyperechoic outer serosa)[178], a precise characterization of the depth of invasion of a tumor lesion cannot be achieved by US, therefore staging cannot be performed by US. Muscle invasion is suspected when the hypoechoic middle layer, corresponding to the detrusor muscle, appears interrupted. Moreover, an intrinsic limitation of US is its operator-dependency that limits reproducibility.

CT scan represents the currently most used imaging technique for the detection, staging and follow-up of bladder cancer patients. CT urography, a three-phase CT scan technique in which acquisitions are performed before and after administration of an intravenous contrast agent, is generally used to assess at the same time the bladder as well as the upper urinary tract. The recommendation for CT urography at baseline for bladder cancer patients is matter of debate, as the risk of concurrent upper tract lesions is ~2%. The risk for upper tract carcinoma increases if the bladder tumor is located at the trigone (~8%) or if the patient has a long story of high-risk, high-grade recurring bladder tumors[179–182]. The first acquisition is called unenhanced phase, and it is mostly used to exclude the presence of blood clots, calcifications, and/or urinary stones. The second acquisition, known as urothelial phase, is taken immediately after the intravenous injection of the contrast agent, when the bladder is not yet filled with contrast[183]. The bladder wall can be visualized as an enhancing rim (due to vascularization) against the low-attenuation urine volume. Similarly, bladder tumors may show an early enhancement, due to their high concentrations of neo-vessels. In the third acquisition, the delayed phase, the bladder lumen is filled by contrast agent and the urothelial tumor appears as a filling, unenhanced defect. For staging, CT scan is most useful to identify tumors that have macroscopically broken into the perivesical fat (T3b) or invaded adjacent organs (T4). On the other hand, CT scan is not reliable to differentiate T1 vs. T2 tumors.

Magnetic resonance imaging (MRI) provides the best resolution and contrast between different tissue structures; therefore, MRI can be used to distinguish between T1 vs. T2 lesions, especially if detrusor muscle invasion is deep and extended. In T2-weighted MRI, the detrusor muscle appears as a hypointense band in between hyperintense intraluminal urine and perivesical fat, while tumors have an intermediate signal intensity. Thus, differences in signal intensity can be used to detect the depth of invasion of tumor lesions in the detrusor and beyond. For precise and appropriate staging of organ-confined T1/T2, the implementation of functional MRI techniques, like diffusion-weighted imaging (DWI) or dynamic contrast-enhanced (DCE) MRI, has been tested, although each technique has its own pros and cons. Therefore, the final staging is often based on the evaluation of different acquisitions.

It is worth mentioning that the current standard imaging technique is CT scan, due to the cost-effectiveness, wide availability, and rapid acquisition time. The role of multi-

parametric MRI is increasing, and its potentialities even in follow-up settings (e.g., to evaluate post-treatment residual disease vs. complete response) are under investigation. However, MRI has not yet a well-established, guideline-recommended role in urothelial carcinoma diagnosis and staging[184–186].

6.1.4 Transurethral resection of bladder tumor

TURB could be at the same time a diagnostic and a therapeutic procedure (therapeutic only for NMIBC), and it is a crucial procedure in the management of urothelial bladder carcinoma. Indeed, incomplete or suboptimal TURB makes the patients potentially facing a double risk: understaging and incomplete tumor resection. To perform an optimal and appropriate TURB, the surgeon must perform a complete exploration of the bladder mucosa and annotate all findings (e.g., number of lesions, position, approximate size, morphological appearance, etc.), proceed with the resection and annotate the adequacy of the resection (including potential visualization of the muscle at the resection bed) and eventual intraoperative complications (e.g., perforation)[187,188]. To perform a correct histological diagnosis, it is of paramount importance to include the detrusor muscle in the resection. Moreover, to facilitate recognition of the detrusor and its potential invasion by bladder cancer cells, it is recommended to perform the resection of the bladder wall containing the muscularis propria in a separate specimen (the so-called "tumor bed") from the potential exophytic, papillary lesion growing above. This is not required if the TURB is performed using the *En-bloc* resection technique[189].

6.2 Principles of therapy in bladder urothelial carcinoma - NMIBC

As mentioned above, TURB is at the same time a diagnostic and therapeutic procedure for NMIBC, and it is considered the mainstay of treatment. Nevertheless, NMIBC lesions have a high rate of recurrence and may eventually progress to invasive disease. Therefore, to consolidate the cancer-free status, after the initial TURB, bladder cancer patients undergo different protocols of adjuvant (mostly intravesical) treatments, together with a strict diagnostic follow-up, according to the estimated risk of recurrence or progression. While a comprehensive discussion of the different risk classes and the therapeutic algorithms is beyond the scope of this thesis, the main treatment options in this setting will be briefly described.

6.2.1 Perioperative intravesical chemotherapy

A potential drawback of TURB is the possibility to release detached cancer cells within the bladder lumen during the endoscopic procedures, with a potential to implant them in the surrounding urothelial mucosa. This mechanism is thought to be responsible for most early recurrences[190–194]. Therefore, an endovesical instillation of chemotherapy immediately after TURB may kill the "floating" urothelial carcinoma cells, preventing cancer recurrence. This approach has been used for several years, with different pharmacological compounds and different protocols. Nowadays, level 1 evidence supports the efficacy of a single-dose of mitomycin C or epirubicin, instilled <6 hours after TURB, in reducing tumor recurrence of solitary, papillary low-grade tumors presenting for the first time[195–197]. The side-effect profile is generally very favorable, with mostly local irritative symptoms that do not require further management. However, serious complications and rare deaths have been reported, especially in patients with perforation of the bladder wall during TURB. Therefore, postoperative intravesical chemotherapy should be avoided in patients with extensive resection, or when there is concern about bladder wall perforation[198,199].

6.2.2. Adjuvant immunotherapy – BCG and novel therapies

The BCG is a live-attenuated mycobacterium that was firstly developed as a vaccine to prevent tuberculosis, but it also showed to have an antitumor activity in different malignancies, including urothelial bladder cancer[200,201]. BCG is administered endovesically ~4 weeks after TURB, to allow complete scarring and re-epithelization of the bladder mucosa, thus decreasing the risk of BCG extravasation and potential serious complications (e.g., BCG sepsis)[202]. After instillation, the patient is instructed to retain the BCG solution within the bladder for at least 1 hour. BCG is used both to treat potential residual disease after TURB (induction protocol) and to maintain a cancer-free status to prevent recurrences (maintenance protocol). Therefore, induction BCG is used to identify the first cycle of BCG immunotherapy (6 weekly administrations) after the initial TURB, while maintenance BCG is used to identify the BCG administration performed after induction to consolidate the cancer-free status and avoid recurrences.

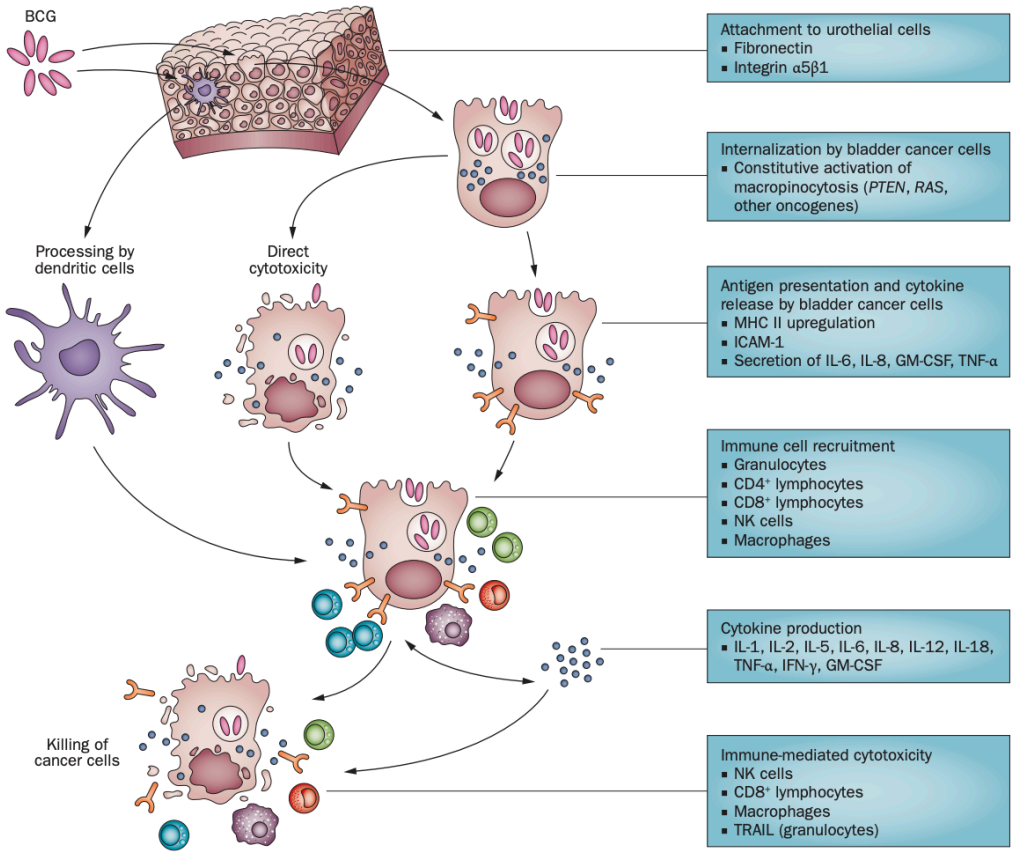


Figure 12 – An overview of the different mechanisms of BCG immunotherapy. (Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nature Reviews Urology, "The mechanism of action of BCG therapy for bladder cancer—a current perspective" by Gil Redelman-Sidi *et al.*[203], © 2014 All rights reserved).

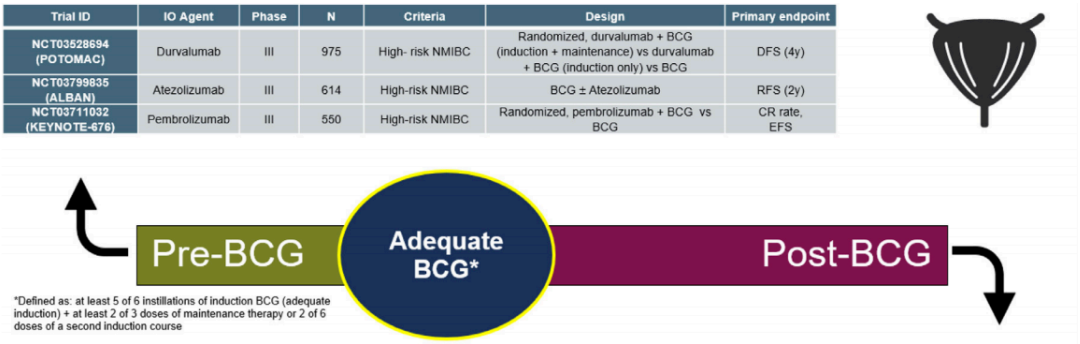
The mechanisms that mediate the BCG antitumor immunotherapy activity are several and not yet completely understood (**Figure 12**)[203]. The first step is the binding of BCG to the bladder wall through fibronectin residues, stimulating a direct cell-mediated immunological response and the release of many cytokines and chemokines involved in inflammation, including granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and several interleukins (ILs)[204]. The main cytokines released after BCG administration – IFN-γ, IL-2, and IL-12 – suggest a preferential activation of the T-helper type-1 response. At the end, the result of the cytokine release caused by BCG is the activation of cell-mediated cytotoxic mechanisms against eventual residual urothelial cancer cells, thus preventing potential recurrences and progression[203–205]. Of note, BCG may at the same time induce the release of IL-10, resulting in the suppressive T-helper type-2 response.

Several trials and meta-analyses confirmed that BCG immunotherapy (induction+maintenance) after TURB is superior to other therapeutic strategies (e.g., endovesical chemotherapy, TURB alone, etc.) to prevent tumor recurrence and progression to MIBC[196,206–211], benefits that are particularly evident in intermediate- and high-risk NMIBC[206,209,212–214].

To date, BCG immunotherapy is the mainstay of conservative therapy (i.e., avoiding radical surgery) in case CIS is diagnosed. In retrospective studies on patients with bladder CIS, a complete response was reached in 48% of patients treated with intravesical chemotherapy, while in 72-93% treated with BCG[215–219]. A meta-analysis of clinical trials comparing BCG to intravesical chemotherapy in patients with CIS-only reported a significantly higher response rate in the BCG cohort, as well as a 59% decrease in the odds of treatment failure if BCG was administered compared to chemotherapy[220].

In addition to BCG, several novel agents have been studied or are currently under investigation. For instance, intravesical IFN-α has been studied to treat NMIBC, based on the antitumor activity played by INFs in physiology. Administered alone, IFN was not superior to BCG or intravesical chemotherapy in eradicating potential residual tumor cells and preventing disease recurrence, and at the same time it was more expensive than established treatment protocols[221–223]. Therefore, combination strategies have been tested, with a particular emphasis on the BCG+IFN combination. A first study found that 63% of patients were tumor-free at 1 year and 53% at 2 years when treated with the combina-

Trial ID	IO Agent	Phase	N	Criteria	Design	Primary endpoint
NCT03528694 (POTOMAC)	Durvalumab	III	975	High- risk NMIBC	Randomized, durvalumab + BCG (induction + maintenance) vs durvalumab + BCG (induction only) vs BCG	DFS (4y)
NCT03799835 (ALBAN)	Atezolizumab	III	614	High-risk NMIBC	BCG ± Atezolizumab	RFS (2y)
NCT03711032 (KEYNOTE-676)	Pembrolizumab	III	550	High-risk NMIBC	Randomized, pembrolizumab + BCG vs BCG	CR rate, EFS



Trial ID	IO Agent	Phase	N	Criteria	Design	Primary endpoint
NCT03519256 (CheckMate 9UT)	Nivolumab	II	436	NMIBC unresponsive to BCG	Randomized, nivolumab vs nivolumab + BMS-986205 vs nivolumab + BCG vs nivolumab + BMS-986205 + BCG	CIS with CR and DOCR, EFS non-CIS
NCT04172675 (THOR-2)	Erdafitinib	II	280	NMIBC unresponsive to BCG	Randomized, erdafitinib vs investigator choice intravesical therapy	RFS

Figure 13 – Main current clinical trials testing immunotherapy strategies in non-muscle invasive bladder cancer. Reproduced from Basile, Pederzoli *et al.*, "Intermediate- and high-risk nonmuscle invasive bladder cancer: Where do we stand?",Urologic Oncology 2021[127].

tion therapy[224]. However, a following randomized trial in BCG-naïve patients did not find a superiority of combination regimens over BCG alone[225].

Based on the promising results of systemic immune checkpoint inhibitors in advanced bladder cancer, a few trials are currently testing these drugs also in NMIBC (**Figure 13**). The scientific rationale mainly derives from the hypothesis that at least a proportion of high-risk NMIBC disease shares a biology similar to MIBC[226], and from the data that NMIBC shows a favorable immunogenic profile[127,227,228]. A brief overview of the key phase II/III trials in this setting follows, but a more comprehensive review can be found here[127].

The most important study to date investigating systemic checkpoint inhibitors in BCG-unresponsive NMIBC is the phase II KEYNOTE-057 trial, testing pembrolizumab in patients with high-risk BCG-unresponsive disease (CIS +/- papillary tumors; cohort A), who refused or were not fit for radical surgery[229,230]. Results on 96 patients reported a 3-month complete response rate of 41%, with a median duration of response of 16.2 months. After trial discontinuation, 36 patients underwent surgery (i.e., radical cystectomy, the complete removal of the bladder and surrounding structures), and only 3 (8.4%) had pathologic upstaging to MIBC. Following these encouraging results, in January 2020 the FDA approved pembrolizumab as a novel therapeutic option for NMIBC patients with CIS[231]. In addition to the intravenous administration, studies are ongoing to investigate the potential activity of immune checkpoint inhibitors by intravesical administration. To this end, intravesical pembrolizumab is being explored in preinduction, induction, and maintenance together with BCG (NCT02808143) or gemcitabine (NCT04164082). Moreover, the NCT03892642 trial is testing intravesical avelumab+BCG (induction and maintenance courses) in patients with BCG-unresponsive NMIBC. A few trials are also testing immune checkpoint inhibitors in BCG-naïve patients, given the good results obtained in BCG-refractory patients. In this setting, pembrolizumab is investigated as single-agent (NCT03504163) and in combination (NCT03711032), as well as the ALBAN, a phase III trial (NCT03799835), is testing atezolizumab+BCG vs. BCG alone.

6.2.3. Intravesical chemotherapy

As previously mentioned, a single post-operative instillation of chemotherapy is used to decrease early recurrences due to floating cancer cells released in the bladder lumen

during TURB. However, patients who are not in the low-risk category need further adjuvant intravesical chemotherapy, similarly to the induction/maintenance scheme presented for BCG. The duration and frequency of repeated chemotherapy instillations is still matter of debate, but it should not be longer than 1 year[232]. Mitomycin C (MMC) is an alkylating agent that inhibits DNA synthesis, and it is probably the most used intravesical chemotherapy for induction/maintenance protocols. Although MMC is not as effective as BCG in reducing tumor recurrence, a meta-analysis found that MMC was associated with a 38% reduction in tumor recurrence, therefore it may be an acceptable therapeutic option, especially in low-risk patients[233].

6.2.4. "Early" cystectomy

Despite local combined therapy with TURB and intravesical chemotherapy/immunotherapy, a good proportion of high-grade NMIBC patients would eventually progress to MIBC. Indeed, about a fifth of patients with a diagnosis of CIS dies because of urothelial bladder cancer within 10 years from diagnosis, and residual tumor found after the initial TURB in high-risk patients increases the risk of progression to muscle invasive disease[234–236]. Moreover, even if the term non-invasive suggests a favorable condition, the intrinsic risk of the disease should not be underestimated, as up to half of patients with presumed non–muscle invasive high-grade disease is actually diagnosed with muscle invasive disease at cystectomy[237]. Those data are worrying and highlight a potential underestimation of the disease extend and depth of invasion in a good proportion of high-risk patients, a situation that ultimately translates into more cancer-specific deaths among them. Therefore, those considerations supported the "early radical cystectomy" approach, in which high-risk patients are counselled for the surgical removal of the bladder to avoid potential disease progression and, ultimately, death because of urothelial carcinoma. A study found that 92% patients treated with early radical cystectomy for T1 tumors were alive and disease-free after 10 years, while the disease-free survival rate for comparable T1 patients with a delayed cystectomy after conservative management was only 64%[238]. It is evident that radical cystectomy is a major surgical procedure with a strong impact on the quality of life and morbidity of patients. Therefore, a careful discussion with patients is mandatory before such a surgical intervention, to clearly explain potential benefits and risks of all the available

possibilities. The European Association of Urology (EAU) guidelines recommend early cystectomy in patients with BCG unresponsive tumors and in patients with relapsing high-grade urothelial carcinoma after BCG[197].

6.3 Principles of therapy in bladder urothelial carcinoma – MIBC

The mainstay of therapy for muscle invasive bladder cancer is represented by the surgical removal of the bladder (radical cystectomy) and surrounding structures, preceded by neoadjuvant chemotherapy.

6.3.1 Radical cystectomy

For patients with muscle invasive bladder cancer (cT2–T4a, N0, M0 disease), radical cystectomy with bilateral lymph node dissection is the gold standard radical treatment. In men, radical cystectomy includes, in addition to bilateral pelvic lymphadenectomy, removal of the prostate and seminal vesicles, while in women it includes removal of the ovaries, uterus with cervix, and anterior vagina. Randomized trial data showed superior outcomes when neoadjuvant systemic chemotherapy is performed before radical cystectomy (refer to section 6.3.2 for neoadjuvant chemotherapy). Timing from diagnosis to radical surgery plays an important role in oncological outcomes. A recent meta-analysis based on data from 19 studies confirmed that a longer delay (> 3 months) of radical cystectomy after diagnosis has a negative effect on survival (hazard ratio = 1.34)[239]. Although the definition of "delay" used by the reviewed studies is different, making difficult to clearly define the best timing for radical cystectomy, a significant delay (i.e., months) does appear to negatively affect patient survival.

A precise and complete pelvic lymphadenectomy is of paramount importance. About one fourth of MIBC patients has nodal disease at cystectomy, and positive pN disease is one of the major factors driving recurrence and survival[240–242]. Since the first report about the oncological value of lymphadenectomy in radical cystectomy, several studies have confirmed this finding[243–245]. However, what an "adequate lymphadenectomy" is remains unclear and matter of debate.

About the surgical technique used to perform radical cystectomy, several studies, including systematic reviews and clinical trials, found similar results: robot-assisted radical cystectomy has a shorter length of hospital stay and less intraoperative blood loss,

but longer operating times compared to open, laparotomic radical cystectomy[246,247]. Complications, post-operative patient quality of life and oncological outcomes seem comparable between the two approaches.

6.3.2 Neoadjuvant chemotherapy

About half of MIBC patients treated with radical cystectomy alone sooner or later progresses to metastatic urothelial carcinoma, highlighting how surgery *per se* does not offer sufficient tumor control[248,249]. To improve these rates, cisplatin-based chemotherapy has been introduced since the 1980s[250–252]. The main aims to use neoadjuvant chemotherapy are reducing the burden of local bladder lesions (pathologic downstaging) and of disseminated micrometastases, thus increasing the likelihood of complete tumor eradication by radical cystectomy. There are several arguments in favor of neoadjuvant chemotherapy before radical cystectomy in muscle invasive urothelial carcinoma of the bladder (cTanyN0M0). Firstly, the earliest the chemotherapy is administered, the higher the probability that the tumor cells would be largely chemosensitive (i.e., less clonal selection for more aggressive clones) and that the burden of micrometastatic disease would be low. Patients are clearly fitter before radical cystectomy than after surgery, thus increasing patient compliance to the therapy. Moreover, neoadjuvant chemotherapy does not increase the complexity of subsequent surgical procedures or surgical morbidity[253,254]. "Delayed" cystectomy following chemotherapy in patients who did not respond to it does not seem to affect patient outcomes.

