

Polymorphismes des gènes associés à l'inflammation et microenvironnement tumoral lymphocytaire CD8+: valeur pronostique dans les carcinomes urothéliaux de la vessie.

Alexandra Masson Lecomte

▶ To cite this version:

Alexandra Masson Lecomte. Polymorphismes des gènes associés à l'inflammation et microenvironnement tumoral lymphocytaire CD8+: valeur pronostique dans les carcinomes urothéliaux de la vessie.. Médecine humaine et pathologie. Université Paris-Est, 2017. Français. NNT: 2017PESC0073. tel-01939721

HAL Id: tel-01939721 https://theses.hal.science/tel-01939721

Submitted on 29 Nov 2018

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

UNIVERSITÉ PARIS EST

ECOLE DOCTORALE SCIENCES DE LA VIE ET DE LA SANTE

THÈSE DE DOCTORAT

Présentée et soutenue publiquement Le 26 Juin 2017 Par

Mme Alexandra Masson-Lecomte Née le 30 octobre 1983 à Boulogne-Billancourt

POLYMORPHISMES DES GENES ASSOCIÉS A L'INFLAMMATION ET MICROENVIRONNEMENT TUMORAL LYMPHOCYTAIRE CD8+ : VALEUR PRONOSTIQUE DANS LES CARCINOMES UROTHELIAUX DE LA VESSIE

Thèse dirigée par Monsieur Yves ALLORY

JURY <u>Président du Jury</u>: Rapporteurs:

Examinateurs:

Mr François DESGRANDCHAMPS Mme Simone BENHAMOU Mme Kirsten JUNKER Mr Morgan ROUPRÊT Mr François RADVANYI

POLYMORPHISMES DES GENES ASSOCIÉS A L'INFLAMMATION ET MICROENVIRONNEMENT TUMORAL LYMPHOCYTAIRE CD8+ : VALEUR PRONOSTIQUE DANS LES CARCINOMES UROTHELIAUX DE LA VESSIE

<u>RESUMÉ</u>

L'objectif du travail a été d'explorer la valeur pronostique pour les tumeurs de la vessie des polymorphismes de gènes associés à l'inflammation et du microenvironnement tumoral lymphocytaire CD8+. Pour les marqueurs constitutionnels, deux approches ont été conduites concomitamment, l'une explorant de façon globale les gènes associés à l'inflammation, l'autre ciblant un gène inflammatoire d'intérêt, *PDL1*, impliqué dans des points de contrôles immunologiques. A l'échelle du génome, en utilisant des méthodes statistiques soit classiques soit innovantes (dites multi marqueurs), nous avons démontré que les variants (SNP) dans les gènes *TNIP1*, *CD5* et *JAK3* étaient associés au risque de récidive des tumeurs de vessie non invasives du muscle alors que les variants dans les gènes *MASP1*, *AIRE* et *CD3* étaient associés au risque de progression. Dans un deuxième temps, l'association entre variants dans le gène de *PDL1* et pronostic des tumeurs de vessie a été explorée en appliquant une méthode classique « SNP par SNP » et une approche à l'échelle du gène. Nous avons identifié une forte association entre des variants de *PDL1* et le pronostic de tumeurs de vessie envahissant le muscle dans une large cohorte prospective, mais sans pouvoir répliquer ce résultat dans une série issue du consortium TCGA.

Dans les tumeurs n'envahissant pas le muscle, nous avons développé et évalué une méthode d'évaluation standardisée de l'infiltrat lymphocytaire CD8+, cellules T-cytotoxiques impliquées dans la mort des cellules tumorales. L'analyse morphométrique après double immuno-marquage des cellules tumorales et des lymphocytes CD8+ et numérisation a permis d'estimer de façon séparée le compte des cellules inflammatoires dans la tumeur et le stroma,

et d'estimer l'hétérogénéité spatiale intra-tumorale. Nous avons montré que cette hétérogénéité limite les estimations de l'infiltrat CD8+ sur les puces tissulaires (Tissue Micro Array) qui échantillonnent les tumeurs de façon restrictive. Sous cette réserve, nous avons identifié dans les tumeurs n'envahissant pas le muscle une association entre l'infiltrat lymphocytaire CD8+ le stade tumoral Ta/T1, ainsi qu'avec le risque de récidive des tumeurs T1. A l'avenir, variations génétiques constitutionnelles dans les gènes de l'inflammation et évaluation de l'infiltrat tumoral inflammatoire pourraient être intégrées en vue d'améliorer la prédiction du pronostic des tumeurs vésicales.

INFLAMMATORY GENETIC VARIANTS AND CD8+ TUMOR MICROENVIRONMENT: PROGNOSTIC VALUE IN UROTHELIAL CARCINOMA OF THE BLADDER

SUMMARY

The aim of this study was to explore prognostic value for bladder cancer of germline polymorphisms in inflammatory genes and tumor CD8+ lymphocytic microenvironment. For constitutional markers, two approaches were conducted jointly: one genome-based using specific GWAS statistical methods, the other gene-based focusing on *PDL1*, an inflammatory gene implicated in immune checkpoints. At the genome level, using both standard and innovative statistical methods (multi marker methods Bayesian Lasso and Bayes A) we demonstrated that variants (SNPs) in TNIP1, CD5 and JAK3 were associated with the risk of recurrence of non-muscle invasive bladder cancer (NMIBC) while SNPs in MASP1, AIRE and CD3 were associated with risk of progression. Meanwhile, association between *PDL1* and prognosis of NMIBC and muscle invasive BC (MIBC) was explored using classical SNPS by SNP investigations and a gene based approach. We identified a very strong association between PDL1 variants and MIBC prognosis in a large prospective cohort but failed replicating those results in the TCGA consortium series.

In non-muscle invasive bladder cancer, we developed and evaluated a standardized counting approach of CD8+ cells, T-cytotoxic lymphocytes implicated in tumor cells death. Morphometric analysis after double immuno-staining of tumor cells and digitalization allowed separate estimation of CD8+ cells in the tumor and stroma compartment and estimation of spatial intra tumoral heterogeneity. We demonstrated that this heterogeneity compromised CD8+ estimation on Tissue Micro Arrays, which sample the tumors in a restrictive manner. Keeping those limitations in mind, we identified an association between

CD8+ inflammatory infiltrate and both NMIBC stage and T1 tumours risk of recurrence. In the future, germline variation in inflammatory genes and evaluation of tumor inflammatory infiltrate could be integrated for better prediction of bladder cancer prognosis.

Mots clés: tumeur de vessie, inflammation, variants germinaux, infiltrat lymphocytaire, méthodologie et pronostic.

Key words: bladder cancer, inflammation, germline variants, inflammatory infiltrate, methodology and prognosis.

Thèse de Doctorat préparée au sein de l'Unité de Recherche Translationnelle en Oncogenèse Génito-urinaire du Pr Alexandre de la Taille – Institut Mondor de Recherche Biomédicale – Faculté de Médecine de Créteil - 8, rue du Général Sarrail, 94010 Créteil, France et du Genetic and Molecular Epidemiology Group du Dr Nuria Malats – Centro Nacional de Investigaciones Oncologicas – Madrid, Espagne.

REMERCIEMENTS

A mon directeur de Thèse le Professeur Yves Allory.

Je ne peux résumer tout ce que je vous dois. Vous m'avez tellement appris. Vous m'avez tellement soutenue. Sans vous rien n'aurait été possible. Ma reconnaissance est immense.

To my co-director Mrs Nuria Malats

You taught me all about methodology and statistics, about rigor, precision, honest evaluation of the work done. You also taught me the taste for sharing results, networking, discussing and interacting with others. Working in your lab was an incredible opportunity, for everything I learned as well as for the inestimable value of the people I met.

A mon Président de jury le Professeur François Desgrandchamps.

Vous avez cru en moi dans une période où l'avenir s'était assombri, vous m'avez fait une place au sein de votre maison et vous m'aidez chaque jour à votre contact à grandir comme Urologue et comme Chercheuse. Je vous remercie de la chance que vous m'offrez et j'espère être à la hauteur.

A mon directeur de laboratoire le Professeur Alexandre de la Taille.

Merci de m'avoir poussée à travailler sur la vessie, d'avoir insisté sur l'absolue nécessité d'avoir une thématique claire, d'avoir mis à ma disposition les moyens de votre laboratoire pour conduire ce travail de thèse.

Aux membres du jury.

Merci à Mme Simone Benhamou et à Mme Kirsten Junker d'avoir accepter de consacrer le temps précieux qui est le leur à la lecture de ce manuscrit et à son commentaire. Thanks To Mrs Simone Benhamou and Mrs Kirsten Junker for accepting to spend their precious time reading and commenting this manuscript.

Merci à François Radvanyi pour ses conseils toujours pertinents, ses enseignements et la mise à disposition de ses moyens pour m'aider à avancer dans la difficile carrière de chercheuse. Merci à Morgan Rouprêt pour son soutient permanent, ses qualités pédagogiques et humaines et son investissement pour aider et faire grandir les plus jeunes.

To Paulina, Evangelina, Marta and Silvia

I would never have succeeded without you all. I will not forget the incredible generosity you all put in helping me moving on with this work. Your permanent support and kindness has helped me climb a mountain regarding the total absence of knowledge I had in your field when I arrived in Madrid. I wish you all the very best both personally and professionally. Thank you.

A Mme Pascale Maillé

Merci Pascale pour tout le temps passé à m'aider, pour ta gentillesse et ta bonne humeur inamovible.

A Olivier.

Etre une femme chirurgien, engagée dans une carrière universitaire, toujours au travail, le soir, la nuit, le week-end, est impossible sans le soutient solide et permanent de celui qui l'aime. Je n'aurai jamais poursuivi jusqu'ici sans toi, sans la vie personnelle équilibrée que tu m'offres et qui me comble du bonheur indispensable a la persévérance dans cette voie difficile. Chaque jour supplémentaire avec toi est un cadeau.

OUTLINE

II	NTRODUCTION	1
1		1
Ι.	IMMUNITY IN THE NATURAL HISTORY OF BLADDER CANCER	l
	1.1. <u>Natural history of bladder cancer</u>	I
	1.1.1. Incidence	1
	1.1.2. Risk factors	1
	1.1.2.1. Non inflammatory risk factors	1
	1.1.2.2. Inflammation and cancer risk	3
	1.1.2.2.1. NSAIDs/Aspirin	3
	1.1.2.2.2. Germline variation	3
	1.1.2.2.3. Local inflammation.	4
	1.1.3 Evolution and principle of treatment of bladder tumours	5
	1 1 3 1 NMIRC	5
	11311 Recurrence	5
	1 1 3 1 2 Prograssion	5
	1 1 2 1 2 Treatment generality for NIMIDC	5
	1.1.2.2 MIDC	0
	1.1.2.2. MIBC	0
	1.1.3.2.1. Metastasis and mortality rates	6
	1.1.3.2.2. Treatment generality for MIBC	6
	1.2. Classical prognosticators	7
	1.2. <u>Classical prognosticators</u>	
	1.2.1. NWIDC	/
	1.2.2. MIDC	9
		9
	1.2.2.2. Pathological factors	9
	1.3 Molecular subtypes and micro environment	11
	1.3.1 Molecular subtyping of UCB	11
	1.3.2. Micro environmental interactions.	14
2.	IMMUNITY IN THE TREATMENT OF BLADDER CANCER	16
	2.1. Immune system modulators	16
	2.1.1. BCG in NMIBC	16
	2.1.1.1. Mechanism of action	
	2112 Scheme of administration	18
	2.1.2. Others	18
	2.2. Immune checkpoint inhibitors in MIBC 19	
_		
3.	INFLAMMATORY BIOMARKERS IN BLADDER CANCER PROGNOSIS	22
	3.1. <u>Rational</u>	22
	3.1.1. Limitations of the classical prognosticators	22
	3.1.2. Interest in inflammatory biomarkers and methodological limitations	22
	3.2. Germline variants in inflammatory genes and cancer prognosis	24
	2.2 Tumor related inflammation and annear prognessis	25
	5.5. <u>I unior related inframmation and cancer prognosis</u>	23

3.3.1. Cytokines/proteins	
3.3.2. Micro environmental cells	
3.3.2.1. The Immunoscore and colon cancer	
3.3.2.2. Inflammatory infiltrate and bladder cancer	
3.4 Serum inflammation and cancer prognosis	32
3.4.1 Inflammatory proteins	32
3.4.2. Inflammatory cells.	
3.5. Urinary inflammatory response and bladder cancer prognosis	33
5.5. Officially inflaminatory response and bladder cancer prognosis	
4. SUMMARY: IMMUNITY IN THE NATURAL HISTORY AND MA	NAGEMENT
OF BLADDER CANCER	
II OBJECTIVES	
1 HVPOTHESES	37
2 GERMLINE VARIANTS	37
2.1. Genome-based approach.	
2.2. Gene-based approach	
	20
3. TUMOR INFLAMMATORY INFILTRATE	
3.1. <u>Rational for using CD8 as a model marker</u>	
5.2. Methodological validation.	
III RESULTS	
ARTICLE 1. INFLAMMATORY-RELATED CENETIC VARIANT	S IN NON-
MUSCLE INVASIVE BLADDER CANCER PROGNOSIS. A MUL	TI-MARKER
BAYESIAN ASSESSMENT. A Masson-Lecomte et al. CEBP 2016	40
1.1 Abstract	40
1.1. <u>- 1000000</u> .	
1.2. <u>Manuscript</u>	
ARTICLE 2: ASSOCIATION BETWEEN GERMLINE PDL1 VAR	AND 57
DLADDER CANCER I RUGNUSIS	
1.1. <u>Abstract</u>	57
1.2 Manuscript	50
1.2. <u>Ivianuscript</u>	

1.1. <u>Abstract</u>	. 67
1.2. <u>Manuscript</u>	68

IV	<u> DISCUSSION</u>	105
1.	INFLAMMATION RELATED GENETIC POLYMORPHISMS AND BLAI CANCER PROGNOSIS	DDER
	1.1. Different output of candidate-gene or a genome-wide approaches	105
	1.2. Biological relevance as the cornerstone of genetic studies	106
	1.3. The need for integration with the other –omics	107
2.	TISSUE INFLAMMATION ASSESMENT AND ASSOCIATION WITH BLAI CANCER PROGNOSIS	DDER 107
	2.1. <u>Strengths of the results</u>	107
	2.2. <u>Remaining challenges</u>	108
	2.2.1. Compartments	108
	2.2.2. Impact of UCB natural history on tumor inflammation	109
	2.2.3. Consideration of multiples inflammatory cell types	109
3.	SERUM INFLAMMATION IN THE CONTEXT OF BLADDER CANCER	111
	3.1. Serum inflammation in the EPICURO cohort	111
	3.2. <u>Future directions</u>	113
4.	INTEGRATIVE APROACH BETWEEN INFLAMMATION AND MOLECUI SUBTYPES	L AR 114
	4.1. Integration between the different matrices.	114
	4.2. Integration with molecular taxonomy.	115
5.	PREDICTION OF BLADDER CANCER PROGNOSIS AND RESPONSE TO IMMUNOTHERAPY TREATMENT.	116
6.	FUTURE PROJECTS	117
V	<u>CONCLUSION</u>	120
V	I REFERENCES	121
<u>VI</u> rev	<u>I APPENDIX</u> : Inflammatory Biomarkers and Bladder Cancer Prognosis: a system view. A Masson-Lecomte et al. Eur Urol 2014	ematic 145

I INTRODUCTION

1. IMMUNITY IN THE NATURAL HISTORY OF BLADDER CANCER

1.1. Natural history of bladder cancer

1.1.1. Incidence

Each year 2.7 million people are diagnosed with bladder cancer (BC) in the world. Most cases appear after 60 years of age [1]. In France, 12 305 new invasive cases were identified in 2015, mainly in men. Bladder cancer occupies the 4th place in incidence and is the 7th cause of death all cancers included. It constitutes the second urological malignancy after prostate. Incidence is rising by 1% every year, yielding to an important number of prevalent cases [2]. Urothelial carcinoma is the major histological type of bladder cancer (UCB) and is subdivided in two groups according to its invasion of the bladder wall: nonmuscle invasive (NMIBC), accounting for 70-80% of UCB at diagnosis, and muscle invasive (MIBC) accounting for the remaining 20%.

1.1.2. Risk factors

1.1.2.1. Non inflammatory risk factors

Bladder cancer is a complex disease with both environmental and genetic risk factors.

Environmental: Main known risk factors are tobacco use and exposition to aromatic amines [3-5]. Although bladder cancer incidence in smoking men is decreasing [6], trend is inverted in women due to crescent tobacco consumption. Exposure to tobacco, both active and passive, is associated with the occurrence of bladder cancer. In a recent meta-analysis of incidence, the pooled relative risk of BC incidence was 2.58 (2.37-2.80) for all smokers, 3.47 (3.07-3.91) for current smokers, and 2.04 (1.85-2.25) for former smoker. Occupational exposure to aromatic amines, nitrosamines and polycyclic aromatic hydrocarbons has been historically associated with bladder cancer risk. Although in Europeanised countries,

prevention policies led to a reduction of workers' exposure to aromatic amines, the French Institut de Veille Sanitaire estimated in 2001 that, in France, 8 to 14% of incident bladder cancers and 10 to 14% of deaths linked to this type of cancer in men were attributable to occupational exposure, mainly due to polycyclic aromatic hydrocarbons in contemporary context [7]. Some industries are now less exposed because of prevention policies (a recent meta-analysis found only a borderline increase risk for BC in the aluminum production (OR 1.28, 95 % CI 0.98-1.68) and in iron and steel foundries (OR 1.38, 95 % CI 1.00-1.91) [4]) while others still remain at risk nowadays (plastic, chemical and pharmaceutical industries, see specific tables in article [7]). To date, despite the prevention programs that have been implemented for workers exposed, hundreds of annual deaths from bladder cancer are still imputable to occupational exposures.