Several clinical trials tested the benefits associated with neoadjuvant chemotherapy before radical cystectomy, analyzing different chemotherapy regimens (single agents vs. combination regimens, number of cycles, etc.) and different patient populations (e.g., allowed clinical stages, cisplatin-eligibility, etc.)[254–261]. Meta-analyses published in 2005[262] and 2016[263] confirmed the survival benefit associated with neoadjuvant cisplatin-based combinatory regimens. Cisplatin-based neoadjuvant chemotherapy provides a 5-6% survival advantage. Guidelines from several medical societies, including the EAU and National Comprehensive Cancer Network (NCCN), highlight the need for clinicians to discuss and strongly recommend cisplatin-based neoadjuvant chemotherapy for cT2-4aN0M0. However, neoadjuvant chemotherapy is still underused in real-life settings, despite all supporting data. Using the National Bladder Cancer Database, it was found

that only ~1% of the >7000 patients with stage III bladder cancer received neoadjuvant chemotherapy from 1998 to 2003[264]. This figure increased to ~15% in the following 15 years (using data from the 2004-2011 SEER-Medicare), but it is still far from an optimal coverage[265]. Moreover, the SEER-Medicare study reported that ~50% of patients receiving neoadjuvant chemotherapy were administered with a non-standard-of-care regimen (i.e., a non-cisplatin-based combination regimen) for any reason, highlighting the need for a better patient counselling and for the identification of equivalently efficacious options for patients who are not fit for cisplatin.

There seem to be differences in the efficacy and oncological outcomes after neoadjuvant chemotherapy in patients with primary or secondary (i.e., progressing from NMIBC) muscle invasive bladder cancer[266]. However, to date there are no high-quality data to support changes in the actual standards of care.

6.3.3 Neoadjuvant immunotherapy and innovative therapies

Immunotherapy by immune checkpoint inhibition has emerged as a potential gamechanger in the treatment of urothelial carcinoma. Since the first promising results in the metastatic setting, leading to the approval of five anti–PD-(L)1 monoclonal antibodies by the FDA in first- or second-line for advanced urothelial carcinoma, there has been a great push to move immunotherapy also in earlier stages of disease, as the neoadjuvant setting[267–271]. A few phase II clinical trials have reported encouraging results in this setting (**Figure 14**), both for immune checkpoint inhibitors monotherapy and for immunotherapy-chemotherapy combination strategies[272].

The single-arm, phase II ABACUS study (NCT02662309)[273] tested 2x neoadjuvant atezolizumab in 95 patients who were cisplatin ineligible. Pathologic complete response rate was 31%, the treatment was well-tolerated by patients and did not result in delays in radical cystectomy planning. At a median follow-up time of 13.1 months, one-year relapse-free survival was 79%, while 17 patients relapsed and 17 died. Associated biomarker studies pointed out that the quality of the tumor-associate lymphoid infiltrate (i.e., presence of preoperative activated CD8+ T cells within the tumor and cytotoxic T cell gene signature) was associated with pathologic complete response, while traditional immunotherapy biomarkers, like TMB or programmed death-ligand 1 (PD-L1) expression, were not.

The single-arm, phase II PURE-01 study (NCT02736266) tested flat-dose 200mg neoadjuvant pembrolizumab administered 3 times every 21 days[92,115,274–276]. In the overall population of 143 patients, pathologic complete response at radical cystectomy was found in 55 (38.5%) patients. At a median follow-up of 23 months, 12- and 24-month event-free survival rates were 84.5% and 71.7%, respectively. Moreover, lower activity of neoadjuvant pembrolizumab was reported in patients with variant histologies of urothelial carcinoma, even if lymphoepithelioma-like urothelial carcinoma and squamous cell carcinoma showed promising responses (i.e., downstaging to non-muscle invasive disease in 6 out of 7 squamous cell carcinoma patients; pathologic complete response in 2 out of 3 lymphoepithelioma-like urothelial carcinoma). Similarly to the ABACUS trial, neoadjuvant pembrolizumab did not delay radical cystectomy, nor resulted into additional surgical morbidity[277]. Importantly, complete pathologic response was associated with preoperative PD-L1 positive status and with higher levels of pre-existing immune infiltration (in particular, IFN signatures).

After these landmarking initial trials, more studies are ongoing testing different combinations of immunotherapy, chemotherapy, and radiation therapy protocols. Concurrent studies to evaluate established and novel biomarkers of response, including the fecal and urinary microbiota, are largely needed to foster a (near) future of biomarker-based, personalized therapeutic protocols.

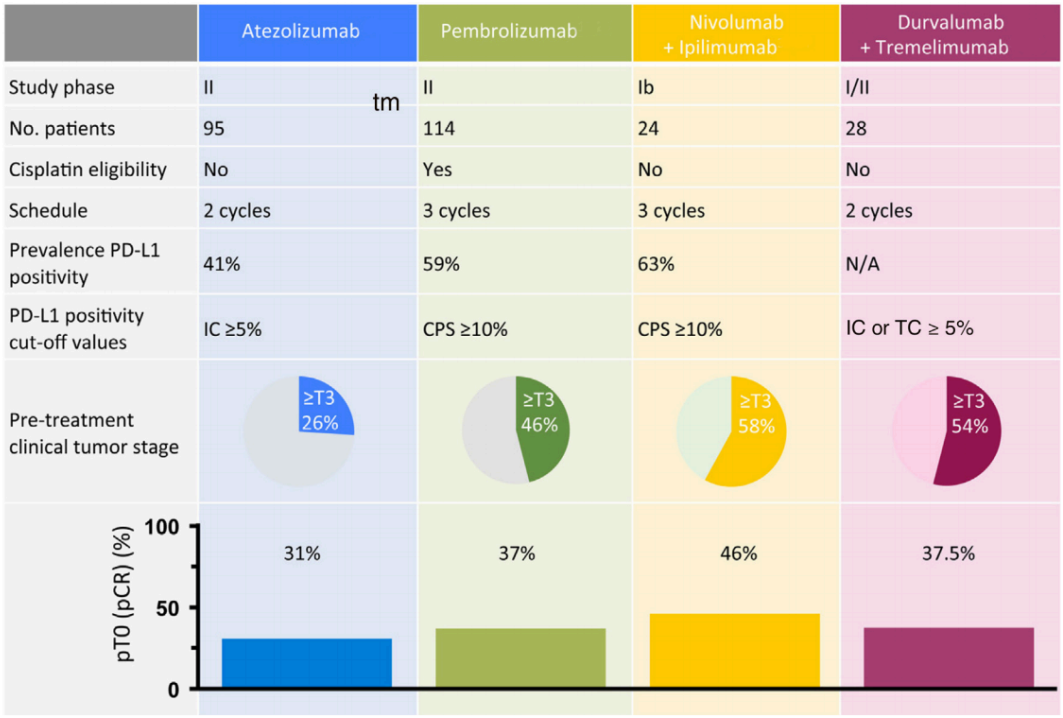


Figure 14 – Main clinical trials testing neoadjuvant monotherapy with immune checkpoint inhibitors in muscle invasive bladder cancer (atezolizumab: NCT02662309; pembrolizumab: NCT02736266; nivolumab + ipilimumab: NCT03387761; durvalumab + tremelimubab: NCT02812420). (Reprinted from European Urology Oncology, Mathieu Rouanne *et al.*[272], "Rationale and Outcomes for Neoadjuvant Immunotherapy in Urothelial Carcinoma of the Bladder", ©2020, with permission from Elsevier.).

7. Introduction – Principles of immunotherapy pathobiology

The introduction into clinical practice of T-cell targeted immunomodulating molecules blocking the immune checkpoints cytotoxic T-lymphocyte antigen 4 (CTLA-4) and PD-1/PD-L1 is probably one of the most important achievements of the last 10 years, as testified by the attribution of the 2018 Nobel Prize in Medicine to the scientists who developed the concept of immune checkpoint inhibition-based immunotherapy, James Allison and Tasuku Honjo. Immune checkpoint inhibitors do not only reinvigorate T cells by releasing their inhibitory breaks, but they also play an effect on other cells of the innate and adaptive immunity (**Figure 15**). Since the game-changing results of the phase III NCT00094653 clinical trial, leading to the FDA approval of ipilimumab to treat metastatic melanoma in 2011[278], several molecules received approval in different stages of disease and for different tumor histologies. I will briefly revise the major biological determinants of immune checkpoint inhibition, with a particular emphasis on PD-1/PD-L1, and its major mechanisms of inhibition. The potential role of the microbiome in mediating immunotherapy efficacy or resistance will be reviewed in the next section.

7.1 Biological bases of PD-1/PD-L1 antitumor activity

The immune system is designed with a series of switch-on and switch-off mechanisms to promote a rapid and massive local activation when needed, and a similarly rapid resolution after completion of its activity. Since the first description of the PD-1 protein in 1992[279], its expression pattern, structure and function on several different cells of the immune system, from T cells to macrophages, from dendritic cells to B cells, have been thoroughly studied[280]. The main effect deriving from the engagement between PD-1 and its ligand PD-L1/PD-L2 is the suppression of the activity of T cells[281,282]. Molecularly, the interaction between PD-1 and PD-L1 causes the phosphorylation of the PD-1 intracellular immunoreceptor tyrosine-based switch motif (ITSM), thus recruiting the Src homology region 2 domain-containing phosphatase-2 (SHP-2), which in turn inhibits the T cells activity by inhibiting several downstream pathways of the T cell receptor (TCR)[283]. The direct inhibition on the TCR pathways, together with a SHP-2-mediated inhibition of cell proliferation and growth (mediated through a modulation of the Ras/MEK/ERK pathway), eventually leads to a dampening of the activated immune response[283–285].

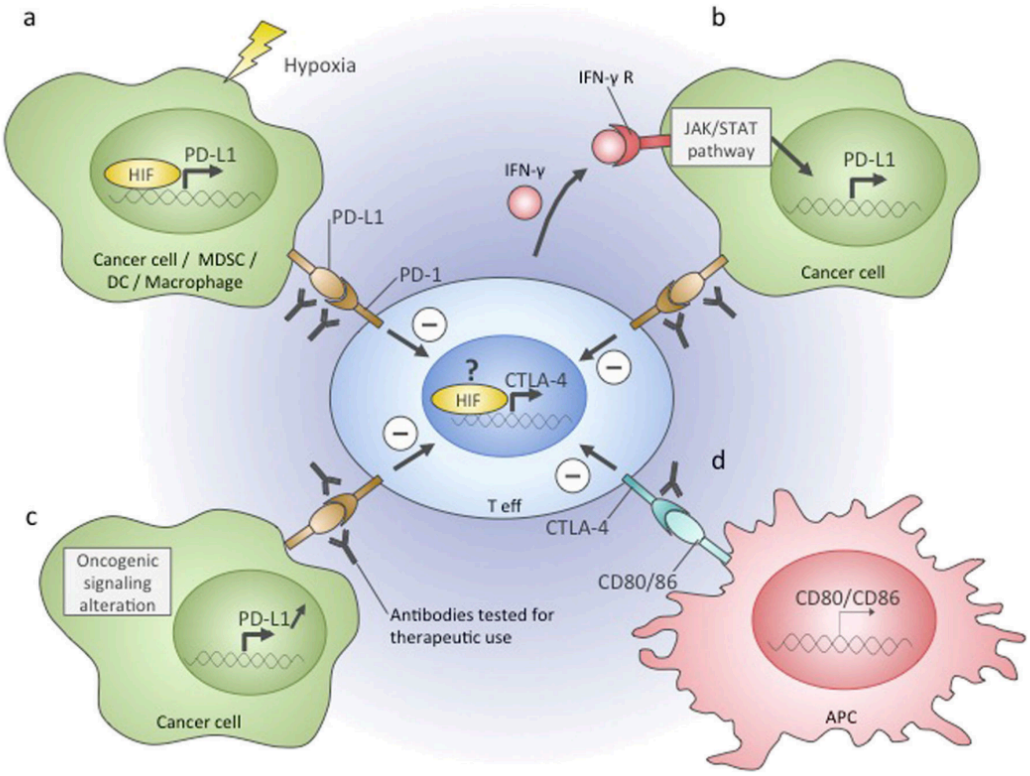


Figure 15 – The targeting of PD-1 and CTLA-4 checkpoint pathways with specific antibodies is a promising therapeutic approach. (T eff = effector T cell; MDSC = myeloid-derived suppressor cell; DC = dendritic cell; IFNγ = interferon gamma; IFN-γ R = interferon gamma receptor; APC = antigen-presenting cell). Reproduced from Petrova *et al.*[286], "The hypoxic tumour microenvironment", Oncogenesis 2018 (the original article is distributed under the terms of the Creative Commons Attribution 4.0 International License).

The utility and efficacy of targeting the PD-1/PD-L1 engagement to increase immune antitumor activity have been demonstrated by a continuously growing number of reports over the last 20 years. A landmark paper[287] published in 2002 showed that the PD-1/PD-L1 engagement was associated with an increased tumor growth, while its inhibition by an anti-PD-L1 antibody resulted in a decreased tumor growth. Similar results were also obtained when tumor cells were implanted in PD-1 knockout mice. These and similar results from preclinical tumor models[288–290], together with the clinical studies showing that tumors expressing higher levels of PD-L1 were linked to worse prognosis than PD-L1-low tumors[291–293], led to the first clinical trials in humans[294–297], paving the way to the wide introduction of immunotherapy in Oncology as it is today.

7.2 Local and systemic changes following immune checkpoint inhibitors

Immune checkpoint inhibitors are generally administered systemically, so that they may impact immune cells not only at the intended location (i.e., the tumor microenvironment, TME), but also everywhere else throughout the body. Indeed, a preclinical study using a mouse model of triple-negative breast cancer showed that, upon administration of immunotherapy, there is a concerted response in the TME and in numerous other organs and tissues, including draining lymph nodes and the bone marrow[298]. Moreover, those "extratumoral" effects on immune cells were fundamental for the immune-mediated anticancer activity. Indeed, treating the animals with a compound (FTY720, Cayman Chemical) that blocks the migration of immune cells from the secondary lymphoid organs invalidated the efficacy of immunotherapy, highlighting that only potentiation of the anticancer immune response at the tumor site is not sufficient to mount an effective tumor clearance. Moreover, a subset population of peripheral $CD4^+$ T cells was expanded in patients responding to immunotherapy, and their transfer into untreated tumor-bearing mice could start an antitumor response. It is clear from this study that engagement of systemic immune cells is as important as engagement at the local tumor site to mount a proper cytotoxic antitumor immune activation.

The evidence derived from preclinical studies – that activation of the immune response at the systemic level is crucial for the efficacy of immune checkpoint inhibitors – brought many clinical investigators to perform immune cell phenotyping in the blood of immunotherapy-treated patients to uncover the main mechanisms of response/resistance

to therapy and to discover potential biomarkers of response. Indeed, lymphocytes (especially T lymphocytes) were the first immune population to be extensively studied in this field, given their central role in the immune-mediated cancer clearance. As expected, several reports confirmed the association between increasing concentrations of peripheral lymphocytes (in particular effector and memory $CD8^+$ T cell and natural killer cells) in blood and immunotherapy efficacy (tumor response and overall survival)[299–303]. A study analyzing matched pre/post immunotherapy blood immunophenotypes in melanoma patients treated with either anti-CTLA-4 or anti-PD-1 highlighted different enriched immune subpopulations based on administered therapy and response[303]. Using mass cytometry (CyTOF) to analyze ~40 markers, the authors found that patients responding to anti-CTLA-4 displayed more $CD45RA^-CD8^+$ T cells and $CD45RA^-CCR7^-$, while patients not responding to anti-PD-1 displayed lower levels of CD69 and MIP-1β expressing natural killer cells.

Based on its target role in immunotherapy, the expression of PD-1 on $CD8^+$ T cells has been studied as a potential marker of response. Indeed, the identification at the tumor lesion (melanoma, non-small cell lung cancer) of $CD8^+$ cells expressing high levels of PD-1 before initiation of immune checkpoint inhibitors was predictive of response and prognostic in terms of overall survival[304,305]. Similarly, other studies focusing on circulating immune cells found that expansion of systemic subsets of $CD8^+PD\text{-}1^{high}$ T cells soon after initiation of immunotherapy was associated with better oncological outcomes[306,307]. Of interest, the importance of $CD8^+PD\text{-}1^{high}$ T cells in orchestrating the immune antitumor activity is not only due to the high expression of PD-1, but also due to the release of the chemokine C-X-C Motif Chemokine Ligand 13 (CXCL13) and CXCL13-mediated recruitment of B cells and T follicular helpers[305].

Even if most of the studies focused on $CD8^+$ T cells, other immune cell subtypes play a central role in immune antitumor activity. For instance, pre-treatment presence of specific $CD4^+$ T cell subsets (e.g., $CD62L^{low}CD4^+$ or memory $CD45RA^-CD4^+$) was associated with response[303,308]. In line with the findings on $CD8^+$ T cells, a study on $CD4^+Foxp3^-PD\text{-}1^{high}$ T cells reported that these cells increase in number in the human and murine melanoma TME as a function of tumor burden, and that their reduction during anti-PD-1 therapy is linked to better oncological outcomes[309]. Within $CD4^+$ T cells, regulatory T cells represent a major subgroup within the TME. The role of Tregs and of specific Tregs

subsets during therapy with immune checkpoint inhibitors is still not completely understood. Some studies reported that a decrease in peripheral and intratumoral Tregs during therapy was linked to a therapeutic success, but others reported that high frequencies of pretreatment peripheral Tregs were associated with better response to ipilimumab[301,310–312]. In a study on a small cohort of patients with localized urothelial bladder cancer treated with anti-CTLA-4[313], the authors found that therapy induced a higher expression of inducible T-cell costimulator (ICOS) on interferon-gamma producing $CD4^+$ T cells both in the TME and in the blood, resulting in an increased ratio of effector/regulatory T cells. Although it was not possible to draw conclusions about the predictive and prognostic values of those findings, the study further highlighted the possibility to use immune phenotyping performed in the blood to monitor changes at the tumor sites.

Other than lymphocytes, macrophages and other cells of the myeloid lineage have been extensively studied in the context of immunotherapy. Preclinical and clinical studies have extensively documented the immunosuppressive role of tumor-associated macrophages and myeloid-derived suppressor cells and their negative effects on the efficacy of immune checkpoint inhibitors, when these immune cells were studied both in the TME and in the peripheral blood.

It emerges evident from the literature briefly reviewed above that immune checkpoint inhibitors promote a perturbation of the immune status of the receiving patients, both at the systemic and at the local levels. Although our knowledge has extensively increased over the last years, the main mechanisms leading different classes of immune cells to response or lack of response after immunotherapy deserve further studies.

7.3 Mechanisms of resistance to immune checkpoint inhibitors

Immunotherapy has revolutionized the therapeutic strategy of a variety of malignancies and offered novel opportunities of a better and longer survival to many patients worldwide. However, a fair proportion of treated patients does not show any benefit from immune checkpoint inhibitors. Understanding the mechanisms of immune checkpoint inhibitor therapy failure may help stratify patients in more effective way, as well as desi-

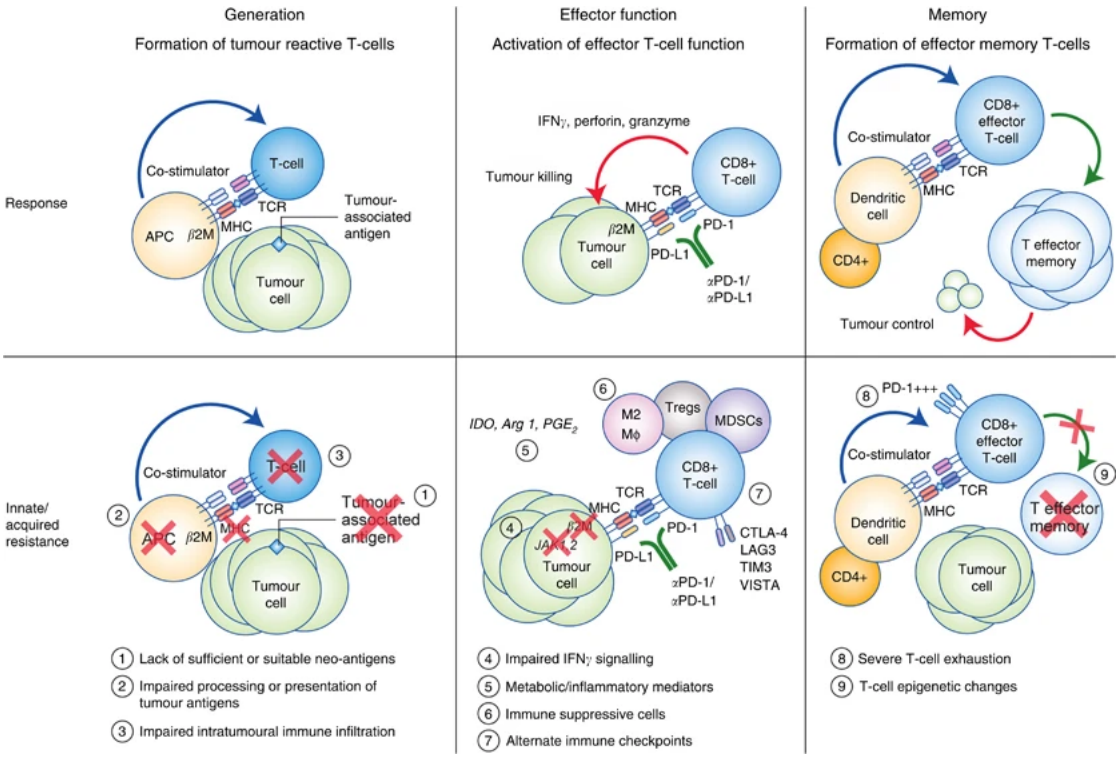


Figure 16 – Response and resistance to ICI therapy. Schematic drawings representing the basic steps involved in generation of tumour-specific T cells, effector T-cell function, and formation of memory T cells (upper panels) and putative mechanisms of innate and/or acquired resistance to immunotherapy (lower panels). Reproduced from Jenkins *et al.*[314], "Mechanisms of resistance to immune checkpoint inhibitors", British Journal of Cancer 2018 (the original article is distributed under the terms of the Creative Commons Attribution 4.0 International License).

gn combinatory strategies to improve response in patients who would otherwise fail immunotherapy (**Figure 16**).

7.3.1 Insufficient generation of antitumor T cells

As the mechanism through which immune checkpoint inhibitors work is the reactivation of antitumor T cells, resistance to this strategy may simply derive from an insufficient *a priori* generation of antitumor T cells. Intrinsic mechanisms leading to impaired antitumor T cell activation include disruption of tumor neoantigens generation, mutations in the antigen presentation apparatus (e.g., class I MHC, b2-microglobulin), and alterations in the intracellular pathways leading to cytotoxic T cell activation[315–318]. Moreover, several extrinsic influences from the TME may impair the correct mounting of the T cell antitumor response, including deleterious signals from cancer-associated stromal cells, exhausted immune cells or local microbiota[315,319]. A higher generation of tumor neoantigens has been associated with a higher genomic instability and higher levels of nonsynonymous mutations in tumor cells, which in turn are generally linked to higher TMB values and/or presence of altered DNA-mismatch repair genes leading to increased microsatellite instability[316,320–322].

During tumor growth, the expression of efficient neoantigens for the cytotoxic T cell tumor clearance can be dampened by the predominant growth of tumor clones that are poorly neo-antigenic or that have acquired genetic mutations leading to the loss of expression of the T-cell-targeted neoantigens[323,324]. The centrality of neoantigens presentation in immune-based therapies is highlighted by the several strategies designed to enhance cell death and antigen presentation (e.g., combined chemo-immunotherapy or radio-immunotherapy protocols, oncolytic viral therapy, type I interferon administration, boosting of dendritic cell maturation, etc.)[315,325].

Not all tumors are equally infiltrated by immune cells, and this difference may also be partially explained by different genomic alterations and intracellular pathway rewiring in cancer cells. For instance, phosphatase and tensin homolog (PTEN) loss in preclinical models of melanoma promoted an immunosuppressive TME by increasing the release of immunosuppressive cytokines (e.g., vascular endothelial growth factor, VEGF), thus leading to less T cell infiltration and T cell-mediated death in tumors[326]. In line with the preclinical findings, PTEN loss in humans led to decreased T cell infiltration in the tumor

lesions, thus resulting in inferior responses to immune checkpoint inhibitors. A striking demonstration of the importance of PTEN loss in mediating response to immunotherapy comes from the case of a patient with uterine leiomyosarcoma, who showed almost complete response in all tumor lesions except for the only, isolated mass with PTEN loss[327]. Furthermore, specific genomic alterations have also an effect on the type of immune cells recruited at the tumor site. For instance, KRAS-mutated tumors with loss of serine/threonine kinase 11/liver kinase B1 (STK11/LKB1) release higher concentrations of IL-6, with a consequent increase in neutrophils mediating T-cell-suppressive effects, and an associated higher expression of T-cell exhaustion markers and pro-tumoral cytokines[328].

Epigenetic modifications in tumor cells result into changes in the expression profile of the cells themselves, including genes potentially involved in antigen processing and presentation, cytokine expression or neoantigens production[329]. Indeed, similar effects can also be obtained using drugs targeting epigenetic modifications. For instance, treatment with DNA methyltransferase or enhancer of zeste homolog 2 (EZH2) inhibitors induces the production and release of Th1 cytokines, enhancing immune checkpoint inhibitors efficacy[330]. Epigenetic modifying compounds (e.g., hypomethylating agents) may also favor immunotherapy activity by interacting with endogenous retroviral elements in non-coding regions of the genome[331]. Notably, tumor-specific endogenous retroviral elements have been linked to antitumor activity and immune cell infiltration, although the precise mechanisms through which endogenous retroviral elements activation may lead to enhanced immune checkpoint inhibitors activity are largely unknown but actively investigated[332,333].

7.3.2 Inadequate cytotoxic T cell activity

After the successful priming and expansion, tumor-specific cytotoxic T cells must overcome several detrimental signals and resistances coming from the TME in order to properly carry out their antitumor activity[315,334].

For an effective antitumor clearance, the pro-death intracellular signaling pathways within tumor cells must work properly; if a mutation occurs in one of the mediators involved in those pathways blocking the activation of the pro-death pathway, the cytotoxic T cell-mediated activity is abrogated[318,335]. NGS studies on tumor tissues from

four melanoma patients who developed delayed resistance to immune checkpoint inhibitors after a first response reported alterations in the interferon-receptor-associated Janus kinases 1 and 2 (JAK1/JAK2), which, together with a concurrent deletion of the wild-type allele, led to complete loss of response to interferon gamma and of its antiproliferative effects[318]. In line with this finding from patient biopsies, resistance to immune checkpoint inhibitors following Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-induced mutations in the interferon gamma receptors and associated proteins (like JAK1/JAK2) were shown in an animal model of melanoma[336]. Notably, pharmacological inhibition of the JAK/STAT signaling in an immune-competent model of melanoma resolved the resistance to immune checkpoint inhibitors, highlighting a still-to-be-understood complexity of the JAK/STAT signaling in the context of immunotherapy[337,338]. Moreover, a patient from the previously mentioned study[318] developed a truncating mutation in the gene for the antigen-presenting protein beta-2-microglobulin (B2M) in the context of delayed, acquired resistance to pembrolizumab, mutation that eventually led to loss of expression of major histocompatibility complex class I, thus impairing antigen presentation.

Expression of PD-L1 in the TME has been extensively investigated in preclinical and clinical studies, especially in the tireless search for a predictive biomarker of response to immune checkpoint inhibitors[339]. Even if PD-L1 expression was found higher in responders by some authors, its predictive and prognostic values are still unclear[296,340]. At the pathobiological level, immune checkpoints are meant to control the extent of the immune response and associated inflammation, to avoid improper and unwanted activation in space and time. Several stimuli can induce the expression of PD-L1 in the tumor microenvironment, including interferon-gamma and associated pro-inflammatory cytokines release by CD8+ T cells themselves, suggesting that the expression of PD-L1 may be intrinsically promoted by the immune cells rather than cancer cells[341]. Moreover, as PD-L1 is expressed on tumor, stromal and immune cells, the specific contribution of PD-L1 expression from each cell type is still under investigation in preclinical and clinical settings[342–344].

Resistance to cytotoxic T cell killing can also be caused by a variety of inhibitory signals, including immune suppressive cells (e.g., regulatory T cells, myeloid-derived suppressor cells, T helper 2 CD4+ T cells, M2 tumor-associated macrophages, etc.), metabolites and

cytokines[315,325,334]. For instance, indolamine-2,3-dioxygenase released by tumor cells in the TME can transform the essential amino acid tryptophan in kynurenine, which has immunosuppressive effects[345]. An increasing number of metabolic pathways are emerging in association with cytotoxic T cell activity and efficacy of immune checkpoint inhibitors, including glucose and cholesterol metabolisms, and future studies will shed more light on this aspect of tumor immunology and immunotherapy.

7.3.3 Compromised T cell memory

One of the main issues with immune checkpoint inhibitors is to ensure a long-lasting treatment effect even after discontinuation of therapy, effect that in turn translates into a durable survival benefit for patients. To accomplish this aim, it is crucial that effector memory T cells are formed and expanded during therapy. A similar mechanism has been shown after PD-1 inhibition, and it was found more pronounced in responders, suggesting that intratumoral effector memory T cells may have a complex (and not yet fully understood) involvement in the response to immune checkpoint inhibitors[346–348]. In this setting, epigenetics seems to play an important role in promoting exhaustion rather than expansion of effector and/or memory CD8+ T cells[347]. Moreover, these epigenetic modifications appear to restrict the durability of CD8+ T cell activity after immune checkpoint inhibition[348].

7.4 Immune related adverse events (irAEs)

As any treatment, immunotherapy has its specific side effects, linked to a uncontrolled activation of the immune system in non-target organs or tissues, thus resulting in a spectrum of *ex novo* autoimmune disorders[349,350]. Indeed, immune checkpoint inhibitors quite frequently cause irAEs, as it is estimated that 30-60% of treated patients report at least an irAE of any severity, with severe irAEs being reported in 10-20% of treated patients. Notably, the frequency and likelihood of irAEs differ between different immune checkpoint inhibitors. Anti-PD-1 drugs appear safer than other agents responsible for pneumonitis, thyroiditis and nephritis, while anti-CTLA-4 compounds are more frequently associated with irAEs, especially hypophysitis and severe colitis[349–352]. Some authors tried to understand if the onset and the severity of irAEs could be a proxy of immune checkpoint inhibitor efficacy, leading to mixed and not unanimous reports. For

instance, in melanoma patients an association between onset of immune-mediated vitiligo and response to therapy has been observed, potentially explainable by a common immune activation against shared antigens in melanoma cells and benign melanocytes[353–355]. However, this association is not so apparent in other malignancies, and it is less clear by a pathobiological standpoint. Another point linked to the discontinuation of therapy because of an irAE is the efficacy of the antitumor response developed up to that point along the therapy schedule, as well as the impact of drugs (mostly corticosteroids) used to treat the irAE on the oncological response. Notably, current evidence shows that discontinuation of immunotherapy due to irAEs and subsequent administration of immunosuppressive agents to limit irAEs do not negatively impact immunotherapy efficacy, although more studies are needed[356–358].

8. Introduction – The microbiome, cancer and anticancer therapy

As discussed in the previous sections, many mechanisms of tumorigenesis and response to antitumor treatments are mediated by cell-specific (tumor cells, immune cells, etc.) processes and phenotypes. However, the tumor microenvironment is also composed by a variety of extracellular factors mediating the complex interactions between the local cell populations and the surrounding extracellular environment[359]. Among the different components of the extracellular environment, the resident host-associated microorganisms, collectively known as microbiome, have gained increasing momentum as major players in mediating the local homeostasis (and its perturbation) at the cell, tissue, and system levels[360–362]. Since the discovery of the microorganism world by Antonie van Leeuwehhoek in the 17th century, the field of microbiome research has evolved from an amateur discipline to a blooming area of intense investigation and economic investment[363]. Notably, the main conceptual shifts in microbiology and microbiome research, from the Koch's postulates to ecological and functional host-microorganism theories, have been frequently associated with technological advances, like the development of NGS technologies and the discovery of phylogenetic markers for community analyses.

8.1 General concepts in microbiome studies

The first definition of "microbiome" was coined in 1988 by Whipps and colleagues, who combined the words "micro" and "biome" to define a "[…] a characteristic microbial community occupying a reasonably well-defined habitat which has distinct physio-chemical properties. The term thus not only refers to the microorganisms involved but also encompasses their theatre of activity"[364]. Over the years, many different definitions have been proposed, the majority of them based on ecological theories. Among the most famous and used definitions, Lederberg defined the microbiome as the "ecological community of commensal, symbiotic and pathogenic microorganisms within a body space or other environment", while Marchesi and Ravel stated that "this term refers to the entire habitat, including the microorganisms (bacteria, archaea, lower and higher eukaryotes, and viruses), their genomes (i.e., genes), and the surrounding environmental conditions. This definition is based on that of "biome," the biotic and abiotic factors of given environments. […]The microbiome is characterized by the application of one or

combinations of metagenomics, metabolomics, metatranscriptomics, and metaproteomics combined with clinical or environmental metadata"[365,366]. Recent groups have proposed to apply a more holistic approach to the study (and the definition) of the microbiome, in which the microorganisms are not only statically studied in the "theater of activity" but also in their dynamic interactions in space and time in the micro- and macro- ecosystems[367].

It is also worth mentioning that the terms "microbiome" and "microbiota", sometimes used interchangeably as synonyms, have a proper definition and use (**Figure 17**). The microbiota is defined as "the assemblage of living microorganisms present in a defined environment"[367], and it is contained within the concept of microbiome (microbiome = microbiota + "theatre of activity").

8.2 Microbiome and cancer – mechanisms in carcinogenesis and progression

The link between microorganisms and specific cancer types is known and well-established, and it is estimated that ~15% of all cancers diagnosed in 2012 was linked to an infectious agent[368]. The most famous case of a human tumor driven by a bacterium is gastric cancer and *Helicobacter pylori* chronic gastritis, a finding for which the 2005 Nobel Prize for Medicine was awarded to Barry J. Marshall and J. Robin Warren. *H. pylori* infection is the strongest known risk factor for gastric cancer, with an attributable risk of ~75%[369], and the International Agency for Research on Cancer (IARC) added *H. pylori* to the list of known human carcinogens in 1994. Other robust evidence linking single bacterial pathogens and cancers has been shown for *C. psittaci* and ocular adnexal lymphomas, and *H. pylori* and gastric mucosa-associated lymphoid tissue (MALT) lymphomas[370–372]. Based on those observations, it was hypothesized that also specific microbiomes, rather than single pathogens, may contribute to the process of carcinogenesis and tumor progression. Indeed, several studies using NGS techniques to characterize the microbiome reported different microbial communities in tumor vs. controls. In this setting, colorectal cancer is among the most studied malignancies, as it is one of the most common cancers worldwide and the gut is one of the richest and best characterized microbial ecosystems in the human body[373]. The first animal evidence linking colorectal cancer to specific microorganisms dates back to the 1960s[374,375], and after those findings in preclinical models several human studies have shed more light on

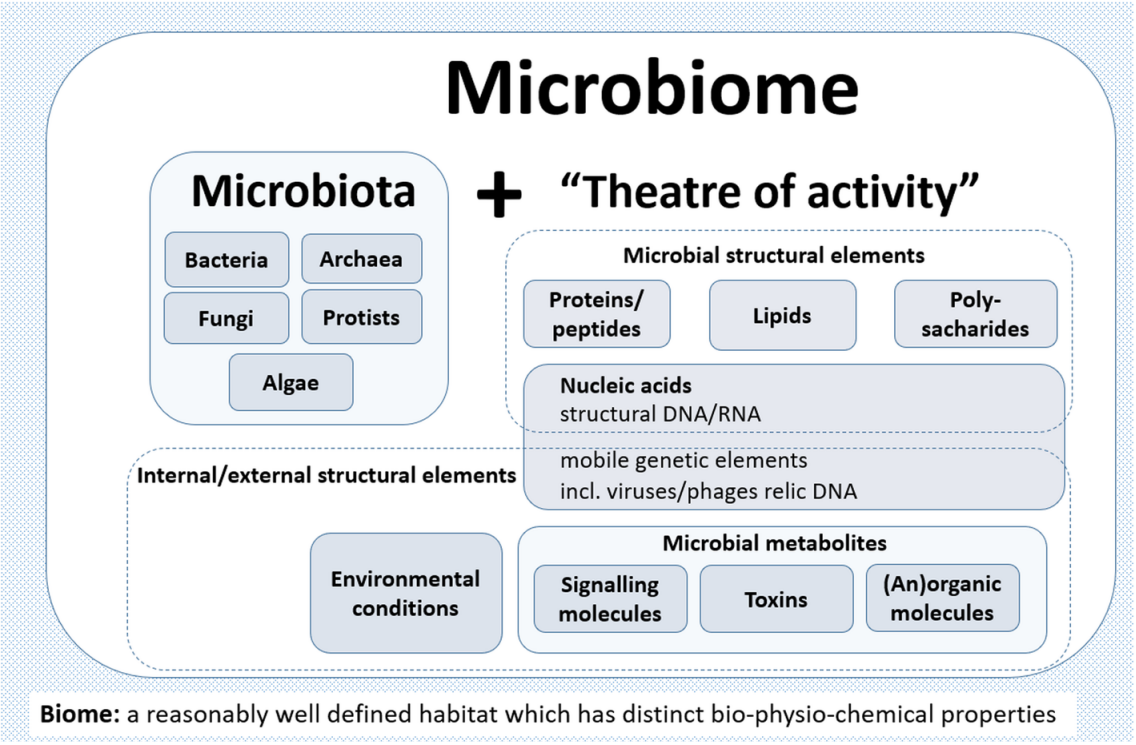


Figure 17 – Schematic representation highlighting the composition of the term microbiome, including both the microbiota and their "theatre of activity". Reproduced from Berg *et al.*[367], "Microbiome definition re-visited: old concepts and new challenges", Microbiome 2020 (the original article is distributed under the terms of the Creative Commons Attribution 4.0 International License).

the role of the gut microbiome in colorectal malignancies. Despite differences in diet, geographical locations, and study populations, meta-analyses of published studies identified a "core" of bacterial species that is consistently more abundant in colorectal cancer patients, including *Bacteroides fragilis*, which has enterotoxigenic properties, *Fusobacterium nucleatum*, and *Prevotella intermedia*[376–379]. Moreover, potentially beneficial bacteria have been found as well, including *Streptococcus thermophilus* and the butyrate-producing *Clostridium butyricum*, which may be implemented in probiotic-based strategies to prevent carcinogenesis or boost anticancer therapy[380,381].

Different microbial mechanisms contributing to colorectal carcinogenesis have been unveiled during the years, and some of them may also have a role in the pathobiology of other malignancies. Firstly, specific microbiome phenotypes may contribute to colorectal cancer development by the establishment and maintenance of a pro-inflammatory environment at the level of the colon mucosa[382,383]. Indeed, chronic colon inflammation is one of the major risk factors for colorectal cancer: patients with ulcerative colitis, an inflammatory bowel disease that causes inflammation and ulcers in the colorectal mucosa, have a 18.4% 30-year cumulative risk to develop colorectal cancer[384,385]. Germ-free mice receiving stools of colorectal cancer patients by gavage showed higher levels of histological (e.g., high-grade dysplasia, macroscopic polyps) and soluble (e.g., C-X-C motif chemokine receptor 2, interleukin 17) signs of gastrointestinal inflammation[386]. Moreover, *F. nucleatum* is able to promote infiltration by myeloid cells of the colonized tissue, thus promoting the formation of a pro-inflammatory environment that could sustain tumor transformation and growth[387,388]. *F. nucleatum* can also promote carcinogenesis in mice by activation of the Toll-like receptor (TLR) 4, which in turn activates the nuclear factor−κB pathway through myeloid differentiation primary response 88 (MYD88), leading to increased proliferation and invasiveness of colorectal tumor cells[389]. Another mechanism through which the microbiome can influence colorectal cancer tumorigenesis is altering the metabolism of dietary and intestinal compounds at the level of the gastrointestinal mucosa. For instance, reduced concentrations of short-chain fatty acids (SCFAs) like propionate or butyrate, molecules that exert anti-inflammatory properties in the gastrointestinal tract, were found in populations at increased risk for colorectal cancer[390,391]. SCFAs are generally produced by intestinal bacteria from the fermentation of nondigestible carbohydrates, such as

vegetal fibers, and mediate their anti-inflammatory activity by inhibiting histone deacetylases and reducing the release of pro-inflammatory cytokines[392–394]. Lastly, specific bacteria may produce genotoxins that directly interfere with host DNA integrity, thus promoting tumorigenesis. For instance, cytolethal distending toxin (CDT) and colibactin, produced by some members of the family Enterobacteriaceae, can induce double-strand DNA breaks in host cells, genomic instability and pro-tumoral effects[395,396]. Interfering with the activity of those genotoxins has shown promising therapeutic effect in preclinical models[397].

Similar mechanisms linking specific bacterial communities to the development and progression of tumor hold true also for other malignancies, potentially including urothelial bladder cancer and other neoplasias arising from body mucosae.

8.3 Microbiome and cancer treatment

Extensive data published over the last decade provided solid evidence that the microbiome plays a central role in the response to anticancer treatments. Moreover, the gut microbiota can also influence the onset and severity of therapy side effects, especially of irAEs. Alexander and colleagues[398] proposed a framework of mechanisms through which the microbiome and chemotherapeutics interact, which they called "TIMER" for Translocation, Immunomodulation, Metabolism, Enzymatic degradation, and Reduced diversity and ecological variation (**Figure 18**). I will now review some of the main mechanisms in the settings of chemotherapy and immunotherapy.

Some studies focused on the interplay between gut microbiota and traditional chemotherapy agents. For instance, the drug cyclophosphamide, an alkylating agent used to treat several hematopoietic and solid malignancies, can induce changes in the gut microbiota and favor translocation of specific Gram-positive bacteria through the gut mucosa into secondary lymphoid organs, where they promote the generation of a peculiar cluster of "pathogenic T helper 17 cells" that mediate the antitumor activity of cyclophosphamide[399]. Nevertheless, the advent of immunotherapy and the strict connection between the immune system and the microbiota have strongly pushed this field of research. The first data were obtained in preclinical models of immunogenic tu-

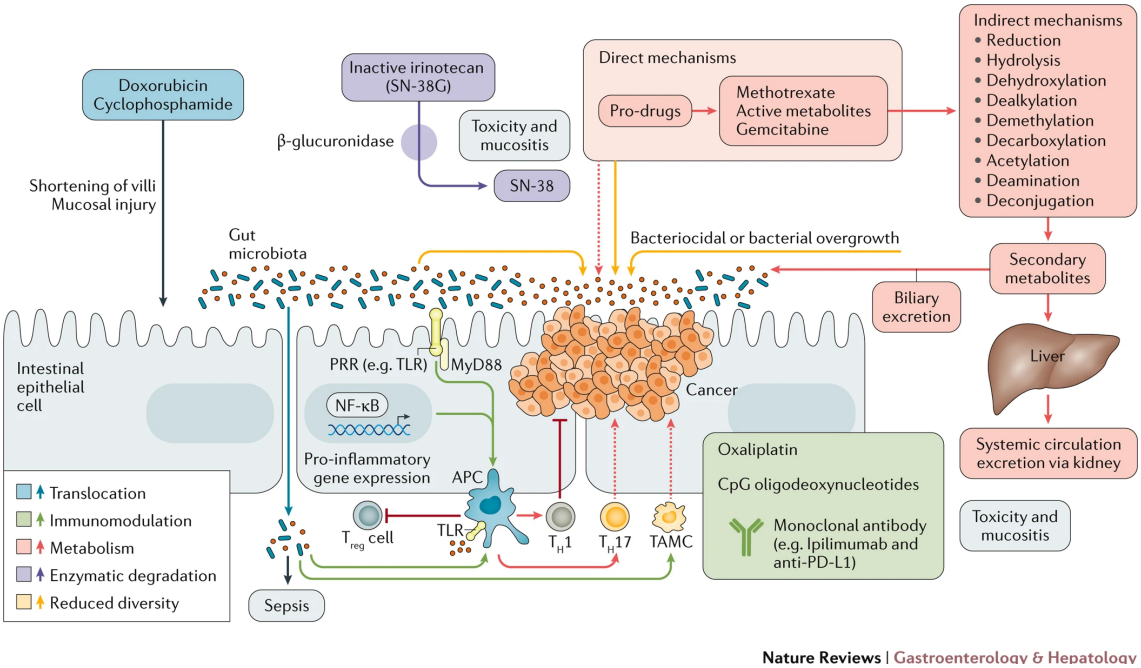


Figure 18 – Multiple mechanisms of influence by chemotherapeutics on microbiota and host metabolism. **Translocation**: cyclophosphamide can cause shortening of the intestinal villi and damage to the mucosal barrier, permitting commensal microorganisms to cross the intestinal barrier and enter secondary lymphoid organs. **Immunomodulation**: intestinal microbiota facilitates a plethora of chemotherapy-induced immune and inflammatory responses. **Metabolism and enzymatic degradation**: direct and indirect bacterial modification of pharmaceuticals might potentiate desirable effects, abrogate efficacy or liberate toxic compounds. **Reduced diversity**: chemotherapy induces changes in the diversity of the mucosal and fecal microbiota through altered biliary excretion and secondary metabolism or associated antibiotic use and dietary modifications. (PRR = pattern-recognition receptor; TAMC = tumour-associated myeloid cell; TLR = Toll-like receptor; Treg cell = regulatory T cell). Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nature Reviews Gastroenterology & Hepatology, "Gut microbiota modulation of chemotherapy efficacy and toxicity" by James L. Alexander *et al.*[398], © 2017 All rights reserved.)

mors like melanoma[400,401]. In one study, the presence in the murine gut microbiome of *Bifidobacterium spp.* increased the spontaneous antitumor activity against melanoma tumors[400]. Moreover, a combination strategy with *Bifidobacterium spp.* administered together with immune checkpoint inhibitors showed greater antitumor activity than each treatment alone, providing an argument in favor of the manipulation of the microbiota as a way to boost immunotherapy. Similar results were obtained by another independent study, showing the beneficial link of anti-CTLA-4 agents and distinct Bacteroides species[401].