Genetic: familial forms of bladder cancer have been described. Most implicated genes have not yet been identified. However, mutations in tumor suppressor *RB1* or oncogene *HRAS*, sometimes as mosaic, can be involved in occurrence of bladder tumors at younger age [8, 9]. Generally, BC is a complex disease and genetic susceptibility is rather due to many Single Nucleotide Polymorphisms (SNPs) with small effect than to a simple SNP/gene disorder. Single Nucleotide Polymorphisms (SNPs) in carcinogen-detoxification genes such as *NAT2* or *GSTM1* have been consistently showed to be associated with BC risk [10]. A recent metaanalysis confirmed the association between *NAT2* slow acetylators status and risk of BC with an OR of 1.31 (1.11-1.55) [11]. Moreover, genetic and environmental factors can interact, such as demonstrated in a genome-wide interaction study of smoking and bladder cancer risk [12]. Garcia Closas et al. explored interaction between smoking and 12 susceptibility loci, individually and combined as a polygenic risk score (PRS) [13]. The 30-year absolute risk of bladder cancer in U.S. males ranged from 2.9% for current smokers in the lowest quartile of the PRS to 9.9% for current smokers in the upper quartile. Risk difference estimates indicated that 8,200 cases would be prevented if elimination of smoking occurred in 100,000 men in the upper PRS quartile compared with 2,000 cases prevented by a similar effort in the lowest PRS quartile.

Integration of both genetic factors and environmental exposure/interactions in the design of bladder cancer prevention program would undoubtedly lead to great reductions in bladder cancer incidence. Cost remains a major limitation.

1.1.2.2. Inflammation and cancer risk

1.1.2.2.1. NSAIDs/Aspirin

The impact of anti-inflammatory agents administration in prevention of cancer strengthens the importance of preexisting inflammation in cancer development[14, 15]. Rothwell *et al.* studied deaths due to cancer during and after randomized trials of daily aspirin versus placebo, done originally for prevention of vascular events. They showed that patients under aspirin treatment had a significantly lower risk of developing all cancers and particularly colorectal cancers than the placebo group (5 years HR 0.66, 95%CI 0.50-0.87, p=0.003). NSAIDs administration effect on bladder cancer risk has been studied in large epidemiological studies with consistent results suggesting reduced risk of developing bladder malignancy under treatment [16]. Oral chemo-prevention by acetyl salicylic acid or COX 2 inhibitors reduced the risk of induced bladder cancer in mouse [17, 18]. Altogether, published studies support the efficacy of anti-inflammatory drugs in preventing cancer in general and bladder cancer in particular. However, adverse effects of long-term administration prevent from recommending such treatments in daily practice [19]

1.1.2.2.2. Germline variation

Polymorphisms in inflammatory genes are known to predispose to or conversely protect against some cancers [20-22]. Epidemiological studies demonstrated that genetic

variations in inflammatory pathways genes affected bladder cancer susceptibility [23]. Cytokines (IL4, IL6, TNF), chemokines and chemokines-receptor genes polymorphisms have been shown to impact bladder cancer development risk in single studies [24, 25]. Yang et al strongly suggested that the most common *COX2* haplotype was associated with increased risk of bladder cancer and that the haplotypes containing at least one variant allele were associated with decreased risk [26]. Moreover, COX2 inhibitor celecoxib demonstrated strong chemoprevention potential in rats exposed to N-butyl-N-(4-hydroxybutyl) nitrosamine [27]. Main limitation of those studies is the lack of external validation of the results in independent cohorts. A the genome wide level, Evangelina Lopez de Maturana et al. explored the association between 886 inflammatory-gene variants and bladder cancer risk in 1,047 cases and 988 controls from the Spanish Bladder Cancer (SBC)/EPICURO Study. Thirteen SNPs in thirteen genes were identified using two different statistical approaches as associated with bladder cancer risk (SNPs in JAK3, IL6R, CD5, ROCK1, CASP3, BIRC5, CD4, CD2, SCARB1, ICAM1, TFF1, AKR1C3). At the genetic level, those results suggested that bladder cancer risk is not driven by a single SNP/gene disorder and is rather influenced by multiples SNPs with small effect.

1.1.2.2.3. Local inflammation

In some type of cancers, chronic local infection/inflammation participate in the initiation of tumorigenesis. The link between Helicobacter Pylori and gastric cancers, Hepatitis C Virus and hepatocellular carcinoma or inflammatory bowel disease and colorectal cancer is very well established [28]. At the bladder level, chronic inflammation is a major risk factor for squamous cell carcinoma but also urothelial carcinoma. Schistosoma haematobium infection of the bladder leads to a Th2 immune response favoring development of bladder malignancy [29]. Patients with neurogenic bladders are at much higher risk then the general population of developing BC, partly due to repeated catheterization and recurrent

urinary tract infections [30]. Inflammatory response is also a major actor of tumor promotion, regardless of pre-existing local inflammatory factors [31]. Aberrant activation of inflammatory pathways triggers the recruitment of inflammatory cells in the tumor microenvironment (macrophages, mastocytes, T cytotoxic, T regulator or B lymphocytes, myeloid-derived suppressor cells) and stimulates cell proliferation, angiogenesis, invasion and metastasis, enabling most of the cancer hallmarks [32]. The role of inflammation has particularly been studied in colorectal cancer, where proinflammatory mediators such as NFkB, IL6, STAT3, TNF, COX 2, and multiples chemokines are known to be major actors of colorectal tumorigenesis [33]. Several inflammatory biomarkers (Inf-BM) have been showed to be useful in clinics, the most widely accepted being IL6, a major mediator of acute inflammation [34]. It has tumor promoting actions and acts as an autocrine and paracrine growth factor [34, 35]. The proliferative effect of IL6 is mainly mediated by the transcription factor STAT3 which is known to be activated in many subtypes of tumor cells [36-39].

1.1.3. Evolution and principle of treatment of bladder tumours

- 1.1.3.1. NMIBC
 - 1.1.3.1.1. Recurrence

Recurrence is defined as the occurrence of a new bladder tumor of the same or lower stage/grade after initial treatment. NMIBC is associated with a very high recurrence rate, between 30% and 70% at five-year according to the EORTC [40]. Consequently follow up of patients is very stringent, leading to alteration of patients' quality of life and high public heath costs.

1.1.3.1.2. Progression

Progression is defined as the occurrence of a tumor of higher stage/grade during follow up, appearance of metastasis or death due to bladder cancer. Overall 15% of NMIBC will progress with major differences between risk groups (ranging from 1% to 50%).

1.1.3.1.3. Treatment generality for NMIBC

NMIBC treatment is based on transurethral resection, alone or followed by adjuvant intra-vesical chemotherapy. Options are intra-vesical chemotherapy using Ametycine or intra-vesical immunotherapy using bacillus Calmette-Guerin (BCG). Choice of treatment depends on the tumor risk of recurrence and progression [41]. Those risks are calculated based on known clinical and pathological prognostic factors (see below).

1.1.3.2. MIBC

1.1.3.2.1. Metastasis and mortality rates

Multi institutional databases regrouping thousands of patients report five-year cancer specific survivals around 60% after radical cystectomy performed for curative intent [42, 43]. However, survival rates differ tremendously according to pathological findings (i.e. lymphovascular invasion, nodal invasion, histological subtypes see below). Consequently, half of the patients will relapse after radical cystectomy with recurrences occurring in the pelvis in 30% of cases or as distant metastasis more frequently [44]. Despite administration of chemotherapy, prognosis of relapsing patients is very poor with five-year survival rates less than 10%. Muscle invasiveness results in poor prognosis, especially in UCB progressing from NMIBC, despite BGC therapy, outlining the need for new accurate prognostic markers in this subgroup of patients [45].

1.1.3.2.2. Treatment generality for MIBC

At diagnosis, 20% of BC are muscle invasive (stage pT2-4N0M0) with potential micro-metastases. Therefore, effective systemic treatment must be associated to surgery. In a neoadjuvant setting, cisplatin based chemotherapy improved the absolute overall survival by 5% and the specific survival by 9%[46]. Various combinations have been assessed and the most efficient so far are either the association of methotrexate, vinblastine, adriamycin, and cisplatin (MVAC), or the less toxic association of gemcitabine and cisplatin (GC). Both are considered equally effective [47]. Neo adjuvant chemotherapy followed by radical cystectomy with extended pelvic lymph node dissection is, to date, the recommended curative treatment for MIBC, with 5-year recurrence free survival approaching 60% [48-50]. Despite NAC, 19.3% of patients are found to have lymph node- positive disease on postoperative pathological analysis [51]. Trials using radio chemotherapy have shown interesting results in selected patients with small, localized muscle invasive UCB allowing long term bladder preservation [52]. The presence of adverse pathological factors after RC, such as lymph node metastases or locally advanced disease are a well-known predictors of overall survival. Consequently, those patients are recommended for adjuvant chemotherapy [53]. Until recently, platin-based chemotherapy has been the only option for metastatic bladder cancer with very poor outcome (overall survival rate < 5% at 5 years). Recently, immunotherapy using anti PDL1 antibodyimmune checkpoints inhibitors (anti PDA, anti PDL1, anti CTLA4 antibodies) has shown promising results and is being tested in clinical trials [54].

1.2. Classical prognosticators

1.2.1. NMIBC

Prediction of risk of recurrence and progression is based on clinico-pathological factors. Two main groups, the EORTC and the CUETO, respectively European and Spanish, have described risk factors for both outcomes using large cohort of thousands of patients [40, 55]. Main difference between both is the administration of maintenance BCG (no maintenance in EORTC trials vs 6 months maintenance in CUETO). Six main clinico-pathological risk factors, divided in categories, are now generally accepted and used in daily clinical practice:

- Number of tumors : single vs multiple
- Tumor diameter : $< 3 \text{ or} \ge \text{to } 3 \text{ cm}$
- \circ Prior recurrence rate: ≤ 1 recurrence / year or > 1 recurrence per year
- Stage: Ta vs T1
- Grade: G1 vs G2 vs G3 (1973 WHO classification) and low vs high grade (2016
 WHO classification) [56]
- Concurrent CIS: no vs yes

Based on those factors patients can be categorized in risk categories (Table 1)

<u>Table 1</u>: Risk group stratification according to EORTC (from the EAU 2016 guidelines). The two WHO grade classifications (1973 and 2016) appear together in the table. This reflects the reality of practice where pathologist and clinicians often use both classifications in daily care.

Risk group stratification	Characteristics Primary, solitary, Ta, G1* (PUNLMP, LG), < 3 cm, no CIS		
Low-risk tumours			
Intermediate-risk tumours	All tumours not defined in the two adjacent categories (between the category of low- and high-risk).		
High-risk tumours	Any of the following: • T1 tumour • G3** (HG) tumour • CIS • Multiple and recurrent and large (> 3 cm) Ta, G1G2 tumours (all conditions must be presented in this point)*		

"Primary, solitary, less than 3 cm TaG1" tumors' risk of recurrence at one and five-year are 15 and 31% respectively while risk of progression at one and five-year are 0.2 and 0.8%

respectively. On the contrary, "more than 3 cm T1G3 plus CIS" tumors' risk of recurrence at one and five-year are 61 and 78% with a risk of progression of 17 and 45% respectively. Risk of progression is probably slightly overestimated in the EORTC cohort due to lack of maintenance BCG.

1.2.2. MIBC

1.2.2.1. Clinical factors

As for NMIBC, main prognostic factors are clinical and pathological. Before radical cystectomy, all localized muscle invasive bladder cancer should receive neo-adjuvant chemotherapy. Markers of response to chemotherapy and identification of pre-operative prognostic markers could help target patients that would benefit most from systemic treatment but none are available yet. Locally advanced diseases defined as suspicion of perivesical fat invasion or hydronephrosis on CT scans are known to have lower survival rates after radical cystectomy. However treatment options for those patients remain the same then for strictly localized disease.

Approximately 5% of MIBC are metastatic at diagnosis [57]. In the setting of local/systemic recurrence, prognosis is very poor and no validated prognostic markers are available. Rink et al published a cohort of 1545 patients from 16 institutions who experienced disease recurrence after radical cystectomy [58]. Advanced age, female gender, RC stage and positive margins were negatively associated with survival.

1.2.2.2. Pathological factors

On RC specimen, pathological prognostic markers have been described and validated in independent cohorts:

• Stage: prognosis is deeply conditioned by stage. Main prognosticator is the absence of residual disease on RC specimen after neoadjuvant chemotherapy

(ypT0) [59]. However, each progression in stage confers poorer survival (pT2 vs pT3 vs pT4).

- Lympho-vascular invasion [60]
- Nodal invasion
- If nodal invasion, number/density of positive nodes and presence of extra-capsular extension [61, 62].

Based on those factors, patients who did not receive neo-adjuvant chemotherapy and who display locally advanced disease or nodal invasion on radical cystectomy specimen should receive adjuvant chemotherapy. As in the neo-adjuvant setting, no predictors of response (clinco-pathological/molecular) are used in daily practice.

• Histological subtypes: Main subtypes are presented in Table 2, extracted from the latest WHO classification (2016). Occurrence of divergent differentiation on radical cystectomy specimen is as high as 30% [56] conferring various prognosis implications. Squamous and glandular differentiations are most commonly observed. Generally, variant histology is associated with advanced disease and worse outcome. [63]. Xylinas et al described in 2013 a cohort of 488 patients with 24.6% of variant histology [64]. Histological UCB variants were associated with advanced tumour stage, lymphovascular invasion and lymph node metastasis (all p-values<0.01) when compared to pure UCB. Association with prognosis was only seen in univariate analysis suggesting that worse outcome was related to advanced stage and adverse pathological features. Table 2: WHO classification of tumors of the urothelial tract. In the latest classification, squamous cell neoplasms are now an independent entity. The squamous differentiation no longer exists.

WHO classification of tumours of the urothelial tract

Urothelial tumours		Neuroendocrine tumours	
Infiltrating urothelial carcinoma	8120/3	Small cell neuroendocrine carcinoma	8041/3
Nested including large nested	0120/0	Large cell neuroendocrine carcinoma	8013/3
Microcystic		Well-differentiated neuroendocrine tumour	8240/3
Micropapillary	8131/3	Paraganglioma	8693/1
l vmphoenithelioma-like	8082/3	i didganghoma	0000,1
Plasmacytoid / signat ring call / diffuse	0002/0	Melanocytic tumours	
Sarcomatoid	8100/3	Melianeotytic tanlours	8720/3
Giant coll	9031/3	Naovue	8720/0
Poorly differentiated	8020/3	Molanosia	0720/0
Lipid rich	0020/0	Widaliusis	
Clear cell		Mesenchymal tumours	
		Bhabdomyosarcoma	8900/3
Non-invasive urothelial neonlasms		Leiomyosarcoma	8890/3
Licotholial carcinoma in situ	8120/2	Anciocarcoma	0120/3
Non invesive popillary urotholial	0120/2	Inflammatory mysfibrablastic tymour	9120/3
non-invasive papiliary dromeliar	0100/0	Periverse var enithelieid cell turnour	0023/1
Non investive popillany uretholial	0130/2	Penvascular epimelioid cell tumour	9714/0
Non-invasive papillary urothelial	0100/0	Maliapont	0714/0
Carcinoma, nign-grade	0130/2	Colitory fibroup turnour	0/ 14/3
Papillary urothelial neoplasm of	0100/1	Solitary librous turnour	8815/1
Iow malignant potential	8130/1	Leiomyoma	8890/0
Urotnellal papilloma	8120/0	Haemangioma	9120/0
Inverted urothelial papilloma	8121/0	Granular cell tumour	9580/0
Urothelial proliferation of uncertain		Neuronbroma	9540/0
malignant potential			
Urothelial dysplasia		Urothelial tract haematopoietic and	
		lymphoid tumours	
Squamous cell neoplasms	10000000000		
Pure squamous cell carcinoma	8070/3	Miscellaneous tumours	Stationary.
Verrucous carcinoma	8051/3	Carcinoma of Skene, Cowper, and Littre glands	8140/3
Squamous cell papilloma	8052/0	Metastatic tumours and tumours extending from other organs	
Glandular neoplasms		Epithelial tumours of the upper urinary tract	
Adenocarcinoma, NOS	8140/3	Tumours arising in a bladder diverticulum	
Enteric	8144/3	Urothelial tumours of the urethra	
Mucinous	8480/3		
Mixed	8140/3		
Villous adenoma	8261/0	The morphology codes are from the International Classification	of Diseases
		for Oncology (ICD-O) (917A). Behaviour is coded /0 for benign	tumours;
Urachal carcinoma	8010/3	/1 for unspecified, borderline, or uncertain behaviour; /2 for carcinoma in	
		situ and grade III intraepithelial neoplasia; and /3 for malignant	tumours.
Tumours of Müllerian type		The classification is modified from the previous WHO classifica	tion (756A),
Clear cell carcinoma	8310/3	taking into account changes in our understanding of these lesis	ons.
Endometrioid carcinoma	8380/3		