Following the exciting results in animal models, several groups focused on the potential clinical and translational relevance in human populations. The main design of these clinical studies is based on the comparison of "responder" vs. "non-responders" cohorts, to identify microbial phenotypes, signatures or isolated species that are enriched in one group with respect to the other, and the findings are sometimes corroborated by animal experiments in which the candidate bacterial communities are transplanted and tested in a controlled setting[402–405]. For instance, Matson and colleagues[402] collected fecal specimens from a cohort of 42 melanoma patients before treatment with PD-1 inhibitors (4 of them received CTLA-4 inhibitors, with similar results to the anti-PD-1 cohort). Overall, they found a 38% response rate, as defined using the Response Evaluation Criteria In Solid Tumors (RECIST) version 1.1 criteria, resulting in 16 responders and 26 nonresponders. They found specific bacterial microbiota phenotypes associated with clinical response; in particular, *Bifidobacterium longum*, *Collinsella aerofaciens*, and *Enterococcus faecium* were enriched in responders. Fecal microbiome transplant (FMT) in tumor-bearing germ-free mice using feces collected from responders was able to improve antitumor immune activity, ultimately resulting in increased tumor control and immunotherapy efficacy. Using a similar design, Gopalakrishnan and colleagues[403] analyzed specimens from 112 melanoma patients treated with anti-PD-1 agents and found bacterial communities enriched in responders (*Clostridiales*, *Ruminococcaceae*, *Faecalibacterium*) or in nonresponders (*Bacteroidales*, *E. coli*). Moreover, the "responders' microbiota" was associated with a higher concentration of effector cytotoxic T cells, while the "nonresponders microbiota" favored increased levels of Tregs and a more inhibitory soluble cytokine profile. Of note, to date there has been little overlap between the different microbiome phenotypes linked to immunotherapy response in the

published studies. More original reports and meta-analyses are needed to expand the field, but it may also be possible that different inhibitors could be associated. With different beneficial immune signatures, so that the hypothesis of a "core immunotherapy microbiome fits all inhibitors" may not be true.

Besides its modulatory effects on the efficacy of anticancer therapy, the microbiome has also been implicated in mediating adverse events and treatment toxicities. For instance, a study in melanoma patients treated with the anti-CTLA-4 ipilimumab found that a gut microbiota rich in *Bacteroidetes* was protective against the development of ipilimumab-induced colitis[406]. This study also suggested that the risk of developing ipilimumab-induced colitis was associated with a decreased microbe-mediated production of B vitamins (thiamine, riboflavin and pantothenic acid) and a decreased bacterial polyamine transport system. Another study on a small cohort of ipilimumab-treated metastatic melanoma patients found that patients with *Firmicutes*-enriched (especially *Faecalibacterium spp.*) gut microbiota showed the best oncological response, as well as the higher rate of therapy-induced colitis[407]. This report highlights that uncoupling the good and bad effects mediated by the microbiome or by single bacterial taxa is not trivial, but it is a fundamental topic to be investigated to confidently manipulate the microbiome to enhance clinical outcomes.

Although less studied, the intratumoral microbiome may contribute to the response to anticancer therapy as well. Indeed, intratumoral bacteria can enzymatically interact with the administered drugs, potentially increasing or decreasing their pharmacological activity and/or local concentrations[408]. For instance, bacteria expressing the enzyme cytidine deaminase can degrade the chemotherapeutic agent gemcitabine into its inactive form. Indeed, presence of cytidine deaminase-expressing *Gammaproteobacteria* in a mouse model of colorectal cancer decreased the efficacy of gemcitabine, efficacy that was rescued by the concurrent administration of antibiotics[409]. Besides enzymatic modifications, other microbiota-mediated mechanisms may alter response to cancer therapies. Members of the *Fusobacterium* genus were found enriched in colorectal cancer tumors resistant to chemotherapy, suggesting a role in resistance to chemotherapy[410]. Indeed, *F. nucleatum*, by activating the TLR4-MYD88 pathway in colorectal cancer cells, promoted the activation of autophagy mechanisms that in turn blocked chemotherapy-

mediated apoptosis. Thus, targeting *F. nucleatum* in colorectal cancer patients may be a strategy to improve response to chemotherapy.

In addition to the mechanisms related to chemotherapy efficacy, intratumoral bacteria may have an immunomodulatory effect (either immunostimulatory or immunosuppressive) on the host immune system and on immunotherapy-mediated anticancer therapies. Intratumoral bacteria may promote local inflammation and immune cell recruitment through the activation of pattern recognition receptors (PPRs) pathways, activating the release of proinflammatory cytokines[411,412]. However, other reports showed that the intratumoral microbiota may also play an immunosuppressive effect. For instance, enterotoxigenic *Bacteroides fragilis*, a potent initiator of IL17-dependent colon tumorigenesis in a mouse model of colorectal cancer, promoted the differentiation of protumoral monocytic- myeloid-derived suppressor cells, and eventually led to the formation of a immunosuppressive TME[413].

8.4 Microbiome as a biomarker

Given the link between microbiome, dysbiosis and cancer, microbial communities and their relative changes are extensively studied as potential predictive and prognostic biomarkers of diseases, both at the level of cancer screening and at the level of therapy efficacy monitoring. In colorectal cancer, a study compared the diagnostic accuracy of the standard fecal occult blood test (FOBT) vs. a metagenomic fecal signature identified in colorectal cancer patients (and validated in its accuracy using an independent cohort) vs. a combination of both tests[414]. Overall, the accuracy of the FOBT or the metagenomic signature alone was similar, while the combination of the 2 tests resulted in a >45% improvement in sensitivity to detect colorectal cancer over the FOBT alone at identical specificity. Another study evaluated the predictive value of some bacterial species commonly enriched in colorectal cancer (*Fusobacterium nucleatum*, *Bacteroides clarus*, *Roseburia intestinalis*, *Clostridium hathewayi*, and one undefined species) as measured by duplex-quantitative polymerase chain reaction (PCR) in stool samples from 203 patients and 236 healthy controls[415]. Areas under the receiver operating characteristic (ROC) curve for *F. nucleatum* alone or the combination of bacteria was similar and ~0.85. Of note, combination of the duplex-quantitative PCR measurements with an approved fecal immunochemical test for the detection of colorectal cancer resulted in good

diagnostic results (sensitivity ~90%, specificity ~80%). Similarly, another group evaluated the diagnostic performance of quantitative PCR for *Fusobacterium nucleatum*, *Peptostreptococcus anaerobius* and *Parvimonas micra* against traditional fecal immunochemical test in a cohort of 309 subjects with colorectal carcinoma at different stages and healthy controls[416]. The quantification of *F. nucleatum* provided the highest accuracy among the selected bacteria and it provided higher sensitivity when combined with the fecal immunochemical test compared to the immunochemical test alone (92% vs. 73%, $p<0.001$) in detecting colorectal cancer. Potential microbiome-based screening strategies have also been tested in other malignancies, like head and neck cancers. In a recent preprint, a machine-learning-based taxonomic and functional metatranscriptomic classifier based on the saliva microbiome showed a sensitivity up to 83% and specificity up to ~98%[417]. Based on the findings from this study, the USA-based microbiome company Viome has realized a clinical diagnostic test that received Breakthrough Device designation from the FDA in May 2021[418]. We are just starting to see the clinical implementation of microbiome-based diagnostics, and studies are ongoing to independently validate microbial signatures of response to therapy in different malignancies and disease stages. Moreover, as suggested by the abovementioned case of Viome, this is an area of great business interest, and in the near future novel microbiome-based diagnostic tests will enter the clinical practice to improve patient stratification and treatment.

8.5 The microbiome as a therapeutic target in cancer therapy

Given the strict connection between microbial communities, cancer, and cancer therapy, it comes with no surprise that different ways to actively modify the microbiome towards a favorable state have been tested in animal models and in humans. The prototypical example in this field is represented by FMT for the treatment of refractory *C. difficile* diarrhea, which was firstly reported in 1958 in contemporary medicine and can be resolutive in up to 80% of affected patients[419,420]. Along this line, the idea to use FMT to improve response to immunotherapy and overcome resistance, thus transplanting an immune favorable microbiota, has been recently tested in two first-in-human clinical trials[421,422]. Davar and colleagues enrolled 15 melanoma patients resistant to anti-PD-1 therapy, defined as no response or progression during immune checkpoint inhibitors

therapy, and the patients firstly underwent FMT using feces from subjects who responded to immunotherapy, then additional cycles of pembrolizumab up to progression[421]. Notably, 6/15 patients showed clinical benefit from the therapeutic protocol (i.e., 3 patients with partial response, 3 patients with stable disease at 1 year), and their gut microbiome was stably and resiliently modified towards a 'immune-beneficial status', with an associated shift towards a favorable blood immune phenotype. Similarly, Baruch and colleagues treated 10 melanoma patients who progressed during or after immunotherapy by nivolumab + recurrent FMT from 2 donor patients who had a complete tumor eradication thanks to immune checkpoint inhibitors[422]. Notably, 3/10 patients showed response to therapy, including one complete response with no residual tumor. Moreover, the 3 patients with clinical benefit showed immune signatures of response in the lamina propria and in the tumor microenvironment, suggesting a microbiome-mediated mechanism behind. Although those two studies reported promising results and acceptable safety profiles, some caution should be considered in the use of FMT, which is classified as a live biological product and a drug by the FDA[423].

Besides FMT, the notion to exploit bacteria to treat cancer dates back several decades[201,424,425]. Indeed, it was shown that some bacteria, if injected in tumor-bearing mouse models, preferentially gather in the TME, probably due to a combination of increased vascularization of the tumor area and changes in the local pH, oxygen concentration and other biochemical parameters[426–428]. Engineered bacteria have been successfully implemented in preclinical models to target tumor lesions and promote tumor clearance[429,430]. For instance, Ho and colleagues designed engineered commensal *E. coli* to express the enzyme myrosinase, which mediates the transformation of dietary glucosinolates to sulphoraphane, an organic small molecule with known anticancer properties[430]. Moreover, the engineered *E. coli* expresses a surface molecule that promotes the attachment of the bacteria to heparan sulphate proteoglycans expressed on colorectal cancer cells, thus increasing the specificity of the treatment. The efficacy of the strategy was demonstrated both *in vitro* and in animal models. In another study, Chowdhury and colleagues employed synthetic biology techniques to engineer a non-pathogenic *Escherichia coli* strain to selectively lyse within the TME and release an encoded nanobody antagonist of CD47 (CD47nb)[431]. Delivery of the engineered *E. coli* to the tumor lesion increased local activation of tumor-infiltrating T-cells, with resulting

decreased tumor volume, metastasis rate and increased tumor survival in murine tumor models. Similar synthetic biology approaches have been used to engineer strains of *Bifidobacterium* to express the enzyme cytosine deaminase to promote *in situ* activation of the nontoxic 5-fluorocytosine into the cytotoxic 5-fluorouracil (5-FU)[432]. Several of these live therapeutics are currently tested in clinical trials for the treatment of solid malignancies (e.g., NCT03847519, NCT04167137, NCT03750071).

Over the last few years, a few biotech companies entered the market with microbiome-based products to improve the efficacy of oncological therapies, and especially of immunotherapy. For instance, 4D pharma plc is a company founded in 2014 that developed a proprietary platform called MicroRx® to identify single-strain bacteria to repurpose as live biotherapeutics based on the biology of the single bacteria and of their interactions with the host[433]. The company has five clinical trials ongoing, three of them (NCT04193904, NCT03934827, NCT03637803) testing their product MRx0518 in solid tumors. MRx0518 is a proprietary single strain live biotherapeutic based on the motile anaerobe *Enterococcus gallinarum*[434]. It is delivered as an oral capsule and stimulates the body's immune system mostly through its flagellin protein, increasing the $CD8^+$/Treg ratio at the tumor site, thus promoting immune-mediated tumor clearance[435]. Among the clinical trials currently testing MRx0518, the most relevant for our discussion is the phase I/II MRx0518-I-002 (NCT03637803) study, testing the safety and efficacy of MRx0518 together with pembrolizumab in up to 132 patients (part A 12, part B 120) with solid tumors (non-small cell lung cancer, renal cell carcinoma, bladder cancer or melanoma) who previously progressed on anti-PD-1/PD-L1 therapy. Among the 12 patients enrolled in part A, there were no treatment-related Grades 4 or 5 serious adverse events, and median progression-free survival was 2.14 months (data cut-off: October 23, 2020)[436].

Another company testing live biotherapeutics is Vedanta Biosciences Inc, which developed a proprietary consortium (called VE800) of 11 clonal human commensal bacteria strains known to potentiate the immune system efficacy and, potentially, cancer immunotherapy[437,438]. VE800 is currently tested in the phase I/II CONSORTIUM-IO (NCT04208958) clinical trial, testing the safety and efficacy of VE800 in combination with nivolumab in patients with advanced/metastatic melanoma, gastric or gastroesophageal junction adenocarcinoma, or microsatellite-stable colorectal carcinoma.

As the interest for microbiome-based approaches to improve cancer treatment is

exponentially growing, more and more clinical trials are expected to open in the near future.

8.6 Microbiome, bladder, and bladder cancer

"*The fresh and healthy urine is perfectly free from bacteria or other minute organisms. The ordinary types of morbid urine, although they may contain blood, pus, or casts of tubes, are equally free from organisms.*" These words uttered by bacteriologist William Roberts in 1881[439] recapitulates the dogma of the "sterility of urine", which held in place for over a century, with only few dissenting opinions[440]. One of the main reasons why the idea of the sterility of urine held stable through the years may be found in the inadequate standard culture technique applied to urinary microbiology and the derived false equation "culture-negative=sterile". Indeed, this conviction has been proven wrong with the implementation of culture-independent methods, such as 16S ribosomal RNA (rRNA) gene amplicon sequencing, applied to urine specimens, and with the introduction of novel culture protocols based on a combination of different growing conditions[441–443]. For example, Hilt and colleagues hypothesized that the lack of growing bacteria in routine urine cultures was not due to the lack of bacteria in the urine, but rather to the low number of inoculated bacteria and/or the need for special culture conditions (e.g., aerobic/anaerobic/microaerophilic growing conditions and longer incubation times)[444]. Applying this differential culture and incubation protocol, which they named "expanded quantitative urine culture" (EQUC), they could grow bacteria in most (52/65, 80%) of the analyzed urine specimens from women with overactive bladder and from healthy controls. Notably, the majority (48/52, 92%) of EQUC-positive samples were reported negative using standard urine culture protocols. Moreover, they showed that many bacteria isolated by EQUC were also identified using metagenomic sequencing of the same specimens, providing evidence of the viability of the bacteria present in the urinary environment. Although the field of urinary microbiota, and especially of bladder microbiota, is still at its dawn compared to other human microbiota, it is arousing growing interest as a potential mediator of therapy efficacy in genitourinary tumors and as a potential biomarker of treatment response.

In bladder malignancies, few studies investigated differential microbial populations in the urine of patients and controls. Chipollini and colleagues reported that urine collected from

patients with invasive bladder cancer showed a significant enrichment of *Bacteroides* and *Faecalibacterium*, while urine from patients with superficial bladder lesions were similar to controls[445]. Wu and colleagues found the *Sphingobacteriaceae* bacterial family more abundant in male bladder cancer patients[446], whereas Popović and colleagues reported an enrichment of *Fusobacterium*, *Actinobaculum*, *Facklamia*, and *Campylobacter* genera in their male bladder cancer patient cohort[447]. Furthermore, Mai and colleagues analyzed a mixed cohort of male and female bladder cancer patients and found *Enterococcus*, *Enterobacteriaceae*, and *Lactobacillaceae* as the most common taxa[448]. Lastly, Oresta and colleagues performed a comprehensive metagenomic analysis to compare different urine collection methods (catheter-collected urines, midstream voided urine and bladder washings) in the setting of bladder cancer[449]. Different bacterial taxa were identified as enriched in bladder cancer patients depending on the different collection method. Altogether, they concluded that catheter-collected and bladder washings were less subjected to contaminations. Moreover, presence of bladder cancer was associated with a decrease in anti-inflammatory bacterial taxa and an increase in opportunistic bacteria. Overall, the studies published about bladder cancer urinary microbiota are far from being concordant on the bacterial taxa associated with cancer. The heterogeneous results from some of the above-mentioned studies can be explained by the different composition of the study cohorts, in terms of patients' race, sex, and extension of the disease (i.e., muscle invasive vs. non–muscle invasive urothelial carcinoma). For example, the reported differences can be partially attributed to sex-specific microbiome composition[152,450].

It is worth mentioning that a bacteria-based treatment (i.e., intravesical BCG immunotherapy) is currently in use to treat bladder cancer and its implementation in the clinical practice dates to the last century. Since the first report by Morales and colleagues in 1976[201], BCG has become the mainstay in the management of high-grade, non-muscle invasive bladder cancer patients. Despite our appreciation of the mechanisms behind BCG anticancer activity is not yet complete[204], it is reasonable to think that the urinary and bladder-tissue microbiota can influence the efficacy of BCG immunotherapy. For instance, the BCG might compete with the local bacterial species in the urothelial microbial niche for adhesion[451] to the extracellular protein fibronectin, the first step needed to mount a BCG-induced antitumor response.

In muscle-invasive urothelial carcinoma of the bladder, neoadjuvant immunotherapy by single-agent immune-checkpoint inhibitor has been proven effective in eradicating the disease, aspiring to become a game changer in the management of bladder cancer patients, as previously reported in this dissertation. Several studies have shown that the efficacy of immunotherapy is strictly linked to the modulation of the enteric commensal bacterial microbiota; therefore, a potential role of the gut microbiota in modulating the efficacy of immune checkpoint inhibitors in urothelial carcinoma is plausible and deserves further investigations. At the same time, it is not known if the urinary and bladder-bound microbiome may play a role in the same setting. Further studies are needed to answer these open questions.

9. Aims of the research project

The aim of this research project is to gain preliminary evidence of the potential role of the microbiome as a biomarker or as an actionable target to improve the management and care of bladder cancer patients. The goal of my thesis was to uncover the role of the microbiome in bladder cancer and during therapy. To this end, my contribution to this project is divided into three main research aims:

A. Characterize the urinary microbiota associated with bladder cancer.

The aim of this first experimental section is to provide a sex-based characterization of the urinary and urothelium-associated microbiome in bladder cancer. For this aim, I used biological specimens from the biobank of the Urological Research Institute (IRCCS Ospedale San Raffaele, Milan) collected from therapy-naïve patients undergoing radical cystectomy.

B. Identify the role of microbiome during neoadjuvant immunotherapy for the treatment of muscle-invasive bladder cancer.

The aim of this experimental section is to identify if the gut microbiome and its perturbations, like due to concurrent antibiotic therapy, may play a role on the response to neoadjuvant pembrolizumab for the treatment of muscle-invasive bladder cancer. In this aim, I analyzed data and specimens from patients included in the phase 2 PURE-01 clinical trial, testing a neoadjuvant immunotherapy protocol (pembrolizumab 200mgx3, every 21 days) for the treatment of muscle-invasive bladder cancer.

C. Design novel models to study the mechanisms played by the microbiota in bladder cancer.

The aim of this experimental section is to design novel *in vitro* and *in vivo* models to study the pathobiological role played by the different microbial communities identified in the previous aims on the normal bladder and bladder cancer cells. To this end, I developed a protocol for mice studies and a novel co-culture system to study cell host-microbiome interactions *in vitro*.

10. Results

10.1 Characterization of the urinary and tissue-bound microbiota associated with bladder cancer

As previously mentioned, bladder cancer displays sex-based differences in its epidemiology and pathobiology, and some of this sex-based divergence may be attributable to a different microbiome in the genitourinary tract. This hypothesis appears plausible, if the anatomical and physiological differences of the genitourinary tract are considered in women and men. Therefore, we firstly aimed to provide a sex-based description of the urinary and urothelium-bound microbiota in therapy-naïve urothelial bladder cancer.

To this end, we profiled by 16s metagenomics the urinary microbiota of 59 healthy controls (34 men and 25 women) and 49 therapy-naïve bladder cancer patients (36 men and 13 women)[452]. General anamnestic details for the study cohorts can be found in **Table 1**. All enrolled subjects provided a morning voided urine specimen, collected into a sterile container, according to standard urinalysis protocols. Bladder cancer patients were asked to collect the urine sample the same day of the radical cystectomy surgical procedure. Using β-diversity (i.e., a measure of the degree to which samples are different among themselves) metrics on the overall cohort, we found distinct clustering of male vs. female urinary samples, highlighting the need for sex stratification (**Figure 19, A-C**). In urine samples, diversity measures (α-/β-diversity) did not show differences between male bladder cancer patients and controls (**Figure 19, D, E**), but β-diversity between the female cohorts (**Figure 19 F, G**). Quantification of 16S rDNA by digital droplet polymerase chain reaction (ddPCR) showed a higher bacterial load in the urine of patients when compared to controls, in both male and female cohorts (**Figure 20, A-B**). In terms of bacterial taxa composition, the urinary microbiome was dominated by the members of the three major bacterial phyla Proteobacteria, Firmicutes, and Bacteroidetes, which accounted for ~80% (male) and ~90% (female) of all identified taxa. Using linear discriminant analyses (LDA), we identified 26 (male) and 48 (female) taxa differently enriched between healthy and bladder cancer cohorts up to the genus level (**Figure 20, C-D**).