1.3. Molecular subtypes and micro environment

1.3.1. Molecular subtyping of UCB

The genomics revolution has provided the opportunity to build tumor molecular "identity

cards" leading to sub-classification based on genetic alterations on top of histological subtypes or clinical presentations. Molecular subtyping of bladder cancer has been on-going in parallel in different groups around the world leading to the release of five UCB molecular classifications [65-69]. Those classifications overlap with some differences (Figure 1), the most comprehensive coming from the work by Sjodahl et al in Lund. Based on genome wide gene expression profiling of 308 UCB covering different stages of the disease (one third Ta, on third T1 and one third \geq T2) they were able to identify five major UCB subtypes (Figure 1, Lund): urobasal A, genomically unstable, urobasal B, squamous cell carcinoma like, and an infiltrated. The Urobasal A tumors display high FGFR3 genes expression signature and low immune gene expression signature. Those tumors are FGFR3 and PI3KCA mutated compared to genomically unstable tumors (55% vs 7% and 25% vs 8%). Urobasal A tumors are mostly Ta, maintain degrees of urothelial stratification (they express tight junction associated genes), express the basal cytokeratin (CK) 5 in cells in contact with the basal membrane, similar to what is seen in the normal urothelium. Genomically unstable tumors mainly differ from Urobasal A by the absence of FGFR3 genes signature and FGFR3 mutations. On the contrary they display a very high amount of TP53 mutations compared to Urobasal A (48% vs 11%). As Urobasal A they display high urothelial differenciation markers expression such as transcription factors GATA3 and FOXA1. Genomically unstable tumors are mainly T1 or \geq T2, G2-G3 tumors. A subset of the urobasal tumors differed from the Urobasal A by their muscle invasive phenotype with increased late cell cycle activity, expression of basal cell related keratins (CK6 and CK14) in suprabasal cell layers and strong immune signature. This group still displays strong FGFR3 signature and high levels of FGFR3 mutations. The squamous cell carcinoma (SCC)-like tumours, commonly called basal tumours in other classifications, are mainly muscle invasive tumors with an aggressive phenotype. The SCC like/basal tumors differed from the other subtypes by showing very

elevated expression of basal cytokeratins (CK6 and CK14, 5- to 31-fold), indicating a keratinized/squamous phenotype and several keratinization-associated genes. Pathologic evaluation of those tumors demonstrated signs of squamous differentiation in 2/3 of the cases. Those tumors also display a very strong immune signature and lack FGFR3 signature/mutations that differentiates them from Urobasal B tumours. Finally, the infiltrated phenotype corresponds to muscle invasive tumors, highly infiltrated with immune cells, displaying strong immune signature but no "basal" phenotype (expression of basal cytokeratins in contact with the basal layer) and no FGFR3 signature.



Figure 1: A: Overlap of molecular subtypes according to the different groups publications. B: Transcription factors, biomarkers and actionable targets according to the Lund subtyping [70].

Using the bladder cancer TCGA data (234 MI tumors) as a reference set (with four molecular groups, TCGA 1, 2, 3 and 4), the Lund group evaluated their five-tiered classification along with two others gene expression-based bladder cancer classification systems: the two-tiered University of North Carolina (UNC), the three-tiered MD Anderson (MDA) (Figure 1, [70]).

This analysis showed that classifications only partially overlap and that substantial biological subgroup heterogeneity remains. Consensus was reached regarding the existence of a group of Basal-Squamous-like tumors characterized by the high expression of CK5/6 and CK14 and low/undetectable expression of FOXA1 and GATA3 [71]. An additional tumor subgroup with urothelial differentiation features was recognized (simplistically called luminal tumors in some studies) whose optimal molecular definition is required. For other subtypes described, more work is needed to determine how robust they are and how to best define them at the molecular level. This is mandatory before molecular classifications can be implemented in translational research and at the end daily practice.

1.3.2. Micro environmental interactions

In the light of recent breakthrough made in metastatic bladder cancer treatment using immunotherapies, interactions between molecular subtypes and tumor inflammatory environment have been explored. Few publications explored those interaction but with consistent and robust results.

The first to describe interactions between molecular subtying and inflammatory cells infiltration was G. Sjodahl in 2014 [72]. Using immunohistochemistry for tumor infiltrated lymphocytes (CD3, CD8, FOXP3) and macrophages (CD68, and CD163) on a series of 296 UCB (all stages) they identified different degrees of immunological responses according to molecular subtypes: the urobasal subtype (TCGA I) induced a weak response, the genomically unstable subtype (TCGA II) induced an intermediate response, and the squamous cell carcinoma-like subtype (TCGA III-IV) induced a strong response.

In the Lancet publication by Rosenberg in 2016 presenting phase 2 results of anti PDL1 Atezolizumab in metastatic bladder cancer, ancillary translational studies have been made to explore interactions between TCGA "4 clusters" molecular subtyping and CD8+ inflammatory infiltrate/response to anti PDL1 drugs [73]. Results are consistent with the ones from Sjodahl et al. with CD8 T-effector gene expression increased in luminal cluster II and basal cluster III or IV and not in luminal cluster I. PDL1 immune cell prevalence was highly enriched in the basal subtype versus the luminal subtype (60% vs 23%) with high immune cells PDL1 expression seen in 15% of the papillary-like luminal cluster I, 34% of the cluster II, 68% of the squamous-like basal cluster III, and 50% of the basal cluster IV subtype.

Concomitantly (2016), Sweis et al explored tumor-oncogenic pathways correlated with the "T cell" phenotype. Using immune gene expression profiling to separate "T cell inflamed" and "non T cell inflamed tumors", they demonstrated that PPARG and FGFR3 pathways were activated in "non T cell inflamed" tumors while up-regulation of genes encoding immune checkpoint proteins was associated with «T cell inflamed tumors » [74]. Those results corroborate the ones from Sjodahl and Rosenberg showing low CD8 infiltration/ effector gene expression in TCGA I luminal tumors.

Altogether, those results support association/interactions between molecular subtyping and inflammatory environment, pinpointing the need for integration of the 2 matrices in the field of translational research but also response to immunotherapies.

2. IMMUNITY IN THE TREATMENT OF BLADDER CANCER

2.1. Immune system modulators

- 2.1.1. BCG in NMIBC
 - 2.1.1.1. Mechanism of action

First report of the use of attenuated BCG strains as a treatment for bladder cancer dates back to 1979 with the publication from Morales et al in Journal of Urology [75]. Authors reported the use of intra vesical and intra dermal BCG strains in 9 patients suffering from NMIBC bladder tumors. According to their finding, the course of the disease was altered favourably with fewer recurrences observed. Since then, treatment of NMIBC using intra vesical BCG stands as one of the most effective demonstration of the benefit of immunotherapy in treatment of cancers.

Mechanism of action of intra vesical BCG has been studied extensively ([76], Figure 2). Briefly, BCG bounds to fibronectin in the bladder lumen and is further internalized by both tumor and normal urothelial cell. After cellular processing, a complex allying BCG and major histocompatibility complex II is exposed at the cell surface triggering the immune reaction. This complex is recognised by CD4+ T cells, leading mainly to a T helper-1 (Th-1) response and production of IL-2, IL-12, interferon (IFN) gamma, and tumour necrosis factor (TNF) beta. Effector cells are cytotoxic CD8 + lymphocytes and Natural Killer cells that display a massive antitumor response after activation. At the same time, a T helper-2 (Th-2) response is induced through production of IL 4, 5, 6, 10, neutrophil and macrophages recruitment and release of TRAIL that appears to be an important predictor of BCG response [77].

Since the 80's, intra vesical administration of BCG has proven its efficacy in reducing both risk of tumor recurrence and progression. Five meta-analyses are available demonstrating that BCG is superior to TURB alone or TURB plus intra vesical chemotherapy in preventing risk of recurrence of NMIBC [44].



Figure 2: Bacillus Calmette-Guerin (BCG)-induced immune response. After internalisation of fibronectin-BCG complexes by normal urothelial and tumour cells, BCG antigens are presented at the cell surface, attracting

CD4+ T cells. Urothelial cells start a T helper-1 (Th-1) response by releasing several inflammatory cytokines (ie, interleukin (IL) 2, IL-8, IL-12, interferon (IFN)g and tumour necrosis factor (TNF)b), which then leads to a cellular response by the recruitment of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. At the same time a Th-2 response is initiated through PMN and macrophages.

APC = antigen-presenting cells; DC = dendritic cell; PMN = polymorphonuclear neutrophil; TRAIL = tumour necrosis factor apoptosis-inducing ligand. From [78]

Moreover, two meta-analyses demonstrated that BCG prevents, or at least delays the risk of tumor progression. However, optimal scheme of administration remains controversial.

2.1.1.2. Scheme of administration

Induction BCG is administrated weekly during 6 weeks as empirically described by Morales in the first published paper [75]. Induction treatment is followed by a maintenance period of time where BCG is administrated for 3 consecutive weeks at 3 months and then every 6 months. Regarding the optimal duration of the maintenance period, a meta-analysis by Böhle et al suggested that at least one year of full dose BCG is necessary for superiority over intra vesical chemotherapy by Mitomycin [79]. Optimal treatment would be 3-years maintenance based on the scheme recommended by Lamm et al in 2000 [80]. However, the inflammatory response induced in the bladder is responsible for mild to intense irritating lower urinary tract symptoms often preventing accomplishment of the full maintenance therapy.

2.1.2. Others

Studies suggested replacing attenuated BCG by BCG parts or recombinant BCG strains. Noteworthy, BCG cell wall skeleton could be used as an alternative leading to the same anti tumor immune reaction without the risk of systemic side effects. Main problem lies in product delivery challenges since it is poorly soluble and has a strong negative charge. Recently, a Japanese team suggested the use of lipid nano particules in order to help dispersion of the treatment [81]. Alternatively, studies suggested to use recombinant BCG able to secrete its own Th1 cytokines, enhancing the anti tumor response. Luo et al reviewed studies of recombinant BCG in the treatment of NMICB. Treatment seems feasible and demonstrated superiority to standard live BGC [82]. Enhanced side effects might however be a limitation. Finally, activating anti tumor immunity using viruses rather then BCG strains has been proposed [83]. This treatment is still at its early stages of development and seems to provoke the same local adverse events then BCG administration. However, viruses could allow virus mediated gene transfer and combination of both immunotherapy and gene therapy [84].

2.2. Immune checkpoint inhibitors in MIBC

As developed before, Immunotherapy has been used for decades in NMIBC. Immunotherapy is especially attractive in urothelial carcinoma because of the genetic instability leading to a high burden of somatic mutations [65]. Consequently, an elevated number of tumor antigens are expressed at the surface. Recently, considerable hope has been raised with the development of monoclonal antibodies regulating the immune checkpoint pathway blockade [85]. It is a fundamental physiological process that regulates the immune response and prevents inappropriate overreaction such as autoimmune disease (Figure 3). Many cancers, including bladder cancer, are controlling host immune system by co-opting certain immune-checkpoint pathways leading to inhibition of T cells that that are specific for tumour antigens and would otherwise have attacked the tumor. Promising results have been observed in metastatic BC with pembrolizumab (MK-3475)/ nivolumab and atezolizumab (MPDL3280A)/durvalmab respectively targeting the Program cell death 1 receptor (PD-1)

and its ligand PD-L1 [54, 73]. Favourable toxicity profile were observed and overall response rates of 24% and 25% were reported respectively in chemoresistant metastatic bladder cancer. Anti tumor effect was often rapid (occurring at the first response assessment (6 weeks)) and prolonged. Stronger responses (up to 50%) were observed for patients displaying elevated PD-L1 expression in tumor-surrounding T cells [86, 87]. Those excellent results led clinicians and researchers to try Immune check points blockade at earlier stages of the disease with on-going or planned trials in the adjuvant, neo adjuvant or even NMIBC setting (Imvigor010 = NCT02450331, PURE 01 = NCT02736266, KEYNOTE 057 = NCT02625961).

Moreover, preclinical data suggested that targeting different immune checkpoints together, such as programmed cell death-1 (PD-1) and cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), could provide for non-redundant pathway blockade and potential synergy. In non small cell lung carcinoma, association of Durvalumab (a selective, high-affinity human IgG1 monoclonal antibody that blocks PD-L1 binding to PD-1 and CD80) and Tremelimumab (a selective human IgG2 monoclonal antibody inhibitor of CTLA-4) has shown a manageable tolerability profile, with anti-tumour activity irrespective of PD-L1 status [88]. Those combinations are about to be tested in bladder cancer clinical trials.



Figure 1 | Multiple co-stimulatory and inhibitory interactions regulate T cell responses. Depicted are various ligand–receptor interactions between T cells and antigen-presenting cells (APCs) that regulate the T cell response to antigen (which is mediated by peptidemajor histocompatibility complex (MHC) molecule complexes that are recognized by the T cell receptor (TCR)). These responses can occur at the initiation of T cell responses in lymph nodes (where the major APCs are dendritic cells) or in peripheral tissues or tumours (where effector responses are regulated). In general, T cells do not respond to these ligand–receptor interactions unless they first recognize their cognate antigen through the TCR. Many of the ligands bind to multiple receptors, some of which deliver co-stimulatory signals and others deliver inhibitory signals. In general, pairs of co-stimulatory-inhibitory receptors that bind the same ligand or ligands — such as CD28 and cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) — display distinct kinetics of expression with the co-stimulatory receptor expressed on naive and resting T cells, but the inhibitory receptor is commonly upregulated after T cell activation. One important family of membrane-bound ligands that bind both co-stimulatory and inhibitory receptors is the B7 family. All of the B7 family members and their known ligands belong to the immunoglobulin superfamily. Many of the receptors for more recently identified B7 family members have not yet been identified. Tumour necrosis factor (TNF) family members that bind to cognate TNF receptor family molecules represent a second family of regulatory ligand-receptor pairs. These receptors predominantly deliver co-stimulatory signals when engaged by their cognate ligands. Another major category of signals that regulate the activation of T cells comes from soluble cytokines in the microenvironment. Communication between T cells and APCs is bidirectional. In some cases, this occurs when ligands themselves signal to the APC. In other cases, activated T cells upregulate ligands, such as CD40L, that engage cognate receptors on APCs. A2aR, adenosine A2a receptor; B7RP1, B7-related protein 1; BTLA, B and T lymphocyte attenuator; GAL9, galectin 9; HVEM, herpesvirus entry mediator; ICOS, inducible T cell co-stimulator; IL, interleukin; KIR, killer cell immunoglobulinlike receptor; LAG3, lymphocyte activation gene 3; PD1, programmed cell death protein 1; PDL, PD1 ligand; TGFβ, transforming growth factor-β; TIM3, T cell membrane protein 3.

Figure 3: Ligand-receptor interactions between Antigen Presenting cells and T cells. From [85]

3. INFLAMMATORY BIOMARKERS IN BLADDER CANCER PROGNOSIS

A systematic review of studies assessing association between germline, serum and tumor inflammatory biomarkers and bladder cancer prognosis has been performed and published in European Urology in 2014. The published manuscript is available in APPENDIX (PMID 25151017).

3.1. Rational

3.1.1. Limitations of the classical prognosticators

At present, the major prognosticators, either for NMIBC and MIBC, remain clinical and pathological factors. The use of promising molecular prognostic markers such as *TP53* or *FGFR3* mutations failed to demonstrate any applicability in daily practice. A new stratification of bladder cancers according to their molecular profile is ongoing [68, 69], aiming to improve outcome through targeted therapies. Whereas optimizing prognostic assessment is required for personalized treatment, most of the prognostic studies in bladder cancer have been limited by sample size, biased populations of patients treated in tertiary expert centers and retrospective design [89].

3.1.2. Interest in inflammatory biomarkers and methodological limitations

Avoiding immune disruption is one of the emerging hallmarks of cancer [90]. It is well established that the inflammatory micro-environment impacts on tumour prognosis, either in a positive or negative manner [91, 92]. How to properly assess the composition and function of the microenvironment is challenging and there is a need of consensus in the field about how to best consider the joint inflammatory response as a component of tumour subclassification [93]. In this regard, pioneer studies were for melanoma, colon, and breast cancer[94-96].

Definition of inflammatory biomarkers (InfBM) is itself challenging. Any molecular compound involved in innate or adaptative immune response, at the gene or protein level, could be considered as so. The list, however, is tremendously large and the edges of the definition are difficult to delineate due to the interaction between inflammatory pathways and other cellular functions. In this introduction, we focus on markers that were directly involved in immune response as first function.

In the field of inflammatory biomarkers, BC remains a neglected disease [97]. This is paradoxical, thinking that BC is one of the few tumours with a longstanding efficient treatment using immunotherapy with BCG. Studies on the prognostic value of inflammationrelated biomarkers (InfBM) in BC have been published since the 70's [98-100]. The infiltration of the tumour by inflammatory cells and its role in prognosis has been explored more extensively than germline DNA polymorphisms in inflammatory genes and blood and urine cytokines. Unfortunately, none of these markers has proven to be sufficiently useful to reach clinical utility. Methodological flaws, technical heterogeneity, and lack of appropriately designed validation studies have been the most important limitations impairing a conclusive research. Guidelines have been published in 2005 to guide researcher in the reporting of prognostic markers but, unfortunately, they are rarely followed [101]. Moreover, most if not all studies investigating inflammatory biomarkers in bladder cancer are performed considering BC as a homogenous disease. This is untrue both at the pathological and molecular level since bladder tumors can display heterogeneous histological subtypes [56] and heterogeneous molecular alterations [102] even in the same tumor. Consequently it is likely that inflammatory infiltrate/inflammatory biomarkers are also heterogeneously
displayed in bladder tumors, yielding potential implications when association with prognosis/treatment response are assessed.

3.2. Germline variants in inflammatory genes and bladder cancer prognosis

Two studies assessed the association between germline IL6 rs1800795 variant and UBC outcome [24, 103]. Ahirwar et al. found a decreased risk of NMIBC recurrence among carriers (Hazard Ratio (HR) = 0.41, 95%CI 0.17-0.94) [24] while Leibovici et al., described a 4.6-fold higher risk of recurrence among high-risk NMIBC patients carriers of the same SNP receiving maintenance BCG therapy (n=38) [103]. For $TNF\alpha$, 2 polymorphisms were studied (rs1800629 and rs1799964) [103, 104]. Both studies found that the $TNF\alpha$ polymorphism considered was associated with a decreased risk of recurrence among patients with NMIBC. However, the findings among patients with MIBC were discordant. One study suggested an association between polymorphisms in the promoter of NFkB gene and NMIBC risk of recurrence [105]. In 2015, Lima et al evaluated association between 42 polymorphisms in 38 genes implicated in BCG mechanism of action and outcome of patients receiving BCG treatment [106]. Using Cox regression adjusted for classical prognosticators they proposed a set of genes significantly associated with BCG response (SNPs in tumour necrosis factor a (rs1799964), interleukin 2 receptor α rs2104286, IL17A (rs2275913), IL17RA (rs4819554), IL18R1 rs3771171, intercellular adhesion molecule 1 (rs5498), Fas ligand (rs763110) and TNF-related apoptosis-inducing ligand receptor 1 (rs79037040)). They proposed to score patients' risk according to their genetic profiling with the low-risk group having a 90% chance of successful treatment, while the high-risk group presented a 75% chance of recurrence after BCG treatment. Gallagher et al. tried to predict response to platin-based chemotherapy administrated to MIBC patients [107]. They described in 2013 seven variants in six gene significantly associated with partial or complete response to chemotherapy.

Among those variants were 2 SNPs in *IL1B*, an important mediator of the inflammatory response. Major limitation of those studies is the absence of replication of the results in independent series. Very recently, Grotenhuis et al. published in Bladder Cancer Journal an attempt of independent replication of published Germline Polymorphisms associated with BC prognosis and treatment response [108]. Out of 114 evaluated SNPs, only six were replicated. One of those SNPs was located in *IL18* (rs187238) and was associated with recurrence after BCG treatment. While the effort deserves encouragements, the cohort used for independent replication has major methodological drawbacks that, again, tremendously limit the applicability of the results.

3.3. Tumor-related inflammation and bladder cancer prognosis

3.3.1. Cytokines/proteins

In many cancers, cytokine expression were shown to be implicated in tumor progression and used as prognostic markers. In lung adenocarcinoma, a 15-cytokine gene expression profile was measured in noncancerous lung tissue and corresponding lung tumor tissue. The cytokine gene signature in noncancerous lung tissue was associated with lymph node status whereas the gene signature of the corresponding lung tumor tissue was associated with cancer survival [109]. In colorectal cancer, NFkB expression has been widely studied and showed to be associated with survival [110]. Regarding BC, in vitro studies showed that chemokine (CCL21/CCR7, CXCL5) expression was critical for tumor growth and progression, through interactions with micro environmental cells but also through direct action on tumor cells [111, 112]. Mukherjee et al. recently published a literature review about the controversial role of NFkB in bladder cancer carcinogenesis and prognosis [113]. STAT3 activation in urothelial stem cells leads to direct progression to invasive bladder cancer in mouse [36]. However, evidence of association of those markers with prognosis in BC is

lacking.

As a prognostic factor, COX2 has been the most studied inflammatory mediator in urothelial carcinoma. COX2 expression has been showed to be weakly associated with outcome as an independent marker [114, 115]. In a large cohort of NMIBC and a metaanalysis, Czachorowski *et al* reported that COX2 tumor expression was associated with advanced pathological stage and grade, but did not independently predict any of the considered outcome: recurrence free, progression free and disease specific survival [114]. In a mouse model of NMIBC, subcutaneous administration of celecoxib (COX 2 selective nonsteroidal anti-inflammatory drug) in association with BCG resulted in an increased infiltration of tumors by CD4+ lymphocytes and improved antitumor effect measured by the mass and number of tumors [116].

Co-stimulatory molecules in tumour cells: B7-H1 (PD-L1) is a T cell co-regulatory molecule. Four studies evaluated this marker in both NMIBC and MIBC (all cohorts were mixed). High PDL1 expression on tumor cells was associated with decreased cancer-specific or overall survival in three studies (two studies after adjustment for classical prognosticators and one in univariable analysis only) [117-119]. In a series of cystectomized patients, Boorjian et al. demonstrated that PDL1 expression (>5% of tumour cells) was associated with a 3.18-fold higher risk of overall mortality compared to those that did not express the marker (95%CI 1.74-5.79, p<0.001) [117]. Bellmunt et al. evaluated PDL1 expression on both tumor cells and mononuclear infiltrating cells. No association was seen between survival and expression on tumor cells. However, positive PD-L1 expression in infiltrating cells was significantly associated with longer survival in metastatic patients [120].

HLA class I molecules are required for the cytotoxic activity of T cells. Loss of HLA class 1 molecules was associated with adverse outcome. Four studies evaluated the impact of HLA class I molecule expression on survival [121-124]. Strong HLA class I expression was

associated with decreased recurrence in a series of NMIBC treated with TURB and induction BCG (HR=0.06, 95%CI 0.01-0.40, p=0.003) [121]. The main limitation of the study was the small sample size (n=30 patients) and number of events. Homma et al. presented a series of 65 patients treated with radical cystectomy with similar results [124]. Patients' whose tumour lost HLA class I expression had a 2.39-fold higher risk of recurrence after RC than those who did not, after adjustment for stage and histological variant.

HSP70 has been shown to have direct implication in pro/anti tumour immunity through its secretion by tumour cells or expression on the tumour cells surface where it can present antigens to immune system [125]. HSP70 has been evaluated twice as a biomarker for survival in UBC [126, 127]. Yu et al. assessed its association with recurrence and progression in a series of 530 patients with NMIBC treated by TURB [127]. Strong expression of HSP70 in tumour cells was independently associated with an increased risk of recurrence (HR=1.52; 95%CI 1.15-2; p<0.001) but there was no independent association with progression in a multivariable analysis. By contrast, HSP27 expression was inversely associated with progression (HR=0.49, 95%CI 0.33-0.73, p<0.0001).

3.3.2. Micro environmental cells

3.3.2.1. The Immunoscore and colon cancer

Main advances in the implementation of inflammatory infiltrate as a component of tumor sub-classification came from the work from Galon *et al* on colorectal cancer. Between 2006 and 2009 two major papers were published demonstrating that the type, location and density of inflammatory infiltrating cells in colorectal carcinoma were better predictors of survival than the commonly used clinical and histo-pathological factors [95, 128]. They defined a score called Immunoscore, aiming at classifying the patients into four distinct prognostic groups based on the density of three different lymphocytic populations (cytotoxic

CD8, CD3 and memory CD45RO lymphocytes) in the center and invasive margin of the resected tumors (Figure 1). The Immunoscore ("I") ranges from 0 ("I" 0), when low densities of both cell types are found in both regions, to 4 ("I" 4), when high densities are found in both regions.



Figure 1: Immunoscoring based on the count of CD8+ and CD3+ or CD45RO+ cells in the centre and at the invasive margin of the tumor. From [129]

After applying the Immunoscore to 602 resected colorectal cancers, Pages et al. demonstrated that only 4.8% of patients with a high "I" 4, relapsed after 5 years when 72% of patients with a low score ("I" 0 and "I" 1) experience tumor recurrence at five years.

While colon cancer is the most advanced in the path toward usage of inflammatory marker in daily practice, other studies suggested associations between immune contexture and different cancer types such as breast, lung or melanoma [130]. Based on those observation, an Immune Task Force has been gathered aiming at validating the Immunoscore in large cohorts of colon cancer patients but also at applying the score to other cancers such as melanoma, breast, ovarian, endometrial and ultimately promoting the worldwide use of the Immunoscore as a routine testing for cancer classification and treatment response prediction [129]. But before, harmonization of inflammatory infiltrate assessment has to be reached, as homogenous methodology is the prerequisite to any application to patients. This is

particularly challenging since the inflammatory infiltrate in tumors is heterogeneous, composed of various cell populations with various functions that can have opposite effects in different cancers. In that regard bladder cancer is particularly challenging since the tumor architecture is disrupted by transurethral resection.

3.3.2.2. Inflammatory infiltrate and bladder cancer

The evidences regarding the prognostic significance of tumor inflammatory infiltrate are very heterogeneous but altogether support the notion that inflammation in bladder tumor microenvironment is of potential prognostic and therapeutic interest. Twenty studies in the literature assessed the association between inflammatory cells in the tumor and BC prognosis [131]. Overall, reporting of techniques and interpretation of results were heterogeneous (see review in appendix).

Tumour infiltration by CD3- and CD8-expressing cells has been showed to be associated with better outcome. All studies showed significant results in univariable analysis. Results were confirmed after adjustment for stage and other prognosticators only in the 2 studies assessing MIBC [123, 132]. Sharma et al. studied CD8 infiltration in a series of 69 patients treated with TURB or RC [123]. HR for overall survival among MIBC patients with strong CD8 cell infiltration in the tumour (>8 CD8+ cells/ field, magnification not precised) on radical cystectomy specimen was 0.3 (95%CI 0.09-0.96). Winerdal et al. considered the impact of CD3 lymphocyte infiltration in the tumour of 37 patients treated by radical cystectomy [132]. Strong infiltration was associated with increased overall survival (HR=0.24, 95%CI 0.08-0.71, p=0.01). The presence of an "inflammatory infiltrate" without further characterization of the lymphocyte markers has also been widely studied [133-137]. The main limitations of those studies were the heterogeneity in the definition of the studied populations and on the "inflammatory infiltrate" term. Three studies including NMIBC and

MIBC patients showed that strong inflammatory infiltrate was an independent good prognosticator for survival after adjustment for stage and other clinico-pathological factors, [133, 135, 138]. Despite some caveats, the available evidence supports the notion that strong inflammatory infiltration in the tumour might be associated with a better prognosis in concordance to CD3 and CD8 study results. Obviously, a precise characterization of the type of inflammatory cells in the tumour is mandatory to draw further conclusions and establish mechanistic hypotheses.

Tumor associated macrophages (TAM) are known to have tumor promoting effect. The presence of a strong TAMs infiltration prior to BCG treatment was associated with recurrence in patients presenting NMIBC treated with maintenance BCG therapy [139]. Maniecki et al investigated the macrophage restricted receptor CD163 in bladder tumor biopsies (all stages and grades merged) and showed that high CD163 mRNA expression was associated with poor outcome. CD163 mRNA expression was significantly increased in muscle invasive and aggressive tumors [140]. Results on CD68 from the available studies are difficult to compare because of the heterogeneity between patients characteristics, tissue location, and methods of quantification. Avari et al. combined the count in the tumour and in the stroma while Takayama et al. did it separately [139, 141, 142]. A total of 3 studies showed statistically significant results in multivariate analysis, with CD68 macrophages strong infiltration being associated with adverse outcome [139, 142, 143]. In 2009, Ayari et al. described that CD68 infiltration in the tumour was associated with a 3.8-fold (95%CI 1.32-11, p=0.013) higher risk of recurrence after TURB and maintenance BCG in a series of NMIBC [139]. This result was not validated by the same author's second publication in 2013 but with a series of patients not treated with BCG therapy [144]. Hanada et al. combined NMIBC and MIBC patients and showed that those presenting strong CD68 infiltration in the tumour had a 5-fold higher risk of mortality [143]. The study, however, lacked of details about patients' management and definition of survival endpoints. Finally, Takayama et al., published a study with 41 carcinoma *in situ* (CIS) treated with TURB and induction BCG [142]. The endpoint was time to recurrence defined by positive cytology. In multivariate analysis adjusted for age and gender, high CD68 count in CIS regions (\geq 4 CD68+ cells) was an independent predictive factor for recurrence (HR=1.7). These authors performed the same analysis using CD68 count in the lamina propria and demonstrated no association with recurrence. Jointly with CD68 analysis, Ayari et al. explored the impact of dendritic cell infiltration defined by CD83 staining, strong CD83 dendritic cells infiltration being associated with adverse outcome - recurrence after maintenance BCG - [139] and progression to muscle invasion [144]. Results were statistically significant but the reliability of the risk estimates by Cox are questionable because of the small number of events (only one tumour recurred in the low CD83 group yielding an upper 95%CI of 85). Altogether these papers pinpoint a possible adverse effect of macrophage infiltration in tumours but evidence remains weak.

The prognostic value of FOXP3 expressing T regulators lymphocytes is controversial. The transcription factor FOXP3 is a master regulator of regulatory T cells (Treg) [145]. In a critical review of the literature, DeLeeuw *et al* concluded that FOXP3+ T cells have very heterogeneous properties and that their biologic effect seems to depend on the tumor site, possibly reflecting differences in the tumor microenvironment [146]. Two studies analyzed the impact of tumour infiltration by FOXP3-positive lymphocytes in both NMIBC and MIBC [121, 147]. Both studies showed better survival when strong infiltration was observed, although the findings were statistically significant, after adjustment, in only one of them (HR=0.17, 95%CI 0.05-0.6, p=0.006, for overall survival) [147].

More than single cells effect, it might be the ratio/interactions between inflammatory cells population that drives the pro/anti tumour effect of the host immune system. Sjodahl et al.

suggested that more than a single population type, the ratio between lymphocytes and macrophages might have higher prognostic significance [72]. In a study on 296 MIBC, the presence of infiltrating CD3+ lymphocytes was significantly associated with good prognosis. This positive association was modulated by the presence of CD68+ TAMs. The strongest association with poor survival was observed for a high ratio between CD68 and CD3 (p-value $7x10^{-5}$).

3.4. Serum inflammation and cancer prognosis

3.4.1. Inflammatory proteins

The prognostic significance of serum levels of proinflammatory mediators such as IL6, CRP (which is synthesized by the hepatocytes after IL6 stimulation), and TNFalpha has been demonstrated in multiple cancers (lung, breast, ovary, colon, prostate, bladder, and urinay tract) [35, 148-154]. CRP was the most widely studied serum marker in BC prognosis. High C-reactive protein (CRP) levels are associated with decreased recurrence free survival, response to chemotherapy and cancer specific survival in patients treated for MIBC [155-159]. Eight studies assessed the association of high CRP levels with overall or cancer-specific survival [115, 157, 160-165]. All studies reported consistent results showing that high CRP levels (>5 mg/L) are associated with adverse outcome (see review in appendix). In one study, the association was only significant in univariable analysis [162]. Another study did not perform multivariable analysis [161]. In the 6 remaining studies, 3 dichotomized CRP levels and 3 used a continuous variable. In all of them, CRP was an independent prognostic factor for both cancer-specific and overall mortality, though variables for adjustment varied widely from one study to another. No study evaluated the prognostic significance of CRP levels on recurrence and progression of NMIBC. Moreover, serum CRP level has never been investigated regarding its impact on response to BCG therapy.

The preoperative plasma level of IL6 has been associated with adverse pathological stage and outcome in patients treated by radical cystectomy [149]. Again, its prognostic significance has not been evaluated in NMIBC.

3.4.2. Inflammatory cells

CD8 cell count was assessed as a serum marker using flow cytometry in one study [166]. Authors observed that serum CD8 was inversely correlated to tumour infiltration with CD8 cells (r^2 = 0.63, p<0.0001). Low levels of CD8 cells in blood were associated with lower intravesical recurrence after TURB applying a multivariable analysis (HR= 0.4, 95%CI 0.17-0.94).

In the past years, efforts have mainly been focusing on demonstrating the prognostic significance of serum Neutrophil to Lymphocytes Ratio (NLR). At the moment, more than 10 studies have described consistent association between high NLR and prognosis of MIBC [167-170] but also upper tract urothelial carcinoma (UTUC) [171-173] or NMIBC [174, 175]. Two studies reported prognostic value for NLR after radical cystectomy for UCB. With a threshold of 2.5, studies in MIBC observed that high NLR was an independent adverse prognostic factor for survival after RC with hazard ration between 1.9 and 2.5. In a meta analysis published in PLoS One in 2014, Wei et al confirmed a consistent association between high NLR and both BC recurrence free survival and cancer specific survival [176]. Most of the published studies lack calculation of the predictive ability of the marker (c-index) that would be necessary to demonstrate clinical benefit. For that reason, and despite the robustness and easiness-to-use of the NLR, no usage in clinical trial/daily practice decision-making has been proposed.