	Male BCa (*N* = 36)	Male controls (*N* = 34)	Female BCa (*N* = 13)	Female controls (*N* = 25)
Age (years), mean (IQR)	69 (67–77)	62 (56–70)	62 (51–70)	59 (56–62)
Smoking status, *N* (%)				
Current smokers	9 (25)	0 (0)	3 (23)	5 (20)
Former smokers	23 (64)	1 (3)	4 (31)	1 (4)
Never smoked	4 (11)	33 (97)	6 (46)	19 (76)
Hydronephrosis (grade I–II), *N* (%)				
No hydronephrosis	24 (67)	–	8 (62)	–
Unilateral	10 (28)	–	4 (31)	–
Bilateral	2 (5)	–	1 (7)	–
pT stage at RC, *N* (%)				
≤pT2	12 (33)	–	4 (31)	–
>pT2	24 (67)	–	9 (69)	–
pN stage at RC, *N* (%)				
pN0	20 (56)	–	7 (54)	–
≥pN1	16 (44)	–	6 (46)	–
Tumor focality at RC, *N* (%)				
Unifocal	11 (31)	–	5 (38)	–
Multifocal	25 (69)	–	8 (62)	–

BCa = bladder cancer; IQR = interquartile range; RC = radical cystectomy.

Table 1 - Main features of therapy-naïve bladder cancer patients and controls.

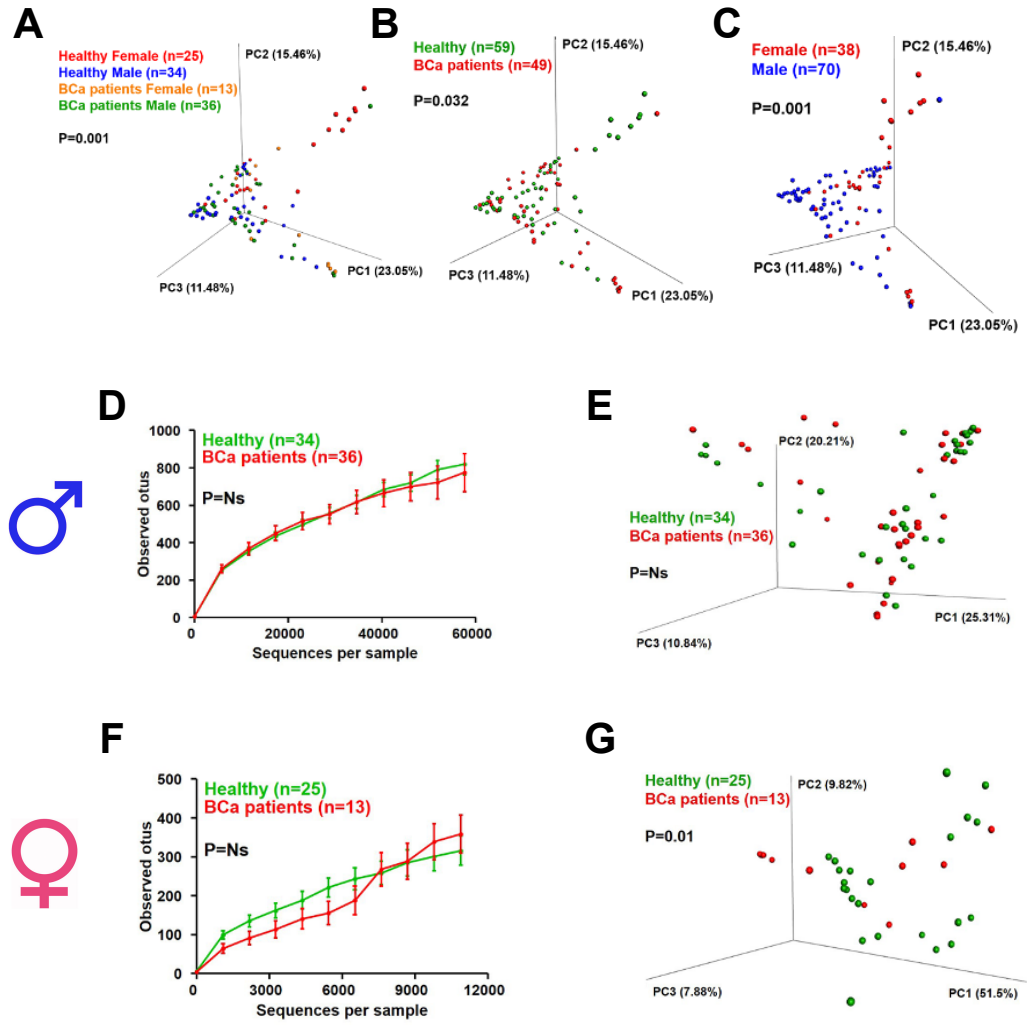


Figure 19 – Urinary microbiome is sex specific. **A-C.** Diversity between study cohorts (β-diversity) was evaluated by principal component analysis using weighted UniFrac distance matrices and represented by weighted variance used to visualize relationships and differences among the urine samples of (A) all tested cohorts (healthy male and female individuals and male and female bladder cancer patients), (B) cohorts stratified for healthy and bladder cancer categories (all healthy individuals vs. all BCa patients), and (C) cohorts stratified for the sex (healthy male individuals and bladder cancer male patients vs. healthy female individuals and bladder cancer female patients). **D-E.** Microbial community diversity between the urine of healthy controls and male bladder cancer patients, evaluated for (D) the diversity within samples (α-diversity) and (E) β-diversity between groups. **F-G.** Microbial community diversity between the urine of healthy controls and female bladder cancer patients, evaluated for (F) the diversity within samples (α-diversity) and (G) β-diversity among groups. (BCa = bladder cancer)

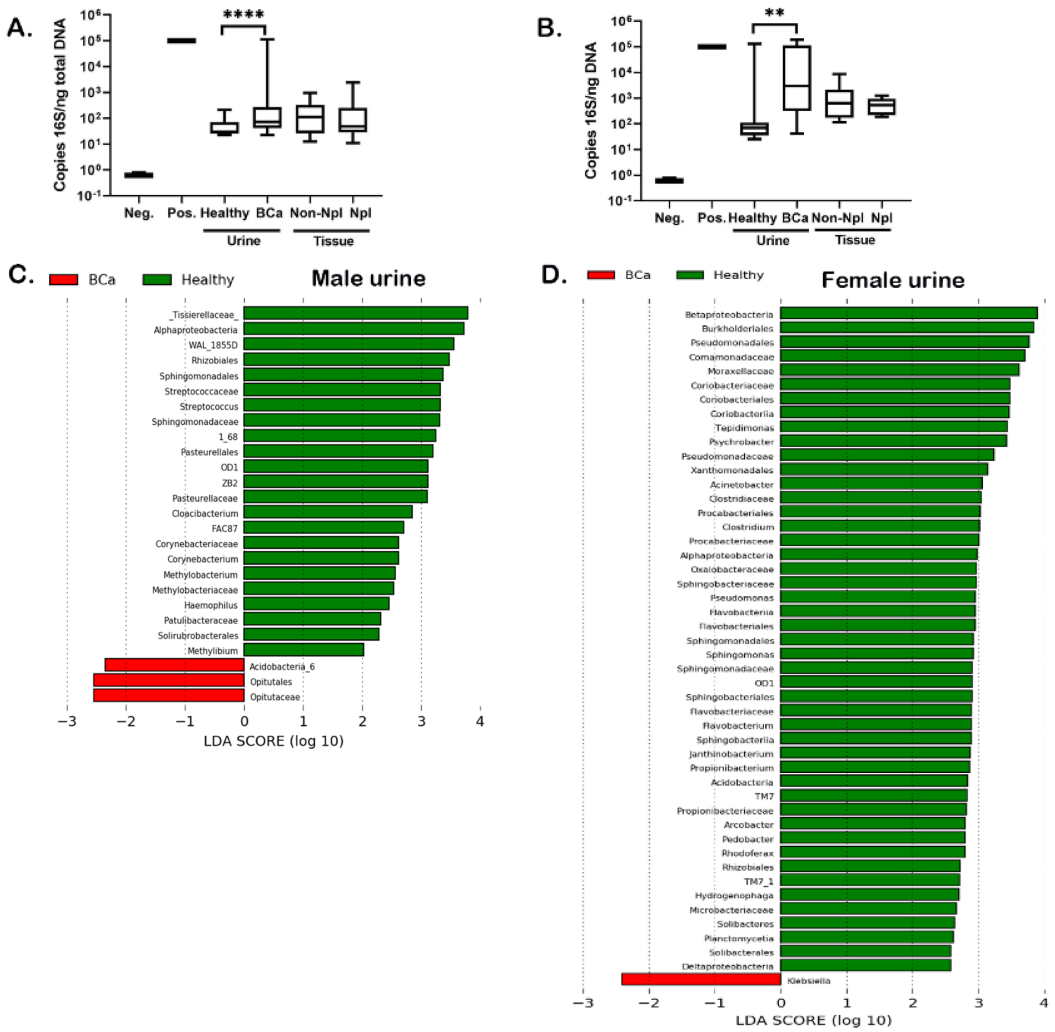


Figure 20 – Urinary microbiome richness in male and female controls and bladder cancer patients. **A**. Whiskers plot (95% confidence interval) of 16S rDNA quantification of the urine and tissue of healthy men (n = 34) and bladder male patients (n = 36), and non-neoplastic (non-Npl; n = 21) and neoplastic (Npl; n = 21) tissues of bladder cancer male patients. **B**. Whiskers plot (95% confidence interval) of 16S rDNA quantification of the urine of healthy women (n = 25), urine of bladder cancer female patients (n = 13), and non-Npl (n = 8) and Npl (n = 8) tissues. **C-D.** Scores of taxonomic biomarkers down to genus level identified by linear discriminant analysis (LDA) using LEfSe in urine of (C) healthy versus bladder cancer men and (D) healthy versus bladder cancer women. (BCa = bladder cancer; Neg. = negative control, represented by the PC3 cell line cultured in the presence of penicillin/streptomycin; Pos. = positive control, represented by oral mucosal swab; ** $p \leq 0.01$; **** $p \leq 0.0001$)

Then, to compare potential differences between paired neoplastic [Npl] and non-neoplastic [non-Npl] tissues from the same cystectomy specimens, we collected pairs of tissue biopsies from 21 men and 8 women of the original urine cohort. Using β-diversity metrics on the tissue cohort, we found distinct clustering of male vs. female bladder tissues (**Figure 21, A-C**). Quantification of 16S rDNA by ddPCR did not show differences in bacterial load between paired neoplastic and non-neoplastic tissues (**Figure 20, A-B**). However, compositionally the Npl tissues were enriched in the class *Betaproteobacteria* and subordinate order *Burkholderiales*, family *Burkholderiaceae*, down to genus *Burkholderia* (**Figure 21, D-E**) in both sexes. Then, we analyzed triplets of specimens (i.e., paired urine, Npl tissue, and non-Npl tissue) from 21 male and 8 female patients. There was distinct clustering of urinary vs. tissue-associated microbiome in both sexes (**Figure 21, F-I**), suggesting that the two environments have a different bacterial composition. At the family level, 34 taxa (male, representing ~81% of the urinary bacterial abundance) and 16 taxa (female, representing ~98% of the urinary bacterial abundance) were shared between urine and tissues (Npl + non-Npl considered together), corresponding to the male and female "shared bladder cancer microbiome," respectively (**Figure 22**).

The results presented in Section 10.1 have been published in the following publication: Pederzoli F. *et al.*, "Sex-specific Alterations in the Urinary and Tissue Microbiome in Therapy-naïve Urothelial Bladder Cancer Patients", European Urology Oncology 2020 (reference 452 in the present thesis).

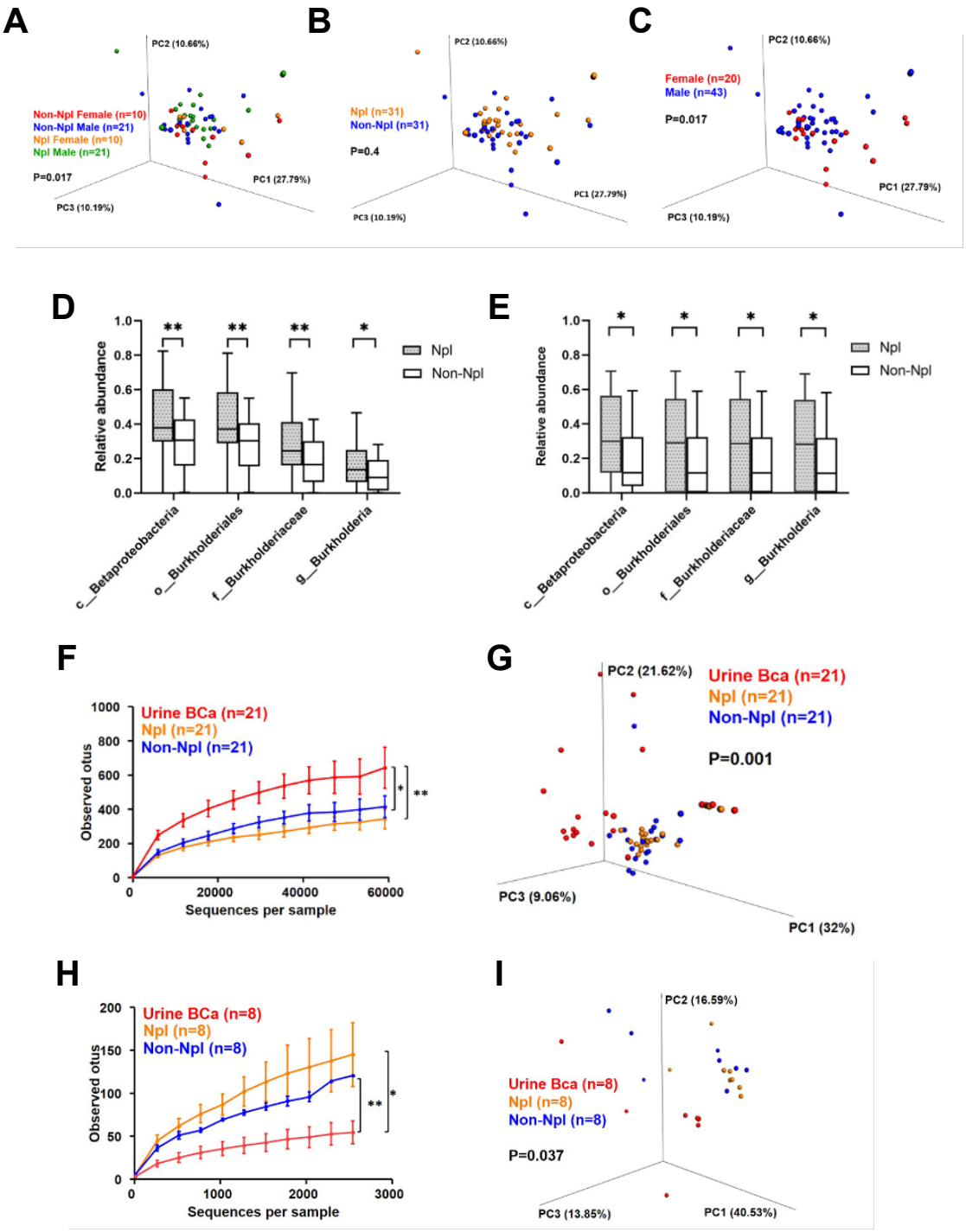


Figure 21 – Bladder-associated microbiome is sex specific. **A-C.** Beta-diversity was evaluated for (A) paired non-neoplastic and neoplastic tissues among all cohorts (from both male and female bladder cancer patients), (B) non-neoplastic versus neoplastic tissue (both male and female bladder cancer patients), and (C) cohorts stratified for sex. **D-E.** Compositional enrichment of the class *Betaproteobacteria*, order *Burkholderiales*, family *Burkholderiaceae*, and genus *Burkholderia* in Npl vs. non-Npl tissue of (D) 21 male and (E) 8 female bladder cancer patients. **F-G.** Microbial community diversity among the urine, and non-neoplastic and neoplastic tissue

of male bladder cancer patients, evaluated for (F) the diversity within samples (α-diversity) and (G) β-diversity among groups. **H-I.** Microbial community diversity among the urine, and non-neoplastic and neoplastic tissue of female bladder cancer patients, evaluated for (H) the diversity within samples (α-diversity) and (I) β-diversity among groups. (BCa = bladder cancer; Npl = neoplastic; *$p<0.05$; ** $p\leq0.01$).

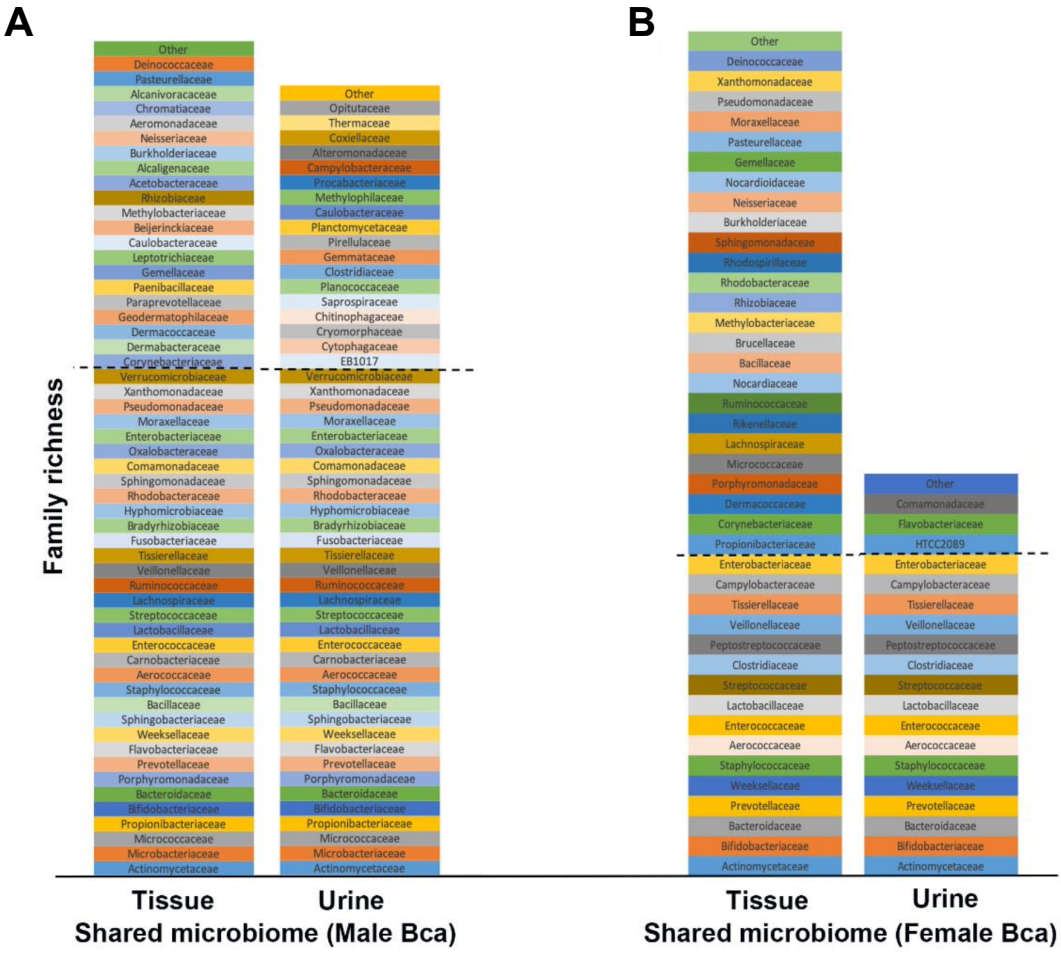


Figure 22 – Sex specific "shared bladder cancer microbiome". **A-B.** Families of bacteria shared between urine and bladder tissues in (A) 21 male patients and (B) 8 female patients (Bca = bladder cancer).

10.2 Identify the role of microbiome during neoadjuvant immunotherapy for the treatment of muscle-invasive bladder cancer

After the preliminary data obtained in therapy-naïve patients, we aimed to deep our understanding of the role of microbiota in the setting of therapeutic treatment for bladder cancer. As previously mentioned, a strict link exists between cancer, cancer immunotherapy and microbiota. Therefore, we started a collaboration with the team of Prof. Andrea Necchi, the Principal Investigator of the phase 2 PURE-01 clinical trial (NCT02736266), to study the potential role of microbiota in the context of neoadjuvant immunotherapy by single-agent pembrolizumab to treat urothelial carcinoma of the bladder. Firstly, I tried to gain some indirect evidence analyzing the impact of antibiotic (ATB) therapy on the response to pembrolizumab in the PURE-01 patient cohort. Those analyses were suggested by some previous works, which found that ATB therapy had a negative effect on immunotherapy efficacy in locally advanced and metastatic tumors, including urothelial carcinoma[453–456]. Indeed, a *post hoc* analysis of pooled data from the IMvigor210 and IMvigor211 trials showed that patients receiving ATBs concomitantly with the immunotherapy molecule (i.e., atezolizumab) had worse overall and progression-free survival[453].

Therefore, it is plausible to hypothesize that any disruptions of the "cancer-cancer immunotherapy-microbiota" triad would result in a perturbation of their reciprocal relationships. To this end, I conducted a *post hoc* analysis of the cohort of patients (n = 149) of the PURE-01 study[457]. A total of 48 patients (32%) were treated with ATB therapy concomitantly to immunotherapy. The main descriptive variables of the study population are listed in **Table 2**. Median TMB (9.3 vs. 11.4 mutation/Mb; p = 0.005) and combined positive score (CPS; 9.5% vs. 20%; p = 0.04) were lower in the ATB cohort. The rate of irAEs was not different between the two cohorts (p = 0.7). Of the 149 patients treated with neoadjuvant pembrolizumab, 141 (94%) underwent radical cystectomy. The most administered class of antibiotics was fluoroquinolones (n = 16; 33%), followed by beta-lactams (n = 14; 29%), trimethoprim/sulfamethoxazole (n = 5; 10%), fosfomycin (n = 5; 10%), and other compounds (n = 8; 17%). Among the 48 patients, 36 (75%) received ATBs for urinary tract infections, 7 (15%) for periprocedural prophylaxis, and 5 (10%) for other reasons.