3.5. Urinary inflammatory response and bladder cancer prognosis

Intra-vesical administration of Bacillus Calmette-Guerin leads to both local and systemic "anti-tumor" immune response [177]. The type and intensity of this response can be used as a surrogate marker for treatment efficacy. Local inflammation can be easily assessed by urine cytokine measurements that reflect the level of parietal inflammatory response. In the early years of 2000, Saint et al repeatedly demonstrated that a T helper 1 lymphocyte response (measured by a high release of IL2 and IFN gamma) was necessary for BCG treatment efficacy [178, 179]. Furthermore they demonstrated in 2003 that failure to detect urinary IL-2 during BCG induction course correlated with time to recurrence (p = 0.01) and progression (p = 0.01). In a large literature review published in 2012 in European Urology, Zuiveroon et al. detailed all available evidence on markers predicting response to BCG Immunotherapy [78]. From the work from Saint et al, urinary IL2 seems one of the most promising marker. Other cytokines have been studies such as IL8 or IL18 or TRAIL (tumour necrosis factor apoptosis-inducing ligand) with contradictory results.

More recently, Rigaud et al looked in a prospective manner at urinary cytokines profiles of 23 patients treated with 6 "induction" BCG courses [180]. Five of them were BCG refractory/recurred after treatment. They observed very different urinary cytokines profiles between the patients. Recurring patients showed a trend toward high baseline IL-6 levels followed by low IL-6 levels before the instillations; low baseline IL-2 levels with only minor changes during treatment; absence of IFN γ and IL-17 production and a low peak of cytokine production at the end of treatment.

Main limitations of those studies come from the very small number of patients included in the series and the lack of external validations. However urologists need to question themselves why those markers have not been investigated deeper in order to integrate them to daily practice. To date no molecular predictive marker is available and clinicians still rely on clinical characteristics to decide weather to guide patient to BCG therapy or radical cystectomy. With the extent of researches conducted in the field, it is worth underscoring that no prospective studies have been designed to assess inflammatory response during treatment. All the more so as studies demonstrated that progression under BCG treatment is associated with very poor prognosis [45, 181].

4. SUMMARY: IMMUNITY IN THE NATURAL HISTORY AND MANAGMENT OF BLADDER CANCER

Along this introduction, we reviewed know associations between inflammation/immunity and bladder cancer occurrence, development, prognosis and treatment. Some of the main associations are summarized in the Figure 4.



Figure 4 : Immunity in the natural history and management of bladder cancer (ref)

II OBJECTIVES

1. HYPOTHESES

Our study was built on the following two fundamental hypotheses:

- 1) Inflammation/Immunity are associated with BC prognosis and response to treatment
- Integration of Inflammatory biomarkers will result in superior accuracy in predicting BC prognosis than the classical clinico-pathological prognostic factors.

2. GERMLINE VARIANTS

2.1. Genome-based approach

The first approach conducted herein aimed at assessing the association between germline variation in inflammatory genes and BC prognosis. We decided to focus first on NMIBC because of the long-lasting evidence of effective treatment with immunotherapy. At the time of the initiation of the project the effect of anti PDL1 therapies in the treatment of MIBC had not been released. The objective of this study was to evaluate the association of SNPs in inflammation-related genes with the risk of NMIBC to recur and/or progress. We applied both classical cox regression and extended the application of innovative multi-marker approaches to the prognostic field for the first time.

2.2. Gene-based approach

While the project started focusing in NMIBC, the field of metastatic bladder cancer treatment was revolutionized by the results of randomized controlled trials demonstrating the efficacy of immune checkpoints inhibitors. Based on excellent and prolonged responses to anti PDL1 therapies, multiples trials tried to extend the use of those drugs to earlier stages of the disease. Authors suggested that the expression of PDL1 in the tumor was associated with prognosis but none had investigated the association between germline variations in *PDL1*

gene and prognosis or anti PDL1 treatment response. Consequently we decided, in parallel to the work conducted on NMIBC to explore the potential association between germline *PDL1* variation and UBC prognosis.

3. TUMOR INFLAMMATORY INFILTRATE

3.1. Rational for using CD8 as a model marker

Cytotoxic lymphocytes are one of the main effectors of anti tumor immunity. (Re)Activation of CD8 lymphocytes is implicated in both BCG response and immune checkpoint inhibitor mechanism of action. From the literature review CD8 was one of the most studied tumor marker in both NMIBC and MIBC. The immunohistochemistry technique is very robust and used in daily clinical practice in most pathology departments. Consequently, we chose CD8 as a model marker for tumor inflammatory infiltrate assessment.

3.2. Methodological validation

Based on the literature review, we identified many methodological drawbacks in both the design of studies and the reporting of potential association between tumor inflammatory biomarkers and bladder cancer prognosis. Limitations were even more patent in NMIBC because of the potential heterogeneity of the tumor emphasized by the disruption inducted by trans urethral resection. Consequently we decided to conduct a first study aiming at assessing heterogeneity in the inflammatory infiltrate and set up a standardized methodology for inflammatory infiltrate assessment in NMIBC. The final aim was to identify potential association between CD8 infiltration in the tumor and NMIBC prognosis. After digitalization and automatic counting, we explored the association between CD8 lymphocytes associated with the tumor compartment and NMIBC risk of recurrence and progression.

III RESULTS

ARTICLE 1: INFLAMMATORY-RELATED GENETIC VARIANTS IN NON-MUSCLE INVASIVE BLADDER CANCER PROGNOSIS. A MULTI-MARKER BAYESIAN ASSESSMENT. A Masson-Lecomte et al. CEBP 2016

1.1. Abstract

Background. Raising evidence points to the role of tumor immunological environment on urothelial bladder cancer prognosis. This effect might be partly dependent on the host genetic context. We evaluated the association of SNPs in inflammation-related genes with non-muscle invasive bladder cancer (NMIBC) risk-of-recurrence and risk-of-progression.

Methods. We considered 822 NMIBC included in the SBC/EPICURO Study followed-up >10 years. We selected 1,679 SNPs belonging to 251 inflammatory genes. The association of SNPs with risk-of-recurrence and risk-of-progression was assessed using Cox regression single-marker (SMM) and multi-marker methods (MMM) Bayes A and Bayesian LASSO. Discriminative abilities of the models were calculated using the *c*-index and validated with bootstrap cross-validation procedures.

Results. While no SNP was found to be associated with risk-of-recurrence using SMM, 3 SNPs in *TNIP1*, *CD5* and *JAK3* showed very strong association with posterior probabilities >90% using MMM. Regarding risk-of-progression, one SNP in *CD3G* was significantly associated using SMM (HR=2.69; *p*-value=1.55x10⁻⁵) and two SNPs in *MASP1* and *AIRE*, showed a posterior probability \geq 80% with MMM. Validated discriminative abilities of the models without and with the SNPs were 58.4% vs. 60.5% and 72.1% vs. 72.8% for risk-ofrecurrence and risk-of-progression, respectively. **Conclusions**. Using innovative analytical approaches, we demonstrated that SNPs in inflammatory-related genes were associated with NMIBC prognosis and that they improve the discriminative ability of prognostic clinical models for NMIBC.

Impact. This study provides proof of concept for the joint effect of genetic variants in improving the discriminative ability of clinical prognostic models. The approach may be extended to other diseases.

1.2. Manuscript

Cancer Epidemiology, Biomarkers & Prevention

Inflammatory-Related Genetic Variants in Non-Muscle-Invasive Bladder Cancer Prognosis: A Multimarker Bayesian Assessment

Alexandra Masson-Lecomte^{1,2}, Evangelina López de Maturana¹, Michael E. Goddard^{3,4}, Antoni Picornell¹, Marta Rava¹, Anna González-Neira⁵, Mirari Márquez¹, Alfredo Carrato⁶, Adonina Tardon⁷, Josep Lloreta⁸, Montserrat Garcia-Closas⁹, Debra Silverman¹⁰, Nathaniel Rothman¹⁰, Manolis Kogevinas¹¹, Yves Allory¹², Stephen J. Chanock¹⁰, Francisco X. Real^{13,14}, and Núria Malats¹, on behalf of the SBC/EPICURO Study Investigators

Abstract

Background: Increasing evidence points to the role of tumor immunologic environment on urothelial bladder cancer prognosis. This effect might be partly dependent on the host genetic context. We evaluated the association of SNPs in inflammationrelated genes with non-muscle-invasive bladder cancer (NMIBC) risk-of-recurrence and risk-of-progression.

Methods: We considered 822 NMIBC included in the SBC/ EPICURO Study followed-up >10 years. We selected 1,679 SNPs belonging to 251 inflammatory genes. The association of SNPs with risk-of-recurrence and risk-of-progression was assessed using Cox regression single-marker (SMM) and multimarker methods (MMM) Bayes A and Bayesian LASSO. Discriminative abilities of the models were calculated using the *c* index and validated with bootstrap cross-validation procedures.

Results: While no SNP was found to be associated with risk-ofrecurrence using SMM, three SNPs in *TNIP1*, *CD5*, and *JAK3*

Introduction

Urothelial bladder carcinoma (UBC) is the fifth most common neoplasm in terms of incidence in industrialized countries. UBC is a multifactorial complex disease, tobacco and occupation exposure to aromatic amines being the two best established environmental risk factors (1, 2). In addition, UBC has a genetic component, and candidate gene and genome-wide association studies so far have identified 16 loci associated with UBC risk (3–13) showed very strong association with posterior probabilities >90% using MMM. Regarding risk-of-progression, one SNP in *CD3G* was significantly associated using SMM (HR, 2.69; *P* = 1.55×10^{-5}) and two SNPs in *MASP1* and *AIRE*, showed a posterior probability ≥80% with MMM. Validated discriminative abilities of the models without and with the SNPs were 58.4% versus 60.5% and 72.1% versus 72.8% for risk-of-recurrence and risk-of-progression, respectively.

Conclusions: Using innovative analytic approaches, we demonstrated that SNPs in inflammatory-related genes were associated with NMIBC prognosis and that they improve the discriminative ability of prognostic clinical models for NMIBC.

Impact: This study provides proof of concept for the joint effect of genetic variants in improving the discriminative ability of clinical prognostic models. The approach may be extended to other diseases. *Cancer Epidemiol Biomarkers Prev*; 25(7); 1144–50. ©2016 AACR.

The majority of UBC are non-muscle-invasive (NMIBC). These tumors are heterogeneous regarding their clinical, pathologic, molecular, and genetic features. Management of NMIBC poses challenges because of their propensity to recur, requiring a long-term surveillance, and their risk to progress to muscle invasion, showing a poor 5-year survival rate (14). The current prognosticators do not completely discriminate between patients who will suffer from a tumor recurrence/progression and patients

Academic Hospital, Paris Est Créteil University, INSERM, Créteil, France. ¹³Epithelial Carcinogenesis Group, Spanish National Cancer Research Centre (CNIO), Madrid, Spain. ¹⁴Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Barcelona, Spain.

Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

A. Masson-Lecomte and E. López de Maturana contributed equally to this article.

Corresponding Author: Núria Malats, Genetic and Molecular Epidemiology Group, Spanish National Cancer Research Centre (CNIO), C/Melchor Fernández Almagro, 3, Madrid 28029, Spain. Phone: 34912246900 (ext. 3330); Fax: 34912246911; E-mail: nmalats@cnio.es

doi: 10.1158/1055-9965.EPI-15-0894

©2016 American Association for Cancer Research.

1144 Cancer Epidemiol Biomarkers Prev; 25(7) July 2016



¹Genetic and Molecular Epidemiology Group, Spanish National Cancer Research Centre (CNIO), Madrid, Spain.²Urology Department, Henri Mondor Academic Hospital, Paris Est Créteil University, Créteil, France. ³Biosciences Research Division, Department of Environment and Primary Industries, Agribio, Bundoora, Victoria, Australia. ⁴Department of Food and Agricultural Systems, University of Melbourne, Melbourne, Australia. ⁵Human Genotyping-CEGEN Unit, Spanish National Cancer Research Centre (CNIO), Madrid, Spain. ⁶Servicio de Oncologia, Hospital Universitario Ramon y Cajal, Madrid, and Servicio de Oncología, Hospital Universitario de Elche, Elche, Spain. ⁷Department of Preventive Medicine, Universidad de Oviedo, Oviedo, Spain. ⁸Institut Municipal d'Investigació Mèdica - Hospital del Mar and Departament de Patologia, Hospital del Mar - IMAS, Barcelona, Spain. ⁹Division of Genetics and Epidemiology, Institute of Cancer Research, London, United Kingdom. ¹⁰Division of Cancer Epidemiology and Genetics, National Cancer Institute, Department of Health and Human Services, Bethesda, Maryland. ¹¹Centre for Research in Environmental Epidemiology (CREAL) and Institut Municipal d'Investigació Mèdica - Hospital del Mar, Barcelona, Spain. ¹²Pathology Department, Henri Mondor

Inflammatory Variants in Bladder Cancer Prognosis

who will remain stable after the first transurethral resection of the bladder (TURB); thus justifying the need of prognostic biomarkers to guide the clinical management of patients with NMIBC (15).

Inflammation and cancer are deeply intricate. Not only local inflammation can promote tumor development but also systemic or tumor immune reaction has been shown to have either promoting or opposing cancer effects (16–18). These reactions are however dependent on the host genetic context (19). Previous studies have assessed SNPs involved in inflammatory pathways as prognostic markers for UBC (20–22). Those studies have had limited success, as they have applied simplistic models analyzing each SNP individually, therefore ignoring the complexity of the disease likely underlined by many genetic variants with relatively low effects (23). A recent study has shown the usefulness of multi-marker methods (MMM) able to handle large amount of SNPs; often exceeding the number of individuals, to assess associations between SNPs in inflammatory genes and UBC risk (24).

The objective of this study was to evaluate the association of SNPs in inflammation-related genes with the risk of NMIBC to recur and/or progress by extending the application of MMM to the prognostic field for the first time. We compared results with those coming from the classical single-marker method (SMM) accounting for the time-to-event nature of the data.

Materials and Methods

Ethics statement

Informed consent was obtained from study participants in accordance with the Institutional Review Board of the U.S. National Cancer Institute and the Ethics Committees of each participating hospital.

Study population and tissue samples

We primarily considered the 995 newly diagnosed patients with NMIBC included in the Spanish Bladder Cancer (SBC)/ EPICURO Study, a multicenter hospital-based study conducted in 1997-2001 in 18 hospitals (3). Tumors were reviewed and confirmed by trained uropathologists who classified their stage and grade homogeneously using TNM 1997 AJCC and 1973 and 2004 WHO grade classifications. All tumors were transitional cell carcinomas (TCC). Clinical data and information on primary treatment were retrieved from the hospital charts by trained monitors using a structured questionnaire. Patients with NMIBC were classified at high (HiR, n = 284) or low (LR, n = 538) risk of progression according to the EAU guidelines (25). Low-risk patients consisted of PUNLMP, Ta G1, and G2/low grade and high-risk patients included all T1, G2, and G3/high grade and carcinoma in situ (CIS). The intermediate-risk group was not considered herein due to reduced sample size. Patients were followed up for >10 years using both the hospital charts and through direct telephone calls to patients/families. The follow-up rate for patients with NMIBC was 94%.

Gene and SNP selection and genotyping

Germline DNA extracted from blood or saliva, in case blood was not available (4% of the patients), was used for genotyping (3). Genes (n = 251, Supplementary Table S1) were carefully selected according to current available evidence of their involvement in inflammatory processes, favoring those inflam-

matory genes showing association with cancer as described elsewhere (24). TagSNPs covering these genes were identified using SYSNP (26) and genotyped with the GoldenGate Illumina Genotyping Assay platform (27). On the basis of a literature review, we further included 3,628 SNPs in 52 inflammatory genes already genotyped in the same individuals with the Illumina Infinium HumanHap1M array (6). We excluded SNPs with a low genotyping rate (<95%) and minor allele frequency (MAF) <0.05. Missing genotypes were imputed with BEAGLE (28). To reduce both colinearity between variables and number of statistical tests, pairwise linkage disequilibrium (LD) between SNPs was estimated using the R-package GENETICS (http://cran.r-project.org/web/packages/ genetics/index.html). We retained the SNPs with the highest MAF of each LD block when $r^2 > 0.5$. At the end of the quality control process, 822 patients with 1,517 SNP genotypes and complete clinical and pathological information were available for the analysis. These patients were comparable with the whole series (n = 995) for age, gender, area, and tumor stage and grade.

Outcome definition

Time-to-first recurrence (TFR) was defined as time elapsed between first TURB and histologic diagnosis of a new NMIBC of any stage/grade. Time to progression (TP) was defined as time between first TURB and a subsequent histologic diagnosis of a muscle-invasive breast cancer (MIBC), occurrence of metastasis, or death due to bladder cancer.

Statistical analyses

Median follow-up times were obtained by using the reverse Kaplan-Meier method. We applied both an SMM based on a multivariate Cox regression and MMM based on Bayes A (BA) and Bayesian LASSO (BL; Supplementary Fig. S1). Cox proportional hazard regression was used to estimate the HR and 95% confidence intervals (CI) to assess the association between individual SNPs assuming both an additive and a dominant mode of inheritance and the outcomes of interest. Each SNP effect was adjusted for classical clinicopathologic prognosticators for TFR and TP (Supplementary Table S2). TFR analyses did not include patients who received radical cystectomy as first treatment (n =12). TP was analyzed using all available patients with NMIBC and stratified according to HiR/LR. Stratification was not performed for TFR because survival curves of LR and HiR patients overlapped. Analyses were run in R-language (http://www.R-project.org). SNPs with P < 0.05, 2-sided test, after Bonferroni's correction were kept for comparison with the results from the MMM.

Multimarker methods

Both Bayes A (29) and Bayesian LASSO (30) were applied coupled with a sequential threshold model to analyze each time-to-event data (See Supplementary Methods and ref. 31. This model (32) has been previously used in quantitative genetics settings (33, 34). This is the first time the method is applied in the prognosis field. The same clinicopathologic adjusting variables were used in the MMM (Supplementary Table S2). Because BA and BL do not provide P value, the association strength was estimated using a posterior probability that the SNP is associated with the outcome. An arbitrary threshold of 80% was deemed as significant. Analyses were done using an *ad-hoc* made Fortran program.

Masson-Lecomte et al.

	All (N = 822)	Low-risk (<i>n</i> = 538)	High-risk (<i>n</i> = 284)	Р
Age, y				7.6×10^{-5}
Median [IQR]	68 (60-73)	67 (58-73)	69 (63-75)	
Mean (SD)	65.5 (10.2)	64.5 (10.8)	67.5 (8.7)	
Gender				0.49
Male	722 (88%)	469 (87%)	253 (89%)	
Female	100 (12%)	69 (13%)	31 (11%)	
TG				_
PUNLMP	41 (5%)	41 (7.6%)	_	
Ta G1	311 (37.8%)	311 (57.8%)	_	
Ta G2	253 (30.8%)	186 (34.6%)	67 (23.6%)	
Ta G3	85 (10.3%)	_	85 (29.9%)	
T1 G2	20 (2.4%)	_	20 (7.1%)	
T1 G3	106 (13%)	_	106 (37.3%)	
Tis G2	1 (0.1%)	_	1 (0.34%)	
Tis G3	5 (0.6%)	_	5 (1.76%)	
Multiplicity				4.2×10^{-5}
≤3 tumors	543 (66%)	383 (71.2%)	160 (56.3%)	
>3 tumors	236 (29%)	130 (24.2%)	106 (37.3%)	
Missing values	43 (5%)	25 (4.6%)	18 (6.4%)	
Size				9.9×10^{-5}
≤3 cm	480 (58.4%)	341 (63.4%)	139 (48.9%)	
>3 cm	112 (13.6%)	58 (10.8%)	54 (19%)	
Missing values	230 (28%)	139 (25.8%)	91 (32.1%)	
Number of patients with tumor recurrence	268 (32.6)	190 (35.3)	78 (27.5)	
Number of patients with progression	76 (9.2)	24 (4.5)	52 (18.3)	

Abbreviation: IQR, interquartile range.

Discrimination ability of the model calculation and validation The discrimination ability of the models including the SNPs showing association with the different outcomes was evaluated by estimating the c index. Briefly, a Cox model including the clinical variables only was compared through the c index with a model including also the previously associated SNPs for each outcome of interest. The *c* index is the frequency of concordant pairs (i.e., the risk of the event predicted by a model is lower for the patient who experiences the event at a later time point) among all pairs of subjects. Three c indexes were calculated: the apparent c index (calculated with the original data), the bootstrap cross-validation c index (calculated using the observations that are not in the bootstrap sample, obtained through a random sampling with replacement), and the bootstrap alone c index (i.e., a weighted average of the discrimination in the original dataset and the discrimination in the observations that were not included in the m-th bootstrapped sample; as in ref. 35) and R-package 'pec' http://cran.r-project.org/web/packages/pec/pec.pdf).

Results

The median age at diagnosis of the 822 patients with NMIBC was 68 years; 12% of cases were women and 65% of the patients presented LR tumors. Patient and treatment characteristics are displayed in Table 1 and Supplementary Table S3, respectively. Up to July 2007, median follow-up period for the whole series and for

patients "free of disease" were 80.4 and 77.5 months, respectively, with a total of 8 (1.0%) deaths due to UBC as first event. According to the abovementioned definitions, 324 (39.4%) patients suffered, at least, one event. Survival functions for each event are in Supplementary Figs. S2 and S3.

Time to first recurrence

No SNP was found to be associated with TFR by SMM additive model after Bonferroni's correction ($P > 3 \times 10^{-5}$, Supplementary Table S4). Using a dominant mode of inheritance, we found that an SNP in *CARD4/NOD1* (rs10267377) was significantly associated with TRF (HR, 0.58; 95% CI, 0.45–0.75; P = 0.000026, $P_{adjusted} = 0.039$). Using MMM, 43 SNPs had PP > 80% of being associated with TFR (Supplementary Table S5). Among them, three had probabilities >90% using both BA and BL, pointing to a very strong association (*TNIP1*-rs2277940, *CD5*-rs7104333, *JAK3*-rs6523, Table 2). The same SNP in *CARD4* had PP of 88% and 90% of being associated with risk of recurrence using BA and BL, respectively.

Time to progression

Only one SNP identified by SMM additive model showed a significant association after Bonferroni's correction: *CD3G*-rs3212262 (HR, 2.69; 95% CI, 1.72–4.23; $P = 1.55 \times 10^{-5}$, $P_{\text{adjusted}} = 0.023$; Table 3). Five-year progression-free survival rate

Table 2. SNPs with a strong posterior probability (PP > 90%, BA and BL analyses) of being associated with risk of recurrence in NMIBC

			B	A	E	BL	Cox r	egression
Gene	SNP	MAF	HRaa_AA	PP > 90%	HRaa_AA	PP > 90%	HR	Р
JAK3	rs6523ª	0.40	1.69	98	1.17	92	1.20	0.0488
TNIP1	rs2277940	0.07	1.50	91	1.24	94	1.74	0.0001
CD5	rs7104333	0.49	0.72	90	0.86	92	0.81	0.0126

NOTE: The last column displays the adjusted Cox regression results from models including individual SNPs and covariates.

^aPreviously as rs2286662. Analyses were adjusted for geographical area, gender, multiplicity, tumor stage and grade, tumor size, and treatment (see Supplementary Table S2).

1146 Cancer Epidemiol Biomarkers Prev; 25(7) July 2016

Cancer Epidemiology, Biomarkers & Prevention

NMIBC series using multivariable Cox regression additive model					
Gene	SNP	HR	Р	MAF	
CD3G	rs3212262	2.70	1.5×10^{-5}	0.09	
HLA-B	rs9266462	2.67	0.0026	0.06	
CCL-2	rs929259	0.52	0.0081	0.37	
FAS	rs1571014	1.73	0.0089	0.36	
PPARG	rs7626560	1.85	0.0015	0.19	
CXCR4	rs778192	0.55	0.0017	0.38	
SOCS5	rs973491	2.11	0.0025	0.07	
CXCR4	rs16834018	2.10	0.0035	0.08	
CD8B1	rs13024609	1.70	0.0044	0.20	
IL6	rs2069827	2.00	0.0053	0.06	

Table 3. Top 10 autosomal SNPs associated with risk of progression in the whole

NOTE: Analyses were adjusted for geographical area, age, multiplicity, tumor stage and grade, number of recurrences, and treatment (see Supplementary Table S2).

was 92% for the AA genotype versus 84% for the Aa and 71% for the aa (log-rank P = 0.001, Fig. 1). No SNP was associated with TP using a dominant model. Using MMM, 2 additional SNPs had PP \geq 80% with both BA and BL: *MASP1*-rs698079 and *AIRE*rs941405 (Table 4). When assessing only HiR tumors, MMM identified 3 SNPs with PP between 77–80%: *CARD4*-rs2256023, *MAP2K3*-rs9901404, and *TMEM189*-rs2269217 (Supplementary Table S6). Neither SMM nor MMM identified any SNP associated with TP among LR tumors, *CD68*-rs12942088 (Supplementary Table S7) presenting the highest PP (75% with BL). This SNP was one of the top 2 SNPs associated with TP using an additive SMM.

Models' discriminative ability

The clinical parameter model for TFR showed a moderate discriminatory ability (validated *c* index = 0.58; Table 5). By adding the 3 SNPs showing a PP > 90% (*TNIP1*-rs2277940, *CD5*-rs7104333, and *JAK3*-rs6523), the *c* index raised to 0.61. Adding each SNP to the clinical variables increased the predictive ability compared with the model including clinical variables only, showing that the predictive ability of the SNPs does not overlap (see Supplementary Table S8).

The clinical parameter model for TP showed good discrimination ability (validated *c* index = 0.72, Table 5). By adding the 2 SNPs showing PP > 80% (*MASP1*-rs698079 and *AIRE*-rs941405), the *c* index raised to 0.73. The TP predictive ability was also calculated for the HiR and LR subgroups. Adding the SNPs to the clinical variables improved their discrimination ability by 10.5 % for HiR and by 10.2 % for LR in the validation set. As for TFR, the predictive ability of the SNPs did not overlap for TP considering all and HiR patients (see Supplementary Table S8).

Discussion

Classical studies looking for associations between individual SNPs in inflammatory genes and UBC prognosis have had limited success (20–22). While some variants have been previously associated with UBC prognosis (19, 36), significance was in most cases limited to univariate analysis and none of the variant was



Figure 1.

Progression-free survival of the 822 NMIBC according to *CD3G*-rs3212262 genotypes. Five-year progression-free survival was 92% for AA, 85% for Aa, and 71% for aa genotypes (log-rank $P = 8.4 \times 10^{-4}$, adjusted Cox P = 0.023).

replicated in independent studies. Moreover, Cox regression is limited by the number of variables the model permits (37). In general, the lack of associations found by SMM outlines its inefficiency to pinpoint variants with small effects in complex traits. To explore the joint effect of multiple SNPs, we have applied MMM strategies mimicking the polygenic scenario that features UBC prognosis. MMM identified, with strong evidence, inflammatory genes with variants individually conferring a small risk of NMIBC recurrence or progression.

A larger number of inflammatory variants showed association with TFR than with TP: 44 SNPs with PP > 80%, 3 of them with PP > 90%, have been associated with TFR. Among them, JAK3-rs6523 and CD5-rs7104333 were already identified as associated with UBC risk (24). Only 2 SNPs were associated with TP using MMM. This could be explained by the lower rate of progression events (n = 76) compared with the number of recurrences (n = 268) that may affect the power of tests in detecting associations. Most of the SNPs/genes associated with TFR were not associated with TP and vice versa, which may indicate that different inflammatory genes trigger distinct NMIBC outcomes. The small correlation between SNP effects obtained with MMM for TFR and TP (data not shown) also support this hypothesis. However, it is noteworthy that genetic variation in CARD4/NOD1 was both associated with TR of the whole cohort and TP of high-risk patients. Most of the SNPs identified by MMM also ranked in the first positions when Cox regression was applied. Only two SNPs in CD3G and CARD4 identified by SMM, as associated with TP and TR, passed Bonferroni's correction and their PPs were high: $PP_{BA} = 0.76$ and $PP_{BL} = 0.79$ for CD3G and $PP_{BA} = 0.88$ and $PP_{BL} = 0.90$ for CARD4. Potential explanations for the different SNP ranking for CD3G between tests is the small MAF (0.09) of this variant with very few events in the aa genotype group (Fig. 1; ref. 38) and the adjustment by other SNPs included in the MMMs.

Table 4. SNPs with strong posterior probability (PP > 80%, BA and BL analyses) of being associated with risk of progression in patients with NMIBC

			E	BA	E	BL	Cox re	egression
Gene	SNP	MAF	HRaa_AA	PP > 80%	HRaa_AA	PP > 80%	HR	Р
MASP1	rs698079	0.18	1.31	83	1.10	80	1.58	0.0178
AIRE	rs941405	0.37	1.31	86	1.11	83	1.56	0.0116
NOTE: Analy	yses were adjusted fo	r geographical a	rea, age, multiplicity,	tumor stage and grad	e, number of recurren	ces, and treatment (se	e Supplementa	ary Table S2).

The last column displays the adjusted Cox regression results from models including individual SNPs and covariates.

www.aacrjournals.org

Cancer Epidemiol Biomarkers Prev; 25(7) July 2016 1147

Masson-Lecomte et al.

	Apparent	Bootstrap alone	Bootstrap cross-
	c index	c index	validation c index
Recurrence ALL ($N = 268$ events)			
CV	62.6	60.1	58.4
CV + SNPs (JAK3, TNIP1,CD5)	65	62.5	60.5
Progression ALL ($n = 76$ events)			
CV	77.5	74.7	72.1
CV + SNPs (MASP1, AIRE)	78.7	75.4	72.8
Progression HiR ($n = 52$ events)			
CV	69.4	63.6	60.1
CV + SNPs (TMEM189, MAP2K3, CARD4)	76	70.1	66.4
Progression LR ($n = 24$ events)			
CV	74.2	66.6	62.6
CV + SNPs (CD68)	80.8	73.5	69

Table 5. Apparent and validated c index using Bootstrap alone or 1,000 bootstrap cross-validation indicating the discriminatory ability of the models for each outcome of interest

Abbreviations: CV, clinical variable; n, number of events.

Inflammatory SNPs were not strongly associated with outcome in both HiR and LR subcohorts, probably because of the limited sample size, too. Polymorphisms in inflammatory genes were differently associated with TP in patients at HiR versus LR. Correlation between SMM and MMM estimates of TP in both subcohorts disagreed (Pearson correlation between SNPs effect estimates = -0.01 for BA and -0.03 for Cox regression), this suggesting that the difference in prognosis of both groups may, at least, partially be mediated by inflammatory genes. Risk of TP is highly influenced by Bacillus Calmette–Guérin (BCG) administration (39). Focusing on the HiR subcohort, no BCG*SNP interaction using MMMs was found (results not shown). Larger cohorts might be needed to pinpoint these potential interactions.

SNPs were included as they tagged the selected inflammatory genes, what does not imply a potential function. Since it is difficult to know whether those variants identified are causative or are in high LD with the real ones, they should merely be considered as biomarkers of prognosis. While most of the selected SNPs tagged genes of particular interest in cancer biology, the position of some of the genes changed according to the new version of the human genome release, a fact that misplaced SNPs from the initial selection window (i.e., rs2269217). Noteworthy, most of the significant variants are placed in genes involved in immune tolerance processes: Janus kinase 3 (JAK3) participates in intracellular signal transduction after activation of immune cells. Lower levels of JAK3 might be responsible for the defective reactivity of T lymphocytes in patients with cancer (40). CD5 is known as a negative regulator of T- and B-cell receptor signaling. Its expression has been shown to be implicated in T lymphocytes tolerance toward tumor cells (41). AIRE encodes a transcription factor that regulates the expression of tissue antigens in the thymus and plays an important role in the development of organ-specific T regulatory lymphocytes (42). Those T regulators are thought to be major barrier impeding antitumor immune response (42). In melanoma, polymorphisms in AIRE may variably affect the selection of melanoma-associated antigen-specific thymocytes, generating T-cell repertoires protecting or predisposing individuals to cancer (43). CARD4/NOD1 is a member of the NOD receptor family that plays a major role in innate and adaptive immunities. Polymorphisms in those genes have been shown to be associated with multiple cancer risk including UBC (22, 44). NOD receptors were demonstrated to be involved in antitumor cytotoxicity through the potentiation of human natural killer cells and macrophage activities (45). MAP2K3 pathways

play a critical role in carcinogenesis. *MAP2K3* has been shown to suppress the growth of breast cancer cells (46) and alterations in its pathway are frequent in UBC (47). Finally, the protein encoded by *CD3G* is part of the T-cell receptor–CD3 complex. Prognostic impact of T lymphocytes' infiltration is now being investigated in multiple cancers, and *CD3* expression has been shown to be associated with UBC risk of recurrence and mortality (48).

Statistically significant results and potential biologic relevance are not enough criteria to reach clinical utility. Marker(s) have to be clinically actionable and cost efficient. While we identified SNPs strongly associated with NMIBC outcomes, they contributed little (<3.6%) to the higher event prediction ability provided by clinicopathologic variables. Furthermore, these estimates have to be taken cautiously, as their added value might be overestimated mainly because of the relatively low number of progression events in the whole cohort and mainly in the HiR/LR subcohorts. We cannot discard that the performances of the models are still magnified even though their predictive ability was tested by applying bootstrapped cross-validation samples (49). Therefore, an external validation would be advisable to confirm the added predictive value of the identified SNPs. However, heterogeneity across studies regarding patient recruitment, treatment, patient management, or availability of the genotypes data for the same set of SNPs limit the potential success of the replication stage. Furthermore, the definition of inflammatory genes was itself challenging. The list of potential genes is tremendously large, and the edges of the definition are difficult to delineate due to the crosstalk between inflammatory pathways and other cellular functions. It is possible that potential susceptibility markers identified in other studies were not included here, although we estimate these are a minority. Moreover, incomplete mapping of the genes might have occurred as a result of using a previous HapMap genome reference release or filtering by LD, what might have led to missing SNPs of interest. Finally, we did not explore all genetic mechanisms sustaining UBC prognosis, as the genetic architecture and correlations between genes involving complex interactions and epigenetic regulation are still unknown (50).

Despite that, this study reports valuable findings and has noteworthy strengths. The cohort used was built upon strong methodology. All the patients had complete and homogeneously collected clinical, pathologic, and genetic information, with long enough follow-up to investigate NMIBC prognosis. Using innovative MMM that identified many SNPs in inflammatory genes, we provide further evidence of the complex and heterogeneous

Inflammatory Variants in Bladder Cancer Prognosis

nature of UBC prognosis and enable to find associations that were not found by applying restrictive SMM.