Characteristic	Overall cohort	No ATB treatment	ATB treatment	*p* value
Patients (*n*)	149	101	48	
Median age, yr (IQR)	67.5 (61.8–73.2)	68 (63–75)	66.5 (59.8–71.2)	0.05
Sex, *n* (%)				0.4
Male	130 (87)	86 (85)	44 (92)	
Female	19 (13)	15 (15)	4 (8)	
ECOG PS 0, *n* (%)	133 (89)	89 (88)	44 (92)	0.5
Clinical T stage, *n* (%)				0.08
cT2N0M0	70 (47)	53 (53)	17 (35)	
cT3–4N0M0	79 (53)	48 (47)	31 (65)	
RC not performed, n (%)	8 (5.4)	6 (5.9)	2 (4.2)	0.9
Concomitant CIS, n (%)	24 (16)	13 (13)	11 (23)	0.11
Previous BCG instillations, n (%)	19 (13)	11 (11)	8 (17)	0.3
Median TMB, mutations/Mb (IQR)	10.5 (6.1-14.9)	11.4 (7-17.6)	9.3 (5.3-11.4)	0.005
Median CPS, % (IQR)	10 (3-50)	20 (5-58)	9.5 (1.5-25)	0.04
Sequential neoadjuvant therapy, *n* (%)	14 (9.4)	7 (6.9)	7 (15)	0.2
Complete pathologic response, *n* (%)	57 (38)	50 (50)	7 (15)	<0.001

BCG = bacillus Calmette-Guérin; CIS = carcinoma in situ; CPS = combined positive score; ECOG PS = Eastern Cooperative Oncology Group performance status; IQR = interquartile range; TMB = tumor mutational burden; RC = radical cystectomy.

Table 2 – Baseline patient and disease characteristics (n = 149).

In the antibiotic cohort, 7 patients (15%) reached complete pathologic response (i.e., ypT0N0), compared to 50 (50%; p < 0.001) in the untreated group. On multivariable logistic regression (MLR) analyses with adjustment for known predictive factors (**Table 3, A**), ATB administration was negatively associated with ypT0N0 status (odds ratio 0.18, 95% confidence interval [CI] 0.05–0.48; p = 0.001).

The 12-months recurrence-free survival (RFS) rate was 81% (95% CI 70–93%) in the antibiotic cohort, and 95% (95% CI 91–99%) in the cohort not treated with concurrent ATB (**Figure 23, A**), with corresponding 24-months RFS rates of 63% (95% CI 48–83%) and 90% (95% CI 83–97%). The total number of events was 23, and the median follow-up was 20 months (interquartile range 12–27) for the event-free population. Multivariable Cox regression (MCR) analyses showed that the use of ATB was associated with higher rates of recurrence (hazard ratio [HR] 2.64, 95% CI 1.08–6.50; p = 0.03; **Table 3, B**).

Sub-analyses according to the administered antibiotic class are shown in **Figure 23, B**. The 12-month RFS rate according to ATB classes was 95% (95% CI 91–99%) in the untreated cohort, 86% (95% CI 69–100%) in the beta-lactam cohort, 80% (95% CI 52–100%) in the fosfomycin cohort, 75% (95% CI 43–100%) in the trimethoprim/sulfamethoxazole cohort, 74% (95% CI 55–100%) in the fluoroquinolone cohort, and 88% (95% CI 67–100%) in the other-ATBs cohort. Among the different ATB classes, exploratory MCR analyses showed that fluoroquinolones were associated with a higher rate of recurrence (HR 3.28, 95% CI 1.12–9.60 vs no ATBs; p = 0.03; **Table 4**).

Following those initial, positive results, I focused my attention on studying the potential role of pre-therapy gut microbiome in a group of patients enrolled in the PURE-01 trial. For these analyses, fecal pre-therapy specimens were available from 42 patients, of which 23 patients showed a pathologic response (21 complete responses + 2 partial responses) and 19 did not respond (**Table 5**). Alpha diversity metrics did not differ between responders and non-responders (only observed OTUs and Shannon indexes are reported in **Figure 24, A**), as did not beta diversity (**Figure 24, B**). As the samples were sequenced in two distinct runs, we investigated the potential impact of the batch effect on our results by visually inspecting the data at different taxonomic levels (**Figure 24, C-D**), without the identification of major clustering explainable by a technical batch. Therefore, no batch correction was performed.

A

Factor	OR (95% CI)	*p* value
Tumor mutational burden (mutations/Mb; continuous)	1.05 (1.00–1.11)	0.09
Combined positive score (%; continuous)	1.02 (1.00–1.03)	0.03
cT3–4N0 at diagnosis	0.66 (0.28–1.52)	0.3
Salvage chemotherapy	0.22 (0.01–1.40)	0.17
Antibiotic use	0.18 (0.05–0.48)	0.001

CI = confidence interval; OR = odds ratio.

B

Factor	HR (95% CI)	*p* value
Tumor mutational burden (mutations/Mb; continuous)	0.94 (0.86–1.02)	0.15
Combined positive score (%; continuous)	0.98 (0.96–1.00)	0.06
cT3–4N0 at diagnosis	1.19 (0.49–2.89)	0.7
Antibiotic use	2.64 (1.08–6.50)	0.03

CI = confidence interval; HR = hazard ratio.

Table 3 – **A.** MLR analyses predicting pathologic complete response at radical cystectomy. **B.** MCR model predicting recurrence after pembrolizumab.

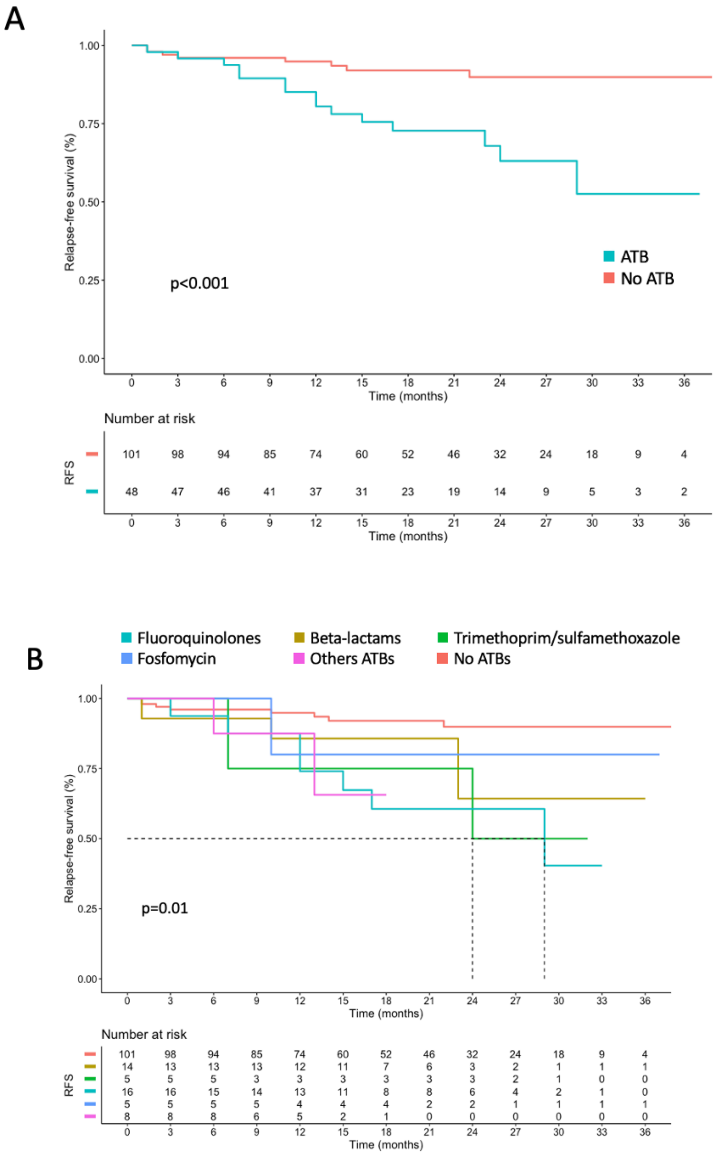


Figure 23 – The effects of concurrent antibiotics in the PURE-01 trial. **A.** Kaplan-Meier curves of relapse-free survival (RFS) stratified by use of antibiotics (ATB). **B.** Kaplan-Meier curves of relapse-free survival stratified by classes of antibiotics.

Factor	Univariable analyses		Multivariable analyses	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Beta-lactams	2.71 (0.72–10.2)	0.14	2.28 (0.59–8.84)	0.23
Trimethoprim/sulfamethoxazole	4.89 (1.03–23.1)	0.05	1.61 (0.19–13.7)	0.66
Fluoroquinolones	5.30 (1.92–14.6)	0.001	3.28 (1.12–9.60)	0.03
Fosfomycin	2.25 (0.28–18.0)	0.44	1.45 (0.18–11.7)	0.73
Other antibiotics	4.52 (0.94–21.7)	0.06	3.79 (0.77–18.7)	0.10
cT3–4 at diagnosis	1.82 (0.77–4.29)	0.17	1.32 (0.53–3.32)	0.55
TMB (mutations/Mb; continuous)	0.93 (0.86–1.00)	0.06	0.94 (0.86–1.02)	0.14
CPS (%; continuous)	0.97 (0.95–0.99)	0.02	0.98 (0.96–1.00)	0.08

CI = confidence interval; CPS = combined positive score; HR = hazard ratio; TMB = tumor mutational burden.

Table 4 – Univariable and multivariable Cox regression models predicting recurrence according to the different antibiotic classes vs. no antibiotics.

Characteristic	Response	No response
Patients (*n*)	23	19
Median age, year	68	69
Sex, *n* (%)		
Male	22 (96)	18 (95)
Female	1 (4)	1 (5)
Smoking status, *n* (%)		
Never	3 (13)	4 (21)
Former	17 (74)	10 (53)
Current	3 (13)	5 (26)
Concomitant ATB, n (%)	4 (17)	6 (32)

ATB = antibiotic.

Table 5 – Main baseline characteristics for PURE-01 patients enrolled in microbiome analysis (n = 42).

At the level of taxonomic analysis, Linear Discriminant Analysis (LDA) Effect Size (LEfSe)[458] has been performed to characterize the bacterial taxa differently expressed between responders and non-responders. LEfSe identified 16 bacterial taxa with a LDA score ≥2.5 that were differently enriched between responders and non-responders (**Figure 25, A**). Among them, I identified the genus *Sutterella* enriched in responders ($p = 0.02$), while the species *Ruminococcus bromii* was enriched in non-responders ($p = 0.02$). Similar results have also been confirmed using an additional method (Differential Abundance Analysis using *EdgeR*) mediated from RNASeq Differential Expression Analysis (**Figure 25, B**).

Part of the results presented in Section 10.2 has been published in the following publication: Pederzoli *et al.*, "Is There a Detrimental Effect of Antibiotic Therapy in Patients with Muscle-invasive Bladder Cancer Treated with Neoadjuvant Pembrolizumab?", European Urology 2021 (reference 457 in the present thesis).

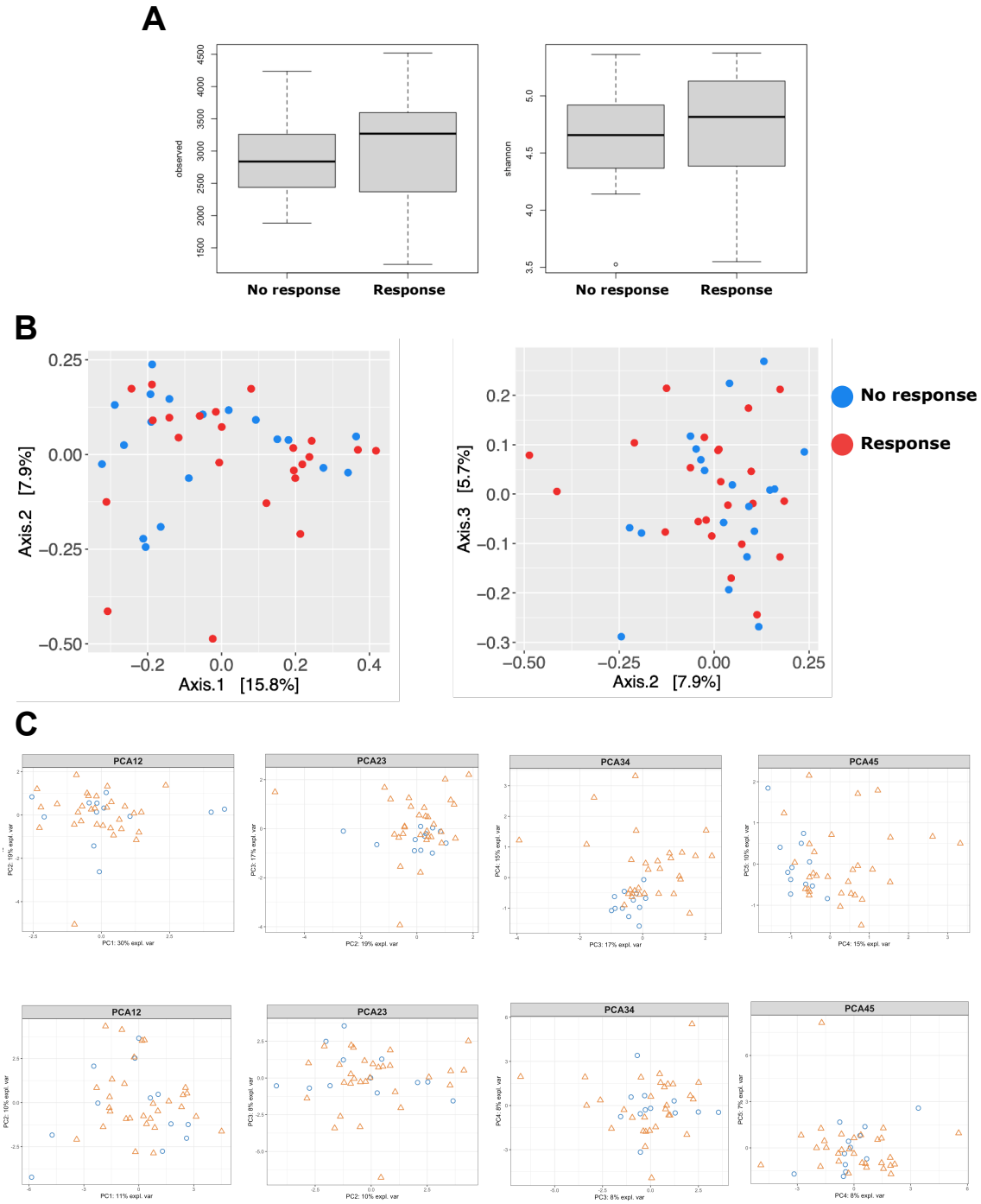


Figure 24 – Gut microbiome in the PURE-01 trial. **A.** Representative gut microbiome alpha diversity metrics (observed OTUs and Shannon Index) comparing responders vs. non-responders (all p >0.05). **B.** Beta-diversity principal coordinate analysis plots based on Bray-Curtis distance (Adonis test, p>0.05). **C.** Principal component analysis (PCA) at different taxonomic levels (Phylum L2, above; Species L7, below) to investigate the potential presence of a batch effect (triangle and circle represents two different sequencing runs).

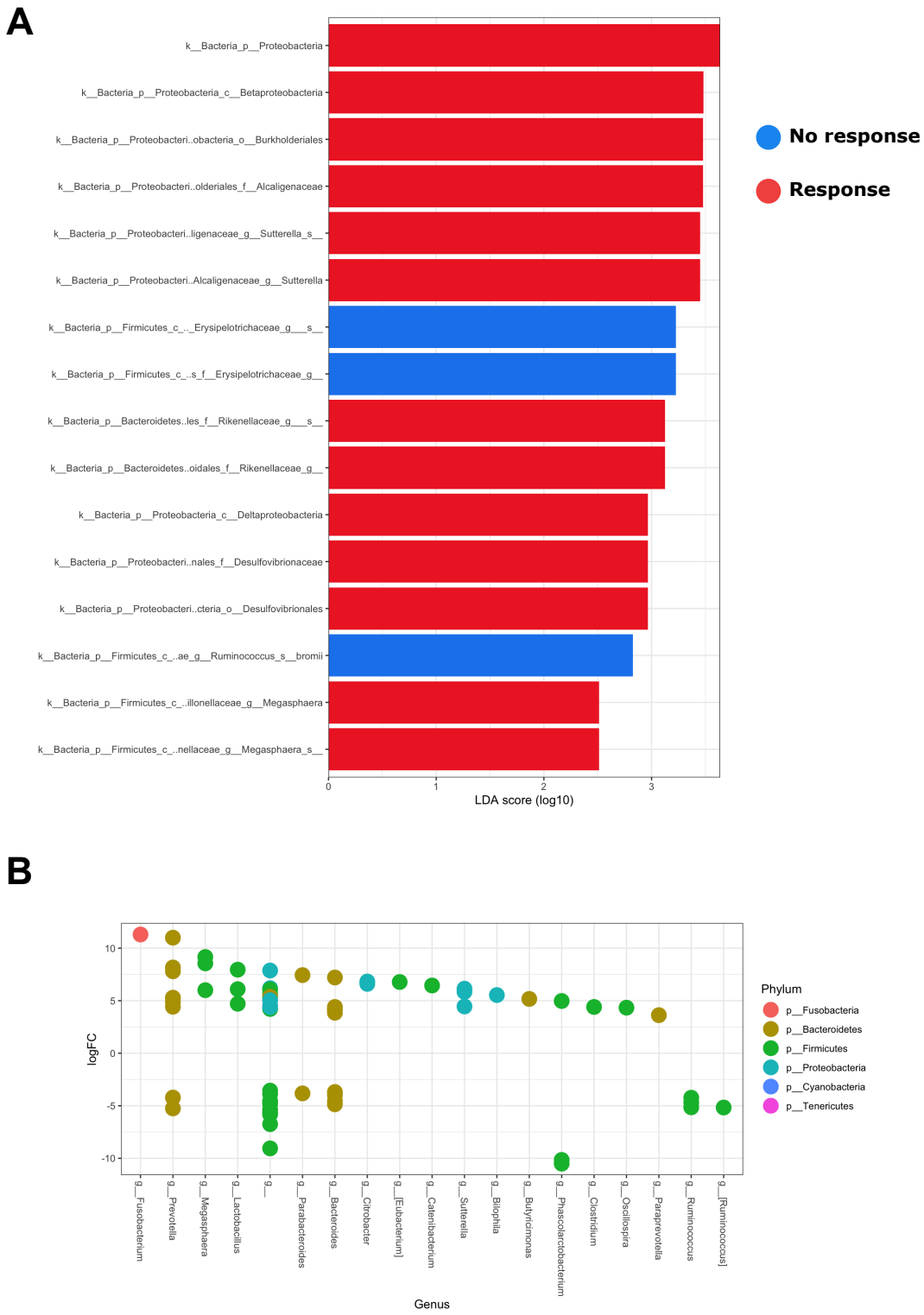


Figure 25 – Differentially expressed intestinal taxa in the PURE-01 study. **A.** LEfSe analysis of gut microbiota changes in responders vs. non-responders. **B.** Top differentially expressed genera using EdgeR.

10.3 Design novel models to study the mechanisms played by the microbiota in bladder cancer

After the important findings described in sections 10.1 and 10.2, I turned my attention to design *in vivo* and *in vitro* models to study the interplay between host and microbiome in a more controlled way. Indeed, those models will be of paramount importance to move from the associations I found in the human microbiome analyses to a more mechanistic understanding of the host-microbiome crosstalk. Here, I will describe some preliminary experiments performed using a syngeneic, orthotopic animal model of bladder cancer and the creation of a novel cell co-culture system to recreate the bladder microenvironment *in vitro*.

10.3.1 Mice experiments

Firstly, I aimed to understand if the most used murine model of bladder cancer, the syngeneic orthotopic MB49 model, could be a valid prototype to study the microbiome and the response to immunotherapy (**Figure 26, A-B**). Therefore, I tested if the MB49 mouse model could be an immunogenic model, simulating in mice the findings of bladder cancer immunotherapy described in human.

The syngeneic, orthotopic MB49 mouse model is obtained by instilling tumoral MB49 cells in the bladder of C57BL/6J mice. MB49 cells were derived by feeding 7,12-dimethylbenzanthracene to the C57BL/6J mouse strain[459]. In general, this syngeneic bladder cancer model is cost-effective, rather quick to establish and reproducible, making it the first choice compared to other models (e.g., carcinogen-induced or xenograft models)[460]. For all those reasons, I wanted to test if the MB49 model could be a reliable proxy in mice of the behavior of bladder cancer immunotherapy in human. Moreover, I used a MB49 cell line genetically engineered to express the luciferase reporter gene. Luciferase-expressing cell lines are widely used for animal experiments because they allow for non-invasive tracking of tumor development and burden by enzymatic luminescence. Indeed, these cells express the enzyme luciferase, derived from fireflies (*Photinus pyralis*), which oxides the substrate luciferin in the presence of molecular oxygen, magnesium and adenosine triphosphate (delivered to mice as an intraperitoneal injection) to produce a yellow-green light detectable at 560 nm[461] from an external detector like a IVIS SpectrumCT scanner (PerkinElmer).