Conclusion

Considering multiple genetic information jointly is key to understand its influence on complex traits such as UBC outcome. Innovative analytic approaches were essential to demonstrate that several SNPs in inflammatory genes were differently associated with risk of TFR and TP in NMIBC. Although external validation is warranted, this study provides proof of concept for the joint effect of few genetic variants in improving the discriminative ability of clinical prognostic models.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A. Masson-Lecomte, E. López de Maturana, M.E. Goddard, N. Rothman, M. Kogevinas, Y. Allory, N. Malats

Development of methodology: A. Masson-Lecomte, E. López de Maturana, M.E. Goddard, A. Tardon, J. Lloreta, N. Malats

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Gonzalez-Neira, M. Márquez, J. Lloreta, M. Garcia-Closas, D. Silverman, N. Rothman, M. Kogevinas, S.J. Chanock, F.X. Real, N. Malats

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Masson-Lecomte, E. López de Maturana, M.E. Goddard, A. Picornell, M. Rava, A. Carrato, A. Tardon, J. Lloreta, F.X. Real, N. Malats

Writing, review, and/or revision of the manuscript: A. Masson-Lecomte, E. López de Maturana, A. Picornell, M. Rava, A. Carrato, A. Tardon, J. Lloreta, M. Garcia-Closas, D. Silverman, N. Rothman, M. Kogevinas, Y. Allory, F.X. Real, N. Malats, N. Malats

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Masson-Lecomte, M. Márquez Study supervision: A. Masson-Lecomte, M. Márquez, N. Malats

Acknowledgments

The authors thank the coordinators, field and administrative workers, technicians, secretaries, and study participants of the Spanish Bladder Cancer/EPICURO study.

Spanish Bladder Cancer (SBC)/EPICURO Investigators: Institut Municipal d'Investigació Mèdica, Universitat Pompeu Fabra, Barcelona—Coordinating

References

- Samanic C, Kogevinas M, Dosemeci M, Malats N, Real FX, Garcia-Closas M, et al. Smoking and bladder cancer in Spain: effects of tobacco type, timing, environmental tobacco smoke, and gender. Cancer Epidemiol Biomarkers Prev 2006;15:1348–54.
- Silverman D, Devesa S, Morore L, Rothman N. Bladder cancer. Cancer Epidemiol Prev 2006;1101–27.
- García-Closas M, Malats N, Silverman D, Dosemeci M, Kogevinas M, Hein DW, et al. NAT2 slow acetylation, GSTM1 null genotype, and risk of bladder cancer: results from the Spanish Bladder Cancer Study and meta-analyses. Lancet 2006;366:649–59.
- Garcia-Closas M, Ye Y, Rothman N, Figueroa JD, Malats N, Dinney CP, et al. A genome-wide association study of bladder cancer identifies a new susceptibility locus within SLC14A1, a urea transporter gene on chromosome 18q12.3. Hum Mol Genet 2011;20:4282–9.
- Kiemeney LA, Thorlacius S, Sulem P, Geller F, Aben KKH, Stacey SN, et al. Sequence variant on 8q24 confers susceptibility to urinary bladder cancer. Nat Genet 2008;40:1307–12.
- Rothman N, Garcia-Closas M, Chatterjee N, Malats N, Wu X, Figueroa JD, et al. A multi-stage genome-wide association study of bladder cancer identifies multiple susceptibility loci. Nat Genet 2010;42: 978–84.

Center (M. Kogevinas, N. Malats, F.X. Real, M. Sala, G. Castaño, M. Torà, D. Puente, C. Villanueva, C. Murta-Nascimento, J. Fortuny, E. López, S. Hernández, R. Jaramillo, G. Vellalta, L. Palencia, F. Fermández, A. Amorós, A. Alfaro, G. Carretero); Hospital del Mar, Universitat Autònoma de Barcelona, Barcelona (J. Lloreta, S. Serrano, L. Ferrer, A. Gelabert, J. Carles, O. Bielsa, K. Villadiego); Hospital Germans Trias i Pujol, Badalona, Barcelona (L. Cecchini, J.M. Saladié, L. Ibarz); Hospital de Sant Boi, Sant Boi de Llobregat, Barcelona (M. Céspedes); Consorci Hospitalari Parc Taulí, Sabadell (C. Serra, D. García, J. Pujadas, R. Hernando, A. Cabezuelo, C. Abad, A. Prera, J. Prat); Centre Hospitalari i Cardiològic, Manresa, Barcelona (M. Domènech, J. Badal, J. Malet); Hospital Universitario de Canarias, La Laguna, Tenerife (R. García-Closas, J. Rodríguez de Vera, A.I. Martín); Hospital Universitario Nuestra Señora de la Candelaria, Tenerife (J. Taño, F. Cáceres); Hospital General Universitario de Elche, Universidad Miguel Hernández, Elche, Alicante (A. Carrato, F. García-López, M. Ull, A. Teruel, E. Andrada, A. Bustos, A. Castillejo, J.L. Soto); Universidad de Oviedo, Oviedo, Asturias (A. Tardón); Hospital San Agustín, Avilés, Asturias (J.L. Guate, J.M. Lanzas, J. Velasco); Hospital Central Covadonga, Oviedo, Asturias (J.M. Fernández, J.J. Rodríguez, A. Herrero); Hospital Central General, Oviedo, Asturias (R. Abascal, C. Manzano, T. Miralles); Hospital de Cabueñes, Gijón, Asturias (M. Rivas, M. Arguelles); Hospital de Jove, Gijón, Asturias (M. Díaz, J. Sánchez, O. González); Hospital de Cruz Roja, Gijón, Asturias (A. Mateos, V. Frade); Hospital Alvarez-Buylla, Mieres, Asturias (P. Muntañola, C. Pravia); Hospital Jarrio, Coaña, Asturias (A.M. Huescar, F. Huergo); Hospital Carmen y Severo Ochoa, Cangas, Asturias (J. Mosquera).

Grant Support

The project was funded partially by Fondo de Investigaciones Sanitarias (FIS, #PI00-0745, #PI05-1436, and #PI06-1614) and Red Temática de Investigación Cooperativa en Cáncer (RTICC, #RD12/0036/0050 and #RD12/0036/0034), Instituto de Salud Carlos III; and Asociación Española Contra el Cáncer (AECC), Spain; and EU-FP7-HEALTH-F2-2008-201663-UROMOL and EU-FP7-HEALTH-*TransBioBC #601933*. D. Silverman, N. Rothman, and SJ. Chanock received funding from the Intramural Research Program of the Division of Cancer Epidemiology and Genetics, National Cancer Institute (contract NCI NO2-CP-11015). A. Masson-Lecomte was awarded with a fellowship of the European Urological Scholarship Program for Research (EUSP Scholarship S-01-2013) and E. López de Maturana with a Sara Borrell fellowship, Instituto de Salud Carlos III, Spain.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 21, 2015; revised April 21, 2016; accepted April 22, 2016; published OnlineFirst May 6, 2016.

- Wu X, Ye Y, Kiemeney LA, Sulem P, Rafnar T, Matullo G, et al. Genetic variation in the prostate stem cell antigen gene PSCA confers susceptibility to urinary bladder cancer. Nat Genet 2009;41:991–5.
- Kiemeney LA, Sulem P, Besenbacher S, Vermeulen SH, Sigurdsson A, Thorleifsson G, et al. A sequence variant at 4p16.3 confers susceptibility to urinary bladder cancer. Nat Genet 2010;42:415–9.
- Rafnar T, Vermeulen SH, Sulem P, Thorleifsson G, Aben KK, Witjes JA, et al. European genome-wide association study identifies SLC14A1 as a new urinary bladder cancer susceptibility gene. Hum Mol Genet 2011;20: 4268–81.
- Tang W, Fu Y-P, Figueroa JD, Malats N, Garcia-Closas M, Chatterjee N, et al. Mapping of the UGT1A locus identifies an uncommon coding variant that affects mRNA expression and protects from bladder cancer. Hum Mol Genet 2012;21:1918–30.
- Rafnar T, Sulem P, Stacey SN, Geller F, Gudmundsson J, Sigurdsson A, et al. Sequence variants at the TERT-CLPTM1L locus associate with many cancer types. Nat Genet 2009;41:221–7.
- Rafnar T, Sulem P, Thorleifsson G, Vermeulen SH, Helgason H, Saemundsdottir J, et al. Genome-wide association study yields variants at 20p12.2 that associate with urinary bladder cancer. Hum Mol Genet 2014;23:5545–57.

www.aacrjournals.org

Cancer Epidemiol Biomarkers Prev; 25(7) July 2016 1149

Masson-Lecomte et al.

- Figueroa JD, Ye Y, Siddiq A, Garcia-closas M, Chatterjee N, Prokuninaolsson L, et al. Genome-wide association study identifies multiple loci associated with bladder cancer risk. Hum Mol Genet 2014;23:1387–98.
- Schrier BP, Hollander MP, Van Rhijn BWG, Kiemeney LA, Witjes JA. Prognosis of muscle-invasive bladder cancer: difference between primary and progressive tumours and implications for therapy. Eur Urol 2004; 45:292–6.
- Di Martino E, Tomlinson DC, Knowles MA. A decade of FGF receptor research in bladder cancer: Past, present, and future challenges. Adv Urol 2012;2012:429213.
- Allin KH, Bojesen SE, Nordestgaard BG. Baseline C-reactive protein is associated with incident cancer and survival in patients with cancer. J Clin Oncol 2009;27:2217–24.
- Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pagès C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 2006;313:1960–4.
- Viers BR, Boorjian SA, Frank I, Tarrell RF, Thapa P, Karnes RJ, et al. Pretreatment neutrophil-to-lymphocyte ratio is associated with advanced pathologic tumor stage and increased cancer-specific mortality among patients with urothelial carcinoma of the bladder undergoing radical cystectomy. Eur Urol 2014;66:1157–64.
- Leibovici D, Grossman HB, Dinney CP, Millikan RE, Lerner S, Wang Y, et al. Polymorphisms in inflammation genes and bladder cancer: from initiation to recurrence, progression, and survival. J Clin Oncol 2005; 23:5746–56.
- Ahirwar DK, Agrahari A, Mandhani A, Mittal RD. Cytokine gene polymorphisms are associated with risk of urinary bladder cancer and recurrence after BCG immunotherapy. Biomarkers 2009;14:213–8.
- Andrew AS, Gui J, Sanderson AC, Mason RA, Morlock EV, Schned AR, et al. Bladder cancer SNP panel predicts susceptibility and survival. Hum Genet 2009;125:527–39.
- Guirado M, Gil H, Saenz-Lopez P, Reinboth J, Garrido F, Cozar JM, et al. Association between C13ORF31, NOD2, RIPK2 and TLR10 polymorphisms and urothelial bladder cancer. Hum Immunol 2012;73:668–72.
- Malats N. Genetic epidemiology of bladder cancer: scaling up in the identification of low-penetrance genetic markers of bladder cancer risk and progression. Scand J Urol Nephrol Suppl 2008;42:131–40.
- de Maturana EL, Ye Y, Calle ML, Rothman N, Urrea V, Kogevinas M, et al. Application of multi-SNP approaches Bayesian LASSO and AUC-RF to detect main effects of inflammatory-gene variants associated with bladder cancer risk. PLoS One 2013;8:e83745.
- Babjuk M, Burger M, Zigeuner R, Shariat SF, van Rhijn BWG, Compérat E, et al. EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder: update 2013. Eur Urol 2013;64:639–53.
- Galdos BL, Medina I, Suarez CM, Heredia T, Torres ÁC, Sangrós R, et al. Select your SNPs (SYSNPs): a web tool for automatic and massive selection of SNPs. Int J Data Min Bioinform 2012;6:324–34.
- García-Closas M, Malats N, Real FX, Yeager M, Welch R, Silverman D, et al. Large-scale evaluation of candidate genes identifies associations between VEGF polymorphisms and bladder cancer risk. PLoS Genet 2007;3:0287–93.
- Browning SR, Browning BL. Rapid and accurate haplotype phasing and missing-data inference for whole-genome association studies by use of localized haplotype clustering. Am J Hum Genet 2007;81: 1084–97.
- Meuwissen THE, Hayes BJ, Goddard ME. Prediction of total genetic value using genome-wide dense marker maps. Genetics 2001;157:1819–29.
- 30. Park T, Casella G. The Bayesian Lasso. J Am Stat Assoc 2008;103:681-6.
- López de Maturana E, Ibáñez-Escriche N, González-Recio Ó, Marenne G, Mehrban H, Chanock SJ, et al. Next generation modeling in GWAS:

comparing different genetic architectures. Hum Genet 2014;133: 1235-53.

- 32. Albert JH, Chib S. Sequential ordinal modeling with applications to survival data. Biometrics 2001;57:829–36.
- González-Recio O, Gianola D, Long N, Weigel KA, Rosa GJM, Avendaño S. Nonparametric methods for incorporating genomic information into genetic evaluations: an application to mortality in broilers. Genetics 2008; 178:2305–13.
- Goddard ME. Optimal effective population size for the global population of black and white dairy cattle. J Dairy Sci 1992;75:2902–11.
- Gerds TA, Kattan MW, Schumacher M, Yu C. Estimating a time-dependent concordance index for survival prediction models with covariate dependent censoring. Stat Med 2013;32:2173–84.
- 36. Ahirwar D, Kesarwani P, Manchanda PK, Mandhani A, Mittal RD. Anti- and proinflammatory cytokine gene polymorphism and genetic predisposition: association with smoking, tumor stage and grade, and bacillus Calmette-Guérin immunotherapy in bladder cancer. Cancer Genet Cytogenet 2008;184:1–8.
- 37. Vittinghoff E, McCulloch CE. Relaxing the rule of ten events per variable in logistic and cox regression. Am J Epidemiol 2007;165:710–8.
- Peduzzi P, Concato J, Feinstein AR, Holford TR. Importance of events per independent variable in proportional hazards regression analysis II. Accuracy and precision of regression estimates. J Clin Epidemiol 1995;48:1503–10.
- Lamm DL, Blumenstein BA, Crissman JD, Montie JE, Gottesmann JE, Lowe BA, et al. Maintenance bacillus calmette-guerin immunotherapy for recurrent Ta, T1 and carcinoma in situ of the bladder: A Randomized Southwest Oncology Group Study. J Urol 2000;163:1124–9.
- Klink M, Kielbik M, Nowak M, Bednarska K, Sulowska Z. JAK3, STAT3 and CD3-zeta signaling proteins status in regard to the lymphocytes function in patients with ovarian cancer. Immunol Invest 2012;41:382–98.
- Dalloul A. CD5: a safeguard against autoimmunity and a shield for cancer cells. Autoimmun Rev 2009;8:349–53.
- Malchow S, Leventhal DS, Nishi S, Fischer BI, Shen L, Paner GP, et al. Airedependent thymic development of tumor-associated regulatory T cells. J Urol 2013;190:1954.
- Conteduca G, Ferrera F, Pastorino L, Fenoglio D, Negrini S, Sormani MP, et al. The role of AIRE polymorphisms in melanoma. Clin Immunol 2010;136:96–104.
- Kutikhin AG. Role of NOD1/CARD4 and NOD2/CARD15 gene polymorphisms in cancer etiology. Hum Immunol 2011;72:955–68.
- 45. Qiu F, Maniar A, Diaz MQ, Chapoval AI, Medvedev AE. Activation of cytokine-producing and antitumor activities of natural killer cells and macrophages by engagement of Toll-like and NOD-like receptors. Innate Immun 2011;17:375–87.
- MacNeil AJ, Jiao SC, McEachern LA, Yang YJ, Dennis A, Yu H, et al. MAPK kinase 3 Is a tumor suppressor with reduced copy number in breast cancer. Cancer Res 2014;74:162–72.
- Otto KB, Acharya SS, Robinson VL. Stress-activated kinase pathway alteration is a frequent event in bladder cancer. Urol Oncol Semin Orig Investig 2012;30:415–20.
- Otto W, Denzinger S, Wieland WF, Hartmann A. First analysis of immune cell infiltration in stage pT1 urothelial bladder carcinoma: CD3 positivity as a prognostic marker for cancer-specific survival. World J Urol 2012; 30:875–7.
- Bleeker SE, Moll HA, Steyerberg EW, Donders AR, Derksen-Lubsen G, Grobbee DE, et al. External validation is necessary in prediction research: a clinical example. J Clin Epidemiol 2003;56:826–32.
- Besaratinia A, Cockburn M, Tommasi S. Alterations of DNA methylome in human bladder cancer. Epigenetics 2013;8:1013–22.

1150 Cancer Epidemiol Biomarkers Prev; 25(7) July 2016

Cancer Epidemiology, Biomarkers & Prevention

Cancer Epidemiology, Biomarkers & Prevention



Inflammatory-Related Genetic Variants in Non–Muscle-Invasive Bladder Cancer Prognosis: A Multimarker Bayesian Assessment

Alexandra Masson-Lecomte, Evangelina López de Maturana, Michael E. Goddard, et al.

Cancer Epidemiol Biomarkers Prev 2016;25:1144-1150. Published OnlineFirst May 6, 2016.

Updated version	Access the most recent version of this article at: doi:10.1158/1055-9965.EPI-15-0894
Supplementary	Access the most recent supplemental material at:
Material	http://cebp.aacrjournals.org/content/suppl/2016/05/06/1055-9965.EPI-15-0894.DC1

Cited articles	This article cites 49 articles, 13 of which you can access for free at: http://cebp.aacrjournals.org/content/25/7/1144.full.html#ref-list-1	
----------------	--	--

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.