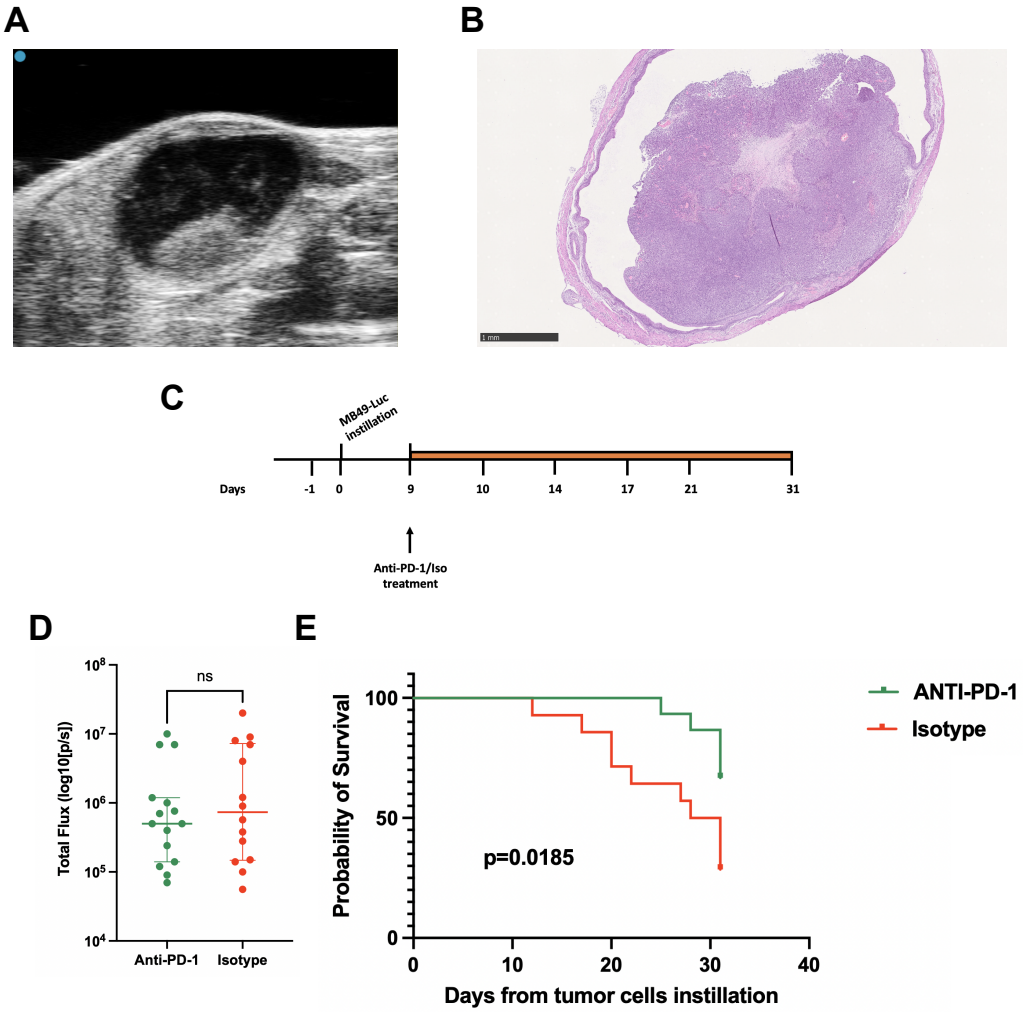


Figure 26 – Mice experimental model. **A, B.** Representative images (A, ultrasound, courtesy of Dr. Elisa Alchera; B, hematoxylin and eosin) of MB49Luc bladder tumors in the mouse. **C.** Schematic representation of the experimental protocol. **D.** IVIS values (total flux, log10(p/s)) measured at the first positive imaging for each mouse and used for randomization in the anti-PD-1 or isotype treatment groups (Mann-Whitney non-parametric tests, $p>0.05$). **E.** Survival curves of the two treatment cohorts.

In this framework, 29 female C57BL/6J mice were instilled with MB49Luc cells and randomized to receive a single injection of either anti-PD-1 (15 mice) or the isotype control (14 mice), when the tumor became evident using IVIS (**Figure 26, C-E**). Then, I followed the animals up to 31 days from instillation of MB49Luc cells, with biweekly monitoring of the wellbeing and health status of the animals. Mice were sacrificed if they lost >20% of the starting weight or in case of evident suffering (rough fur, allodynia, aching posture, etc.), according to institutional standard for animal care. Survival analysis showed a longer survival of mice treated with anti-PD-1 compared to the group treated with the control isotype (p=0.0185), even if a longer observation time is probably necessary to better appreciate the survival benefit.

In conclusion, we demonstrated that the MB49Luc model can be trustworthy used to study response to immunotherapy, paving the way to future experiments to disentangle the complex crosstalk between cancer cells, immune cells and microbiome during therapy with immune checkpoint inhibitors.

10.3.2 Transwell co-culture protocol

Lastly, I designed an innovative co-culture system using the Transwell technology. The Transwell insert defines two environments (a.k.a. inner and outer chambers) separated by the insert membrane, which prohibits the movement of cells and big particles but permits the movement of soluble factors and small nanoparticles (**Figure 27, A**). Therefore, it is possible to co-culture different cell types in distinct but communicating environments, mimicking *in vitro* the *in vivo* physiological conditions.

To mimic the urothelial microenvironment, I cultured human fibroblasts (BJ cell line, ATCC) together with human bladder cells, either the benign/premalignant HBLAK cell line (CELLnTEC) or the malignant 5637 cell line (ATCC). BJ fibroblasts are human normal fibroblasts derived from the skin/foreskin, and they are characterized by a long lifespan in comparison to other normal human fibroblast cell lines[462]. HBLAK cells are thought to be "spontaneously immortalized" benign human urothelial cells, even if a recent characterization of their expression markers, karyotype and typical bladder cancer alterations revealed some genetic alterations thought to contribute to immortalization (*TERT* mutation, $p16^{INK4A}$ loss) of urothelial cells[463]. Therefore, it is likely that HBLAK cells represent a pre-neoplastic state of urothelial cells, being neither *bona fide* benign

nor *bona fide* malignant. Conversely, 5637 cells were derived from a grade 2 tumor lesion of a white, male patient of 68 years of age[464]. At the level of karyotype analysis, 5637 cells have a stemline modal chromosome number of 67, and display a series of chromosomal alterations, including 3q+, 11q+, i(13q), t(9q21q), i(17q), i(21q), der(5)t(5;7)(q31;p11) and 1p. Moreover, they are characterized by mutations in *TP53*, and they do not clearly belong to a single molecular subtype[465].

For the purposes of this study, I designed a novel co-culture protocol (**Figure 27, B**). On day 1, 50000 fibroblasts were seeded on the bottom surface of a tilted Transwell insert and left tilted upside down for 4/5 hours in the incubator, to let the fibroblasts attach to the polyester membrane. Then, the insert was put in the appropriate position within a well, with 1.5mL of DMEM medium in the outer chamber and 0.5mL in the inner chamber. Growth of the fibroblasts as a layer was monitored by visual inspection (**Figure 27, D**) and measuring the diffusion of 70kDa fluorescent dextrans through the chambers. Fibroblast layer was generally formed around day 4/5 after initial seeding. At that point, 20000 bladder cells (either HBLAK or 5637) were seeded on the upper chamber of the Transwell insert. After starting the co-culture, only CnT-PR medium was used in both chambers. Bladder cells generally reached a 70-80% confluence after 3/4 days of co-culture. At that point, the co-culture was stopped, the insert transferred to a new well, washed in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 20 minutes at room temperature, then stored at 4°C in PBS until further use.

To monitor cell growth and the formation of a layer on the Transwell inserts, I visually inspected the insert under light microscopy, and I also measured the permeability of fluorescently labelled dextrans. Dextrans are non-digestible sugars that are not transported by transcytosis, so they can be used to monitor the paracellular route through cell tight junctions over short time periods[466,467]. While a complete stop of the dextrans flux was not achieved, the cells formed distinct layers and the presence of cells decreased the permeability to dextrans.

Lastly, we designed an imaging protocol that allowed the imaging of the insert without detaching the membrane from the plastic support (**Figure 27, D**). The development of such a protocol brings several advantages: the physiological morphology of the cell layers is maintained, as cells are not pushed in between the slide and the coverslip; with minor adaptations, the protocol can be used for vital stainings, which can be repeated at multiple

timepoints as the insert is not destroyed during the staining procedures; the risk of detaching cells or wasting the insert is very low, as all steps are performed with the intact insert, allowing easy manipulation compared to a detached membrane. Using confocal microscopy (**Figure 27, E**), the two cell layers can be easily identified (red: fibroblasts; green: bladder cells) and the polyester membrane dividing the 2 cell populations can be seen in the cross-section image.

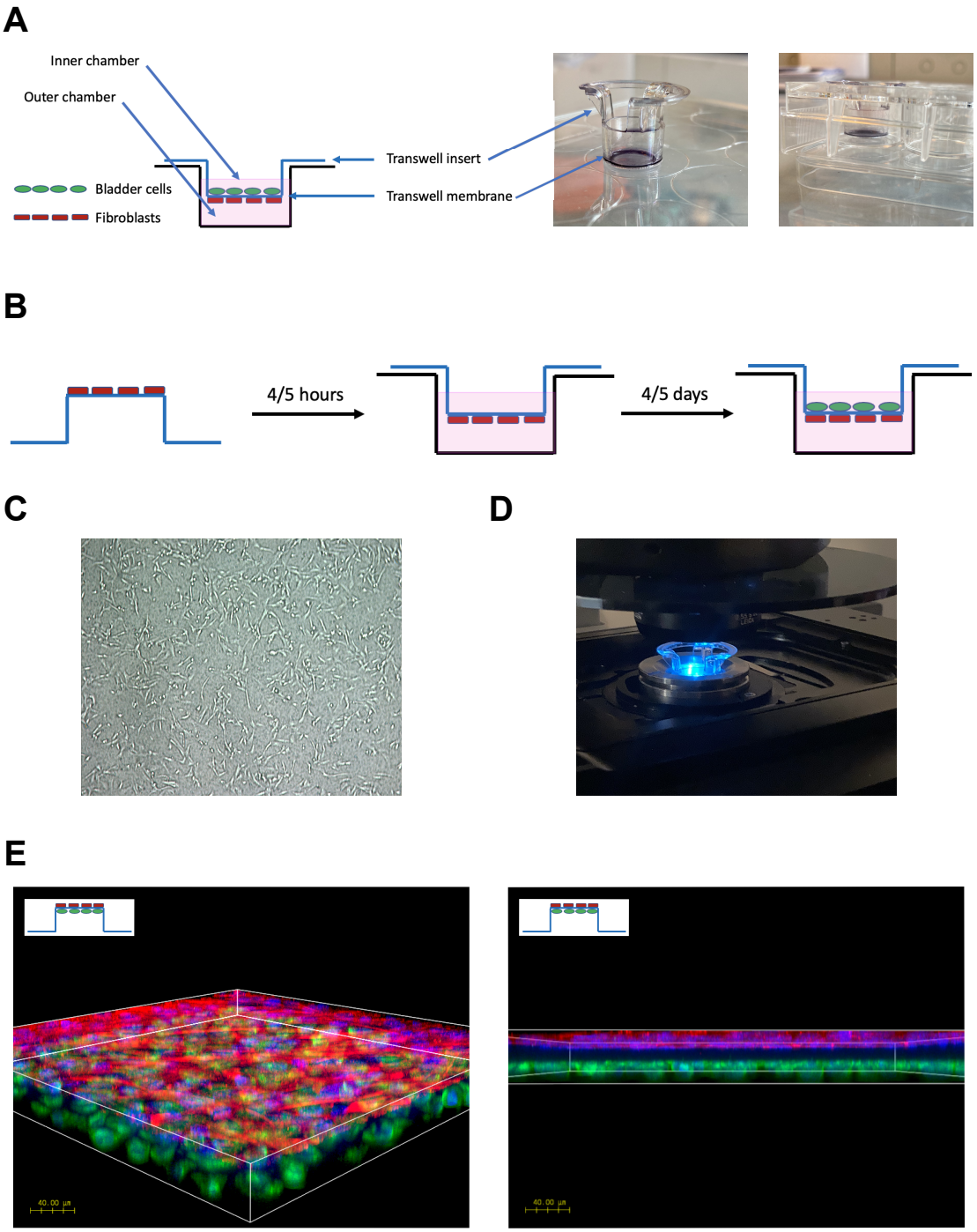


Figure 27 – Novel in vitro co-culture system. **A.** Schematic representation and representative photographs of the Transwell insert and system. **B.** Schematic representation of the co-culture Transwell protocol designed. **C.** Representative image (10x) of BJ fibroblasts growing attached to the bottom of a Transwell insert membrane. **D.** Confocal microscope setting for imaging of intact Transwell inserts. **E.** Co-culture of HBLAK bladder cells (green) and BJ fibroblasts (red). The Transwell membrane can be easily appreciated as a black strip in cross-section (nuclei, blue).

11. Discussion

In the field of bladder cancer, we are just starting to discover the function and role of different microbial communities, either local (urinary and urothelium-bound) or systemic (gut), in bladder carcinogenesis, response to therapy and prognosis. My PhD thesis project followed some steps of this long walk, starting from the evaluation of the microbiome of therapy-naïve bladder cancer patients, moving then to treatment, and eventually trying to fill the gap between association and causation design experiments in more controlled setting (i.e., *in vivo* animal studies and *in vitro* cell studies). As always happens in science, what we have done led us to more questions, more potential experiments, and more work rather than to solid, definitive answers. I will now discuss the results and offer some future implications of the data presented in this work.

The most important and novel concepts we introduced in our analyses on the microbiome of therapy-naïve bladder cancer patients were the sex-based stratification of the microbiome analyses and the proposed concept of a "shared bladder cancer microbiome" by comparing tissue-associated and urinary microbiomes, whose use may be helpful to further dissect and understand the role of the microbiome in the bladder environment[450]. To date, only few small studies regarding the urinary microbiome in bladder cancer are present in the literature, with heterogeneous results and conclusions[445–449]. The heterogenous findings from the above-referenced studies can be explained by the dissimilar composition of the study cohorts, regarding patients' race, sex, lifestyles, and extension of the disease (i.e., muscle invasive vs. non muscle invasive urothelial bladder cancer). Among the differentially enriched bacterial taxa in our groups, we found that urines from both male and female controls were enriched in a common phylogenetic branch composed by the class *Alphaproteobacteria*, order *Sphingomonadales* and family *Sphingomonadaceae*. Of note, *Sphingomonadaceae* are Gram negative bacteria characterized by the presence of sphingolipids in the outer membrane of their cell wall[468], and some members of this family are able to degrade polycyclic aromatic hydrocarbons and aromatic hydrocarbons[469,470]. We can hypothesize that a lower abundancy of *Sphingomonadaceae* in bladder cancer patients may be associated with the carcinogenic process itself, as exposure to aromatic amines is among the well-established risk factors for bladder cancer. Therefore, it may be plausible that subjects with less

Sphingomonadaceae in their urinary microbiome can be at higher risk of developing bladder cancer due to their decreased capacity to degrade aromatic amines. On this line, a study in breast cancer comparing paired neoplastic and normal biopsies found the bacterium *Sphingomonas yanoikuyae* enriched in the non-neoplastic tissues[471]. However, further studies are needed to elucidate the potential role of *Sphingomonadaceae* in bladder carcinogenesis.

We also found several bacterial taxa enriched in the urines of bladder cancer patients according to their biological sex: in men, the order *Opitutales* and subordinate family *Opitutaceae*, together with the isolated class *Acidobacteria-6*; in females, the genus *Klebsiella*. The genus *Klebsiella* belongs to the bacterial family *Enterobacteriaceae*, which has already been reported as enriched in the urine of bladder cancer patients by a previous study[448]. Moreover, *Klebsiella spp.* can produce several genotoxins, like colibactin, which may play a direct role in carcinogenesis, as they can induce DNA strand breaks, genomic instability, and cell cycle arrest. Based on the pro-tumoral mechanism of the colibactin toxin, a potential link between *Klebsiella spp*. and colorectal cancer development has been proposed[472,473].

At the urothelium level, the higher abundance of the genus *Burkholderia* in the neoplastic tissues in both sexes suggests a potential association with bladder cancer pathobiology, as it was recently reported in colorectal cancer[474]. Moreover, a recent study by Oresta et al. found the family *Burkholderiaceae* enriched in bladder washouts obtained from male bladder cancer patients, further corroborating the strict link (physical?) with bladder cancer cells[449]. Strikingly, we identified only minor differences between the paired neoplastic and non-neoplastic tissues. This finding may be explained by the multifocality of the bladder cancer lesions, and therefore the nearby non-neoplastic regions may be influenced by a "cancer field effect" able to induce modifications in the microbiome of those non-neoplastic areas[475]. Another reason may be that the urine shifts bacteria within the bladder according to its movements caused by the daily human activities, thus making the entire urothelial surface almost homogeneous in microbial communities. The genus *Burkholderia* is a complex group of bacteria that comprises more than 120 different species, which are involved in biofilm formation, modulation of the immune system, interaction with host cells and virulence[476]. Of interest, it has been recently showed in a murine model of sarcoma that the efficacy of anti-CTLA-4 immunotherapy is influenced

by the microbiota composition, and specifically by specific bacteria (*B. fragilis* and/or *B. thetaiotaomicron* and *Burkholderiales*)[401]. Indeed, the transplantation of those bacteria into antibiotic-conditioned animals conferred protection against anti-CTLA-4-induced colitis, suggesting a promising role of those taxa as "anticancer probiotics".

The second important concept we introduced in our analyses is the definition and identification of the sex-specific "shared bladder cancer microbiome" comparing the microbiome isolated in urine and bladder tissue. Similarly to the gastrointestinal tract, where fecal microbiome do not recapitulate the gastrointestinal mucosal microbial communities in their entirety[477], voided urine may not completely represent the bladder tissue microbiome, due to potential contaminations from the anatomical structures of the lower genitourinary tract (e.g., prostate and urethra) and due to the preferential affinity of specific bacterial taxa for different ecological microenvironments (urine vs. tissue) and different ability to adhere to the urothelium. Moreover, it has been clearly shown by Oresta et al. that the choice of different sampling techniques for bladder cancer microbiome studies has an impact on the resulting microbial communities[449]. Indeed, they compared paired midstream voided, catheter-derived and bladder washout samples from the same patients and found distinct bacterial taxa enriched or under-represented based on the chosen sampling technique. It is clear that transurethral catheter urine samples have less bacterial contamination from the urethra or other lower genitourinary structures than midstream voided urines. While catheter-derived samples may be easy to collect in the surgical perioperative settings, this is not the case in other situations, like during an oncological treatment or in case of healthy volunteers. Due to these considerations, we preferred to use voided urines for all our analyses, and to control for this source of bias, we compared paired bladder tissue samples, together with the urine samples, to identify the shared bacterial taxa between the two environments. In this context, we observed that 34 and 16 of the bacteria families present in the urine samples of male and female patients were also present in the paired tissues, representing more than 80% of the bacterial abundance in both sexes. Notably, the genus *Klebsiella* (female) was also identified in the tissues, while the family *Opitutaceae* (male) was identified only in the urines and not in the tissues, which might be due to either a contamination from the lower genitourinary tract or the preferential localization of this bacterium within the tumor urinary environment rather than bound to the neoplastic urothelium. We consider that these novel

data are relevant to design and interpret future studies exploiting the urinary microbiome as a potential proxy of the tissue microenvironment, and to properly understand how voided urines may be implemented for the study of the genitourinary microbiome.

With this in mind, we then focused our attention on studying the role of different microbial communities on the response to immunotherapy in bladder cancer patients treated with neoadjuvant pembrolizumab in the PURE-01 clinical trial. To gain a preliminary, indirect evidence of the importance of the microbiome in mediating the antitumor efficacy of pembrolizumab, we firstly analyzed the impact of concurrent antibiotic therapy on the efficacy of pembrolizumab, as measured by pathologic complete response (the primary endpoint of the PURE-01 trial) and by relapse-free survival. In line with previous studies on other malignancies and on metastatic/advances urothelial carcinoma[453–456], we found that concurrent antibiotic use was associated with worse outcomes. This novel information in the neoadjuvant setting is of paramount importance for oncologists and physicians in general, as our study highlights that antibiotic administration during immunotherapy may reduce the survival of an intention-to-cure population, although our findings need to be externally validated in future studies. At a mechanistic level, alterations of the homeostatic microbiome caused by the bactericidal and bacteriostatic effects of antibiotics lead to a state of dysbiosis, characterized by the overgrowth of detrimental bacterial species at the expense of more beneficial commensals. This shift in microbial ecology translates into a negative impact on host anticancer response, as shown by previous studies reviewed above (section 8.3). Although hypothesis-generating, our analyses indicate that not all classes of antibiotics have the same effect on the efficacy of neoadjuvant pembrolizumab. While a decrease in intestinal microbial diversity after any antibiotic therapy is generally reported, the precise effects of different compounds and short- vs. long-lasting effects remain largely unexplored. Recently, a systematic review of the literature found that treatment with the quinolone compound ciprofloxacin caused changes in the intestinal microbiota that were detectable up to 12 months after its use, while other compounds, such as fosfomycin, showed shorter-lasting changes[478]. Similarly, it cannot be excluded that infections, more or less symptomatic, may influence response to immunotherapy, although uncoupling the specific effects of infections from the effects mediated by the drugs prescribed to treat the

infections themselves (= antibiotics) could be difficult[479]. Hopefully, in the near future larger and well-powered studies will be designed to address some key, open questions: what is the impact of the timing of antibiotic administration on the immunotherapy efficacy? Are there any compounds that can be used safely during immunotherapy without risking a detrimental effect on antitumor activity? Until that time, our findings raise a word of caution about the use and choice of antibiotic drug to use in patients undergoing immunotherapy.

To further advance our understanding of the strict interplay between microbiome, bladder cancer and immunotherapy, we profile the gut microbiome of a cohort of patients enrolled in the PURE-01 clinical trial and for which pre-treatment stool samples were available. We found that the gut microbiome of non-responder patients was characterized by a higher relative abundance of *R. bromii*, while patients who benefitted from neoadjuvant pembrolizumab were enriched in the genus *Sutterella*, among other differentially expressed bacteria. Those two bacterial taxa are of particular interest, and they will be now briefly discussed. *Ruminococcaceae* is a family of Gram-positive, obligate anaerobic bacteria that includes many important human gut commensal species, including the *Ruminococcus bromii* and the *Faecalibacterium prausnitzii*, both frequently encountered as "bacteria of interest" in the field of the cancer microbiome. While some studies identified the *Ruminococcus spp.* among the beneficial bacteria enriched in patients responding to immunotherapy protocols[403,404], other reports found it more abundant among patients failing immune checkpoint inhibitors, in line with our findings[402,480]. Moreover, the recent results of one of the first-in-human clinical trial to test whether FMT can affect response to anti–PD-1 immunotherapy further highlighted how the role of *Ruminococcus spp.* is more complex than expected[422]. Indeed, in that trial Baruch et al. treated metastatic melanoma patients with FMT from two different donors before immunotherapy. While patients receiving stools from donor 1 showed objective responses in 3/5 cases, no responses were seen in patients who received FMT from donor 2. Of note, stools from donor 2 used for FMT were enriched in *Ruminococcaceae*, and post-treatment gut microbiome analysis from patients who received FMT from donor 2 found a high relative abundance of *R. bromii*. While the study was not designed to assess efficacy, so it is not possible to confidently state that the lack of objective responses in patients receiving the *Ruminococcaceae*-enriched FMT is significant, those findings are

in line with the inconsistencies of previous reports. On the other hand, the genus *Sutterella*, that we found enriched in responders, is a member of the order *Burkholderiales*, class *Betaproteobacteria*, and currently comprises around 10 confirmed/unconfirmed species[481]. The genus *Sutterella* has gained momentum in the study of inflammatory bowel diseases. Indeed, in a clinical trial testing FMT to treat active ulcerative colitis, the presence of *Fusobacterium spp*. and *Sutterella spp*. was associated with failure of the therapeutic FMT[482]. In line with this finding, a microbiome analysis of pediatric ulcerative colitis patients at diagnosis found that patients with low relative abundance in intestinal *Sutterella spp.* were more likely to achieve corticosteroid-free remission at 52 weeks, after adjusting for other clinical factors[483]. However, similar findings were not confirmed in the other inflammatory bowel disease – Crohn's disease. What is currently known is that an enrichment of *Sutterella* in the gut microenvironment promotes an increased degradation of local immunoglobulin type A (IgA), favoring a potential invasion of epithelial cells by local bacteria[484]. *Sutterella* can also promote the production of interleukin-8 by activating host cells' pattern-recognition receptors, even if this activity is marginal compared to other commensal gut bacteria. The ability of *Sutterella spp.* to adhere to the intestinal epithelium by binding to extracellular matrix proteins, like collagen I, fibronectin and laminin, may suggest they may play an immunomodulatory role[485]. However, much is yet to know about this bacterial genus and its potential role in mediating immunotherapy efficacy. Moreover, reconciling the results from different microbiome studies is one of the priorities in the field, by using metanalytic pipelines to increase the statistical power of the discoveries, and by designing comprehensively mechanistic studies to better understand the role of these bacteria in the context of the gut microbiome[486,487].

I also demonstrated that the syngeneic, orthotopic MB49Luc model can be trustworthy used to study the effect of immunotherapy on bladder cancer. Indeed, the rate of complete response in mice is quite similar to the rate of response to single-agent immune checkpoint inhibitor in human, thus increasing the utility of the model. Moreover, the MB49 bladder cancer model is relatively easy and cost-effective, especially when compared to carcinogen-induced models, time-consuming and potentially dangerous for the research personnel, or to human xenograft models, difficult to manage and resulting

in lower rates of successful tumor engraftment[460]. To sum up, our orthotopic mouse bladder cancer model appears to be a valuable model for further investigations of the crosstalk between the microbiome, bladder cancer and immunotherapy-mediated antitumor activity.

Lastly, the development of the Transwell co-culture system mimicking *in vitro* the bladder microenvironment represents a step forward in our understanding of the mechanistic effects of the microbiome on host cells. Indeed, while the cell co-culture methods offer a simplistic tool to address the complexity of host-microbiome interactions, they allow for a more precise tuning of the experimental conditions and offer an easier platform to deepen the investigations of the molecular interactions between bacteria and human cells[488,489]. For instance, Transwell-based culture systems have been used to study the effects of specific synthetic bacterial consortia or specific bacterial components like lipopolysaccharide (LPS) on *in vitro* models of the small intestine. Moreover, they have also been utilized to study the mechanisms of microbial sampling by dendritic cells in the human gut[490,491]. While the Transwell-based co-culture constitutes a more complex system than single-cell cultures and enables the study of the interactions between different cell type and between cells and microbial products, the culture conditions may be difficult to control and appropriately set due to the growth requirements of the different cultured cell types. Recently, complex *in vitro* and *ex vivo* cell systems, like organoids or organs-on-a-Chip, have entered an increasing number of research laboratories, and will likely revolutionize the way we do *in vitro* research. Those systems have also been used to study host-bacteria crosstalk[492–494]. For instance, Lukovac *et al.* investigated the transcriptomic response of mouse ileal organoids upon stimulation by metabolites derived from two well-known members of the human gut microbiome, *A. muciniphila* and *F. prausnitzii*[494]. They found that the metabolic products from the two bacteria started different transcriptional programs, as *Akkermansia* affected several genes involved in lipid metabolism and cell growth, while *Faecalibacterium* showed more modest effects. Those works, like our efforts to design the Transwell bladder co-culture system, are contributing towards the novel era of microbiome studies, from association to mechanistic understanding of the complex host-bacteria crosstalk. The evidence deriving from those experiments will be fundamental not only to answer important biological questions, but

also to hopefully guide a knowledge-based implementation of microbiome-based technologies in clinical practice, as adjuvant therapeutics or diagnostic tests.

Some limitations to the work here described must be acknowledged. For the microbiome studies, in addition to the intrinsic limitations of the amplicon-based 16s approach, we could not control for patient-specific factors, such as concurrent medications, over-the-counter supplements, diet, or exposure to environmental carcinogens, or disease-specific factors, such as tumor multifocality. Moreover, we could not exclude potential bias introduced by previous diagnostic procedures (e.g., cystoscopies or TURBs) associated with the standard diagnostic work-up for suspected bladder cancer. For the antibiotic analyses in the PURE-01 study, survival analysis is not a prespecified endpoint of the clinical trial, so our analysis could be underpowered and should be considered exploratory. Moreover, it was not possible to accurately assess the duration and the precise timing (e.g., only before pembrolizumab, in between pembrolizumab cycles) of antibiotic therapies. For the mice studies, a potential limitation can be the pre-defined sacrifice at 31 days after MB-49Luc administration, but this endpoint has been set to prevent unnecessary and excessive sufferings to the mice, in line with a human use of research animal models.

To conclude, this thesis work reported innovative data on the role of different microbial communities (urinary/urothelium-bound/fecal) in bladder cancer and bladder cancer therapy, and provided novel *in vivo* and *in vitro* models to validate those finding and uncover the complex microbiome-host cells crosstalk in bladder cancer patients. Our future studies will delve into the role of the urinary microbiome in mediating response to neoadjuvant pembrolizumab, and will try to provide mechanistic explanations of our findings derived from 16S metagenomic analyses.

12. Material and Methods

12.1 Characterization of the urinary and tissue-bound microbiota associated with bladder cancer – Material and Methods

12.1.1 Patient selection and ethical approval

49 patients from the Unit of Urology, IRCCS Ospedale San Raffaele (Milan, Italy), undergoing radical cystectomy for muscle-invasive urothelial bladder cancer (clinical T2–4, N0–3, M0 stage) and 59 healthy volunteers were identified. Healthy volunteers filled a short anamnestic questionnaire about their health status, excluding major conditions that might influence the microbiome (e.g., history of Crohn's disease, etc.). Exclusion criteria included the following: (1) history of recurrent urinary tract infections (two or more physician-diagnosed infections in 6 months or three or more infections in 1 year); (2) positive urine dipstick for nitrites and/or leukocytes and/or recent (≤4 weeks) urinary tract infection; (3) grade III–IV unilateral or bilateral hydronephrosis; (4) recent (≤4 weeks) antibiotic therapy or systemic corticosteroid therapy; and (5) history of intravesical or any neoadjuvant systemic therapy for bladder cancer. Data collection followed the principles outlined in the Declaration of Helsinki; all patients and controls signed an informed consent form agreeing to supply their own anonymous information and tissue specimens for future studies. The study was approved by the Institutional Review Board of IRCCS Ospedale San Raffaele, Milan.

12.1.2. Human urine and bladder tissue collection

Healthy control individuals and bladder cancer patients provided a morning midstream voided urine sample into a sterile container, according to standard urinalysis protocols. Particular emphasis was put on the procedure for clean collection of specimens. Patients were requested to collect a urine sample the same day of radical cystectomy. Thereafter, the sample was immediately refrigerated at 4°C and subsequently centrifuged at 450 *g* for 5 min to pellet the cell component. Under a laminar flow hood, 2 ml of the supernatant was transferred to a sterile container and stored at –80°C until analysis. The entire process, from collection to storage, was performed at 4°C and occurred in <30 min.

Bladder specimens (neoplastic [Npl] vs non-neoplastic [non-Npl] tissues) were available only from patients and were collected using sterile equipment by an experienced

genitourinary pathologist immediately after the surgical removal of the bladder, placed in separate sterile Falcon tubes containing fresh Krebs solution, and processed for storage under sterile conditions within 30 min. Tissues were then included in sterile optimal cutting temperature (OCT) compound and stored at –80°C until analysis. Tissue biopsies were available only when sampling of the bladder for research was considered appropriate, and not affecting the correct pathological diagnosis and staging of bladder cancer. Validation of the bladder specimens was independently carried out by two genitourinary pathologists.

12.1.3. DNA isolation and 16S rDNA amplification

The QIAamp DNA FFPE Tissue Kit (Qiagen, Milan, Italy) was used to isolate DNA from the bladder tissues. The QIAamp Viral RNA Mini Kit (Qiagen) was used to isolate DNA from the urine samples. Bladder tissues and urine samples were processed in parallel. To avoid exogenous contamination, samples were processed under a sterile hood, using disposable materials and avoiding the use of the cryostat.

The V3-V4 region of the 16s rDNA gene was amplified, starting from 200 ng of extracted DNA, using the AccuPrime Taq DNA Polymerase (Invitrogen), using the following primers: V3-16S-Fw: CCT ACG GGN GGC WGC AG; V4-16S-Rev: GAC TAC HVG GGT ATC TAA TCC; and under the following cycling conditions: 94°C for 2 min, 35 cycles of 94°C for 30 s, 56°C for 30 s, and 68°C for 1 min; stored at 4°C.

12.1.4. Quantification of 16S rDNA

Total DNA was amplified for the quantification of 16S copies using TaqMan Gene Expression Master Mix and TaqMan Gene Expression probe for panbacterial detection of 16S rDNA (Ba04230899_s1; Thermo Fisher), according to the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was performed in an ABI Prism 7000 Sequence Detection System thermal cycler (Applied Biosystem). To create the standard curve, a 10-fold dilution series from a well-known concentration of Escherichia coli DH5α was performed, resulting in a set of standards containing 6.81×10^6 - 6.81×10^{11} copies of template (calculated using the following link: https://cels.uri.edu/gsc/cndna.html). A regression line derived from the standard curve

was used to determine the copy number of the unknown samples, and the number of 16S copies was normalized to the total quantity of loaded DNA (in nanograms).

As negative controls, the Krebs solution used for the delivery of the specimens, the freezing solution (10% dimethylsulfoxide/90% fetal bovine serum), and the OCT used for tissue storage were processed in parallel to tissues using the Qiagen kit; because all tested solutions were negative at the DNA quantification, they were not further processed by sequencing. Using the same Qiagen kit, further controls were used, such as DNA isolated from the PC3 cell lines cultivated in the presence of antibiotics (used as the negative control for the quantification of 16S copies) and the DNA from a mucosal swab used as a positive control, using a swab stabilization buffer and buccal-prep DNA isolation kit (Isohelix, UK).

12.1.5. Microbiome analysis

Amplicons were purified using the AMPure XP beads (Beckman Coulter, Brea, CA, USA). A second PCR step was performed for indexing each sample. The Nextera XT Index Kit (Illumina) and the KAPA HiFi HotStart PCR Kit (KAPA Biosystem) were used, according to the following amplification protocol: 95°C for 3 min; eight cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and 72°C for 4 min and then stored at 4°C. A second purification step with AMPure XP beads was performed to clean up the final library. The purified DNA was quantified using the Qubit Fluorometer (Thermo Fisher) and the 2100 Bioanalyzer System (Agilent). Libraries were diluted and mixed: the obtained pool was loaded on the reagent cartridge for sequencing with MiSeq System instrument (Illumina). Sequences with a high-quality score of >Q30 and length of >250 bp were used for the taxonomic analysis and were processed using the Quantitative Insights into Microbial Ecology (QIIME) software package (version 1.9.1)[495]. The sequences were demultiplexed and quality trimmed using default parameters in QIIME script (split_libraries_fastq.py). The filtered sequences were clustered into operational taxonomic units (OTUs) based on a 97% similarity threshold using the UCLUST algorithm. OTUs were picked by the de novo OTU picking method pick_open_reference_otus.py. Representative sequences were aligned with PyNAST against Greengenes database (gg_13_8 release). Taxonomy was assigned to the identified

OTUs using the Ribosomal Database Project classifier. For all samples, a good coverage rate of >99% was obtained.

12.1.6. Statistical analysis

The alpha diversity (i.e., measure of diversity within a sample) was quantified according to the number of observed OTUs in order to analyze microbiome richness. Microbial diversity between samples (beta diversity) was evaluated using the permutational analysis of variance (PERMANOVA) using the distance matrix function ADONIS; principal component analysis using weighted UniFrac distance matrices and represented by weighted variance was used to visualize relationships and differences among groups. At lower taxonomic levels, we performed linear discriminant analysis (LDA). Here, I applied LEfSe with default parameters (alpha value for Wilcoxon tests was set at 0.05; the logarithmic LDA score threshold was set at 2.0) to identify taxonomic biomarkers that characterize the differences between healthy and bladder cancer urinary microbiome. Moreover, two-tailed Wilcoxon signed-rank test was used to compare Npl versus non-Npl specimens. Only taxa present in at least 10% of sample cohorts and with a mean proportional abundance of 0.1% were considered. All statistical tests and enrichments were considered significant at $p \leq 0.05$.

12.2 Identify the role of microbiome during neoadjuvant immunotherapy for the treatment of muscle-invasive bladder cancer – Material and Methods

12.2.1 Antibiotic analysis in the PURE-01 cohort

Concomitant ATB therapy was defined as any ATB administration between 30 days before the first pembrolizumab dose and the radical cystectomy. Kruskal-Wallis and $\chi 2$ tests were used to assess differences between groups treated or not with ATBs, according to baseline characteristics. MLR analyses tested the ATB effect on the probability of achieving a pathologic complete response (i.e., ypT0N0), which is the primary endpoint of the PURE-01 trial. Variables used to adjust in the MLR analyses were chosen based on their clinical relevance, as shown in previous reports[275,276]. We also assessed relapse-free survival (RFS), as previously defined[275], according to ATB use using Kaplan-Meier and MCR analyses, adjusting for baseline T stage (cT2 vs. cT3), PD-L1 expression (CPS

>10% vs. <10%), and TMB. Sub-analyses explored the effects of different ATB classes on RFS.

12.2.2 Stool specimens from the PURE-01 cohort

Pre-immunotherapy stools were available for analysis from 42 patients enrolled in the PURE-01 trial (NCT02736266), testing 3x200mg flat-dose pembrolizumab every 21 days before radical cystectomy. All samples were collected using Stool Nucleic Acid Collection and Preservation Tubes (Norgen) and extracted using the Stool DNA Isolation Kit (Norgen), according to the manufacturer's protocol. 16s sequencing was performed using standardized protocols at the internal facility (Center for Omics Science, COSR) using mock communities and DNA standards (ZymoBIOMICS) to control for extraction and sequencing contaminations, with a comparable pipeline as described above.

A QIIME-based bioinformatic pipeline was used for microbiome analyses. For the purpose of the microbiome analysis, response was defined as complete pathologic response (i.e., ypT0N0) + partial pathologic response (residual <ypT2N0 tumor) at radical cystectomy.

12.3 Design novel models to study the mechanisms played by the microbiota in bladder cancer – Material and Methods

12.3.1 In vivo mice experiments

All procedures involving mice were approved by the Institutional Animal Care and Use Committees of San Raffaele Scientific Institute and performed according to the prescribed guidelines (IACUC, approval number 942 and 1130). Female, 9-week-old C57BL/6J mice (code C57BL/6NCrl) were purchased from Charles River Laboratories (Italy) and housed in the animal facility of San Raffaele Hospital following 12 light/12 dark cycle. For the mouse orthotopic MB49 bladder cancer tumor, murine bioluminescent MB49Luc cells were kindly provided by Prof. Carla Molthoff (VU University Medical Center, The Netherlands) and cultured in DMEM medium (Gibco, ref. 61965-026), supplemented with 10% fetal bovine serum (FBS; Euroclone, ref. ECS0180L) and 1% penicillin/streptomycin (Gibco, ref. 15070-063). For this cell line, a cell bank was prepared and authenticated for lack of cross-contamination by analyzing 9 short tandem

repeats DNA (IDEXX Bioanalytics, Ludwigsburg, Germany). A vial of cell bank was used to start a new experiment. The cells were routinely tested for mycoplasma contamination and cultured for not more than 4 weeks before use[496].

For the instillation of murine bladder cancer cells (M49Luc cells), mice were anesthetized with ketamine (80 mg/kg) and xylazine (15 mg/kg) intra peritoneum and kept in dorsal position on a heated mat and with a water-based gel on the eyes. A 24-gauge catheter (Becton Dickinson, ref. 381212) was inserted in the urethra of animals using Luan gel 2,5% (Lidofast, Angelini SpA, Italy) and urine was vacated gently squeezing the mouse abdomen. Then, the bladder was filled with 100 µl of DPBS with or without $5x10^4$ MB49Luc cells. After 30 minutes, the catheter was gently removed and mice were treated with carprofen (5mg/kg, subcutaneous), allowed to recover in a heated, oxygen-rich environment, and returned to their cage after complete recovery.

The tumor growth was followed through the non-invasive small animal Optical-CT scanner (IVIS SpectrumCT®, Perkin Elmer) in the Preclinical Imaging Facility at IRCCS Ospedale San Raffaele. During these exams, mice were anesthetized with isoflurane (2-4%, 0,3-0,8 L/min) and the hair on the belly was removed with depilatory cream. Luciferine (IVISbrite™ D-luciferin Potassium Salt, PerkinElmer, ref. 122799) was instilled intra peritoneum (15mg/kg) 10 minutes before the IVIS image acquisition.

The use of female mice simplified the procedure and reduced mechanical trauma. The urinary functionality was constantly monitored, and the absence/presence of hematuria was evaluated as a potential sign of sufference. Moreover, the animals were observed for the presence of pain signals, such as weight loss more than 15%, changes in the posture and food and water consumption. Animal wellbeing was comprehensively evaluated using a physical-behavioral score, and they were sacrificed using a carbon dioxide chamber, following best practices for the human use of animal models.

For the immunotherapy treatment experiments, animals were treated with a single injection (10 mg/Kg) of either an anti-PD-1 antibody (InVivoPlus anti-mouse PD-1 (CD279), clone RMP1-14, Bio X Cell) or its control isotype (InVivoPlus rat IgG2a isotype control, clone 2A3, Bio X Cell). Mice were treated when the tumor mass became visible using IVIS, approximatively 7-9 days after MB49Luc instillation. At that point, mice were randomized into the two treatment cohorts and followed by IVIS and visual inspection for a maximum of 1 month. Log-rank (Mantel-Cox) test was used to analyze

survival between the two group cohorts, while nonparametric Mann-Whitney test was used to compare IVIS values (total flux, p/s) before treatment (Prism, Version 9.2.0).

12.3.2 Transwell co-culture experiments

For the Transwell co-culture experiments, 12-wells multiwell plates and related Transwell inserts (Corning Costar ref. 3460 - clear) were used. The diameter of the inserts is 12 mm, resulting in an approximate growth area of 1.12 cm^2; the pore size is 0.4 μm, the membrane material is polyester.

BJ fibroblasts (kindly donated by Dr. Emanuel Della Torre and Dr. Claudia Minici, IRCCS Ospedale San Raffaele) were cultured in DMEM medium, supplemented with 10% FBS and 1% penicillin/streptomycin. HBLAK cells were cultured in CnT-PR medium (CELLnTEC, ref. CNT-PR), supplemented with 1% penicillin/streptomycin. 5637 cells were cultured in RPMI medium (RPMI 1640, Euroclone, ref. ECB2000L), supplemented with 10% FBS and 1% penicillin/streptomycin. All cell lines were maintained in a humified atmosphere (5% CO_2/95% air) at 37°C for all experiments. A vial of cell bank was used to start a new experiment. The cells were routinely tested for mycoplasma contamination and cultured for not more than 4 weeks before use.

To measure dextran permeability, the Transwell insert was transferred to a new 12-well multiwell plate (called receiver plate). 1.5 mL of fresh culture medium was added in the outer chamber of the well. Then, 200 μL of a 1mg/mL solution of rhodamine B-labelled 70kDa dextrans (Sigma-Aldrich, ref. R9379) were put in the inner chamber of the insert, and incubated for 1 hour in the incubator, protected from light. After 1 hour, the permeation was stopped by removing the insert from the receiver plate, and the medium left in the outer chamber of the receiver plate was thoroughly mixed. Then, 3x100 μL of the medium were transferred to a black 96-well opaque plate, and the fluorescence was measured using a Wallac VICTOR^3 Multilabel Plate Reader (PerkinElmer) with the following settings: CW lamp filter FP531, emission filter 595/60, 1 second measurement time.

For immunofluorescence, all steps were performed in a well using intact inserts. The following primary antibodies were used: Alexa Fluor™ 647-conjugated Phalloidin (Thermo Fischer Scientific, ref. A22287), anti-Cytokeratin 5 (Abcam, ref. ab52635) and anti-Cytokeratin 20 (Abcam, ref. ab76126). The secondary antibody used was Alexa

Fluor Plus 488-conjugated ant-rabbit (Thermo Fischer Scientific, ref. A32790), used to label the 2 anti-cytokeratin antibodies at the same time. Phalloidin was used to label the BJ fibroblasts, while cytokeratins were used together to label bladder cells (both HBLAK and 5637). Briefly, cells were permeabilized with a solution of PBS containing 0.2% Triton X-100 (Merck, ref. T8787) for 10 minutes at room temperature. Then, after three washes (5 minutes each) using a solution of PBS containing 0.1% Triton X-100, the insert was blocked for 60 minutes at room temperature using a solution of PBS containing 10% donkey serum (Abcam, ref. ab7475) and 0.1% Triton X-100. After this step, the insert was incubated overnight at 4°C with primary antibodies diluted in a solution of PBS containing 1% donkey serum and 0.1% Triton X-100. The day after, the insert was washed three times (5 minutes each) using a solution of PBS containing 0.1% Triton X-100, followed by incubation (1 hour at room temperature) with secondary antibodies and Hoecst 33342 (Thermo Fisher Scientific, ref. H3570) diluted in a solution of PBS containing 1% donkey serum and 0.1% Triton X-100. The insert was then washed three times (5 minutes each) using a solution of PBS containing 0.1% Triton X-100 and stored at 4°C in PBS until imaging. Imaging was performed using the Leica TCS SP5 Confocal Microscopy, available in the Advanced Light and Electron Microscopy BioImaging Center (ALEMBIC) at IRCCS Ospedale San Raffaele (Milan). The Transwell insert was imaged without detaching the membrane from the rigid plastic support, using a round coverslip and adaptor.

Part of the information reported above in Sections 12.1 and 12.2 has been published in two publications:

- Pederzoli F. *et al.*, “Sex-specific Alterations in the Urinary and Tissue Microbiome in Therapy-naïve Urothelial Bladder Cancer Patients”, European Urology Oncology 2020 (reference 452 in the present thesis);
- Pederzoli F. *et al.*, “Is There a Detrimental Effect of Antibiotic Therapy in Patients with Muscle-invasive Bladder Cancer Treated with Neoadjuvant Pembrolizumab?”, European Urology 2021 (reference 457 in the present thesis).

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