SUPPLEMENTARY MATERIAL AND METHODS

Multi Marker Methods (MMM)

Bayes A (BA) and Bayesian LASSO (BL) were coupled with the sequential threshold model developed by Albert and Chib [182] to handle the large p small n problem accounting for the time to event and censoring. It assumes that for an observation of a patient to be present at a given period of time, he/she must have survived through all previous time periods. Thus, the probability of not presenting the event of interest until interval l, conditional on the event that

the *l*-th interval has been reached, is given by
$$\Pr(y_i = l \mid y_i \ge l - 1, \gamma, \theta) = \Phi\left(\frac{\gamma_l - \mathbf{X}' \theta}{\sigma_e^2}\right)$$
, where

 γ corresponds to unordered cutoff points corresponding to each time interval, **X** corresponds to the incidence matrix of effects (θ) affecting the liability to survive to the next interval given that the present interval has been reached and σ_e^2 is the residual variance, which was set to 1 for identification purposes [183]. Thus, the presence of the event of interest (first recurrence or progression) is checked in each interval, and the outcome in that interval is codified as 0 if present and 1 otherwise. If the data is not censored in that interval, then the patient has data in the next interval and the outcome in that interval is codified as 0 or 1 as before mentioned. If the data is censored in the first interval, then the individual does not have data in the subsequent intervals.

Intervals were defined according to the survival functions for each event (see Figures S2 and S3), considering a minimum of events for interval. For time to first recurrence, 9 follow-up intervals were defined: \leq 3, 4-6, 7-9, 10-12, 13-18, 18-24, 24-36, 36-48 and > 48 months. Regarding time to progression, 4 intervals were defined (4 levels, (\leq 12 months, 13-24 months, 25-48 months and >48 months). When patients were stratified at high/low risk, 4/3 intervals were defined: \leq 6; 7-12; 13-24 and > 24 months; and \leq 24; 25-48 and >48 months, respectively.

As in [184], markers were considered as associated with bladder cancer when both BA_t and BL_t identified them as associated with the outcome. Both BA and BL are characterized by thick-tailed priors, the scaled t and the double exponential (DE), respectively. These densities have higher mass at 0, which shrinks toward 0 the estimates of marker effects with small effects and induces less shrinkage (thicker tails) to markers with larger effects. The prior distributions were Inv $-\chi^2(4.01,0.016)$ (scaled t prior for marker effects) in BA and an exponential density (double exponential prior for marker effects, $\prod_{j=1}^{m} N(\beta_j | 0, \tau_j^2 \sigma_{\varepsilon}^2) \times \prod_{j=1}^{m} \exp(\tau_j^2 | \lambda))$ in BL. Parameter λ in BL controls the shape of the prior

distribution assigned to τ_j^{-2} , assigning more density to small values of τ_j than to large ones, and follows, a priori, a Gamma distribution $G(\lambda^2|10,0.75)$. For each analysis, a unique Markov Chain Monte Carlo of 50,000 iterations was obtained using a Gibbs sampler. The first 20,000 iterations were discarded as burn-in and all the remaining iterations were retained to infer posterior marginal distributions of unknown parameters. Convergence of chains was assessed visually, applying the Geweke criterion [185] and running parallel chains with different initial values. A permutation within Markov Chain Monte Carlo approach [186] was used to determine the markers that were associated with the phenotype for both models. Markers were considered as associated to the trait if the $\max(a,1-a) > t$, where

 $a = \int_{-\infty}^{\beta_p} p(\beta_p | \mathbf{y}_{permu}, \boldsymbol{\beta}_{-p}) d\beta_p, \quad \hat{\beta}_p \text{ is the posterior mean of the SNP } p \text{ after analyzing the}$

original data, and $p(\beta_p | \mathbf{y}_{permu})$ is the posterior distribution of the marker effect given the permuted data (\mathbf{y}_{permu}).

When the existence of BCG*SNP interactions associated with time to progression were explored in the high-risk NMIBC subgroup, we also used MMMs, adding the interaction terms to the model including clinical-pathological parameters and SNPs.

REFERENCES

[1] Albert JH, Chib S. Sequential ordinal modeling with applications to survival data. Biometrics 2001;57:829-36.

[2] Sorensen D, Gianola D. Likelihood, Bayesian, and MCMC Methods in Quantitative Genetics. New York: Springer Science & Business Media; 2002.

[3] Lopez de Maturana E, Ibanez-Escriche N, Gonzalez-Recio O, Marenne G, Mehrban H, Chanock SJ, et al. Next generation modeling in GWAS: comparing different genetic architectures. Hum Genet 2014;133:1235-53.

[4] Geweke J. Bayesian Statistics 1992. p. 169-93.

[5] Che X, Xu S. Significance test and genome selection in bayesian shrinkage analysis. Int J Plant Genomics 2010;2010:893206.

ABCA1	CCR10	CXCR3	IL12A	MAP3K7	SOCS5
ABCA7	CCR2	CXCR4	IL12B	MAP3K7IP2	SOCS6
ABCC4	CCR3	CXCR7	IL13	MAPK14	STAT1
ABCF1	CCR4	EIF2AK2	IL15	MASP1	STAT3
ABO	CCR5	EPHX2	IL15RA	MBL2	TBK1
AICDA	CCR7	EXO1	IL16	MPO	TFF1
AIRE	CCR7	FADD	IL17A	MS4A1	TFF3
AKR1C3	CCR9	FAS	IL17C	MSH2	TGFB1
AKT1	CD14	FAS	IL18	MX1	TGFBR1
ALOX12	CD180	FASLG	IL1A	MYD88	TICAM1
ALOX15	CD2	FCGR2A	IL1B	NBS1	TIRAP
ALOX5	CD274	FOS	IL1RN	NCF2	TLR1
ANPEP	CD28	GATA3	IL2	NFKB1	TLR10
APOA2	CD3	GDF15	IL21	NFKBIA	TLR2
ARHGDIB	CD33	GSK3B	IL21R	NINJ1	TLR4
BCL10	CD4	H2AFX	IL22	NLRP12	TLR6
BCL3	CD40	HAVCR2	IL23	NOD2	TLR9
BCL6	CD45	HDAC5	IL2RA	NOS2A	TMED7
BIRC2	CD5	HDAC7A	IL3	OPRD1	TMEM189
BIRC3	CD68	HFE	IL4	OSCAR	TNF
BIRC5	CD8	HLA-A	IL4R	PARP4	TNFAIP3
BLNK	CD80	HLA-B	IL6	PPARG	TNFRSF10A
CARD15	CD81	HLA-C	IL6R	PRF1	TNFRSF1A
CARD4	CD86	HLA-E	IL7	PRG3	TNFRSF7
CASP1	CDH1	HLA-F	IL7R	PRKRA	TNFSF14
CASP3	CFH	HLA-G	IL8	PTGS1	TNIP1
CASP8	CFLAR	HLA-H	IL8RA	PTGS2	TOLLIP
CASP9	CGB	HSP90AA1	IL8RB	RAG1	TRADD
CBR1	CHKA	HSPA4	IRAK2	RELA	TRAF1
CCL13	CHUK	HSPB1	IRF1	RHOA	TRAF2
CCL17	COX2	HSPD1	IRF3	RIPK1	TRAF5
CCL17	CRP	ICAM1	JAG2	RIPK2	TRAF6
CCL19	CSF1R	ICBR	JAK1	ROCK1	TSLP
CCL2	CSF3	IFNAR2	JAK3	SARM1	UBE2N
CCL21	CTLA4	IFNG	LEPR	SCARB1	ULBP1
CCL22	CX3CL1	IFNGR1	LITAF	SELE	ULBP2
CCL22	CX3CR1	IFNGR2	LTA	SFTPD	ULBP3
CCL28	CXCL10	IKBKB	LY96	SIGIRR	VCAM1
CCL28	CXCL11	IKZF1	MAP2K3	SLAMF1	WWP1
CCL5	CXCL12	IL10	MAP2K4	SLC20A1	XBP1
CCND3	CXCI 9	IL 10R A	MAP3K14	SLC44A2	YY1
	CACLY	ILIUIUI			

 Table S1: List of inflammation-related genes selected for the study.

Table S2:	Variables	used for	adjustment	in the s	ingle and	multimarker	models.
					0		

TFR	Area + gender + multiplicity ^a + TSG^b + tumour size ^c + treatment ^d
ТР	Area + age + multiplicity ^a + TSG ^b + number of recurrences ^e + treatment ^f

TFR = Time to first recurrence; TP= Time to progression : TSG = Tumour stage and grade

^b Divided in 6 categories: 1= PUNLMP+TaG1, 2= TaG2, 3= TaG3, 4= T1G2, 5= T1G3 and 6=TiG2+TiG3.

- ^c Divided in 3 categories: 1 = < or equal 3 cm, 2 = >3 cm and 3 = missing data.
- ^d Divided in 5 categories 1= TURB alone, 2= TURB+intravesical chemotherapy, 3= TURB +Bacillus Calmette Guerin
- (BCG), 4= TURB+intravesical chemotherapy+BCG and 5= Other treatments.
- ^e Divided in 3 categories 1= 1 recurrence, 2= 2 recurrences, 3= 3 recurrences and 4=>3 previous recurrences.

^fDivided in 6 categories as in ^d plus TURB+radical cystectomy.

^a Divided in 3 categories: 1 =One tumour, 2 = >1, and 3 =missing data.

Table S3: Treatment characteristi	ics.
-----------------------------------	------

	Low Risk (N=538)	High Risk (N=284)
	N (%)	N (%)
TURB	274 (51)	78 (27.5)
TURB + IVC	134 (25)	46 (16.2)
TURB + BCG	111 (20.6)	134 (47.3)
TURB + IVC + BCG	12 (2.2)	9 (3.1)
TURB + Cystectomy	4 (0.7)	8 (2.8)
Others	3 (0.5)	9 (3.1)

TURB = Trans Urethral Resection of the Bladder; IVC= Intra-vesical chemotherapy; BCG = Bacillus Calmette et Guerin.

GENE	SNP	HR	p-value	MAF
TNIP1	rs2277940	1.74	0.0001	0.07
CCL28	rs779850	0.61	0.0004	0.17
CCR9	rs2191031	0.62	0.0005	0.17
PPARG	rs3112395	1.73	0.0007	0.05
CCL2	rs10805673	0.75	0.0012	0.49
CARD4	rs10267377	0.71	0.0013	0.29
PTPRC	rs1036332	0.73	0.0029	0.27
RIPK1	rs6596945	0.70	0.0029	0.22
CD3Z	rs1554669	1.50	0.0044	0.07
CCR2	rs3138042	0.76	0.0046	0.34

Table S4: The top 10 autosomal SNPs associated with NMIBC risk of first recurrence using multivariate Cox regression additive model.

Analyses were adjusted for: geographical area, gender, multiplicity, tumour stage&grade, tumour size, and treatment (See, Table S2).

Table S5: SNPs with a strong posterior probability (PP>80%) of being associated with <u>time</u> to first recurrence considering <u>all NMIBC patients</u>. Those with PP in both BA and BL >90% are bold-faced. The last column shows Cox regression results. SNPs in red were also among the top10 SNPs identified by Cox regression.

			BA		BL		Single marker	
							(Cox
Gene	SNP	MAF	HR _{aa_AA}	PP>80%	HR _{aa_AA}	PP>80%	HR	<i>p</i> -
								value
JAK3	rs6523*	0.40	1.69	98	1.17	92	1.20	0.0488
CCL2	rs4497746	0.08	1.59	94	1.14	87	1.31	0.0641
ICEBERG	rs3736149	0.43	1.41	93	1.15	89	1.20	0.0349
CD180	rs5744463	0.11	1.52	93	1.13	86	1.23	0.1230
ICAMI	rs5030390	0.08	0.63	92	0.87	87	0.56	0.0053
CD274	rs7043593	0.23	1.44	92	1.10	81	1.09	0.3827
MAP3K14	rs3785803	0.14	0.67	91	0.88	87	0.68	0.0058
TNIP1	rs2277940	0.07	1.50	91	1.24	94	1.74	0.0001
CD5	rs7104333	0.49	0.72	90	0.86	92	0.81	0.0126
CCL28	rs10805673	0.49	0.73	90	0.84	94	0.75	0.0012
BLNK	rs11188660	0.29	1.42	89	1.16	90	1.27	0.0098
SCARB1	rs3924313	0.33	1.29	89	1.11	84	1.18	0.0628
PTPRC	rs1036332	0.27	0.69	89	0.84	92	0.73	0.0029
BLNK	rs12772113	0.23	0.71	89	0.88	87	0.82	0.0695
CCR9	rs2191031	0.17	0.70	88	0.84	92	0.62	0.0005
CCL2	rs317325	0.37	0.74	88	0.89	87	0.80	0.0179
CARD4	rs10267377	0.29	0.75	88	0.86	90	0.71	0.0013
RIPK1	rs6596945	0.22	0.74	88	0.86	90		
CMKOR1	rs7556982	0.47	1.34	88	1.10	82	1.11	0.2364

Alexandra Masson-Lecomte

IL18	rs11214093	0.43	0.75	87	0.90	85	0.88	0.1541
TNFRSF10A	rs2235126	0.29	0.77	87	0.91	81	0.88	0.2043
CX3CL1	rs2239354	0.10	0.71	87	0.91	81	0.86	0.3187
CD33	rs3865444	0.29	1.30	87	1.09	80	1.12	0.2490
IL6	rs13247988	0.25	1.35	86	1.13	86	1.20	0.0561
CD3Z	rs858553	0.39	1.30	86	1.09	81	1.20	0.0419
CD3D	rs2276424	0.30	0.75	86	0.88	88	0.83	0.0675
CD5	rs7342164	0.14	0.73	86	0.90	83	0.77	0.0628
CD8B1	rs13024609	0.20	1.34	85	1.10	82	1.25	0.0268
HAVCR2	rs919746	0.16	1.30	85	1.12	84	1.27	0.0320
CCR2	rs3138042	0.34	0.78	84	0.89	87	0.76	0.0046
CMKOR1	rs2720100	0.48	0.77	84	0.88	87	0.80	0.0122
CCL28	rs779850	0.17	0.77	84	0.87	89	0.61	0.0004
TNF	rs1799964	0.24	1.30	84	1.14	87	1.28	0.0109
IFNGR1	rs3799488	0.12	0.77	83	0.91	82	0.67	0.0099
CCL21	rs2812377	0.37	0.77	83	0.91	83	0.89	0.1955
CD80	rs9282638	0.16	1.28	83	1.11	83	1.30	0.0180
IFNGR2	rs1059293	0.44	0.81	83	0.90	85	0.86	0.0948
CXCR4	rs543721	0.45	0.77	82	0.90	84	0.86	0.1018
PRKRA	rs2059691	0.35	0.80	82	0.91	84	0.79	0.0147
PPARG	rs3112395	0.05	1.35	81	1.12	83	1.73	0.0007
IL23R	rs10489628	0.35	1.26	81	1.12	85	1.27	0.0050
ABCA1	rs4149313	0.18	1.23	80	1.10	83	1.21	0.0817
BLNK	rs11188661	0.34	1.23	80	1.09	81	1.14	0.1263

* Previously as rs2286662. Analyses were adjusted for: geographical area, gender, multiplicity, tumour stage&grade, tumour size, and treatment (See, Table S2).

Table S6. SNPs with a strong posterior probability (PP>75%) of being associated with <u>time</u> <u>to first progression</u> considering <u>high-risk NMIBC patients</u>. The last column displays the Cox regression results for each SNP + covariates. SNPs in red were also among the top10 SNPs identified with Cox regression.

			BA		BL		Cox regression	
Gene	SNP	MAF	HR _{aa_AA}	PP>75%	HR _{aa_AA}	PP>75%	HR	<i>p</i> -value
TMEM189	rs2269217	0.23	1.31	80	1.10	80	2.41	0.0003
MAP2K3	rs9901404	0.48	1.27	77	1.10	80	1.86	0.0018
CARD4	rs2256023	0.44	1.27	79	1.09	79	1.68	0.0084

Analyses were adjusted for: geographical area, age, multiplicity, tumour stage&grade, number of recurrences, and treatment (See, Table S2).
Table S7: SNPs with a strong posterior probability (PP>75%) of being associated with <u>time</u> <u>to first progression</u> considering <u>low-risk NMIBC patients</u>. The last column displays the Cox regression results for each SNP + covariates. The SNP (in red) was also among the top10 SNPs identified with Cox regression.

			В	A	В	L	Cox	regression
Gene	SNP	MAF	HR _{aa_AA}	PP>75%	$\mathrm{HR}_{\mathrm{aa}_\mathrm{AA}}$	PP>75%	HR	<i>p</i> -value
CD68	rs12942088	0.43			1.08	75	2.91	0.0006

Analyses were adjusted for: geographical area, age, multiplicity, tumour stage&grade, number of recurrences, and treatment (See, Table S2).

Table S8. Apparent and validated c-index using Bootstrap alone or 1000 bootstrap cross validation indicating the discriminatory ability of the models including the clinical variables (CV) and each of the SNPs showing an association with each outcome of interest.

	Apparent <i>c</i> -index	Bootstrap alone <i>c</i> -index	Bootstrap cross validation <i>c</i> -index
Recurrence ALL (N=268 events)			
$CV + rs2277940^{a}$	64	61.3	59.8
CV + rs7104333 ^b	63.2	60.6	59
CV + rs2286662°	63.3	60.5	58.9
CV + rs2277940 + rs7104333	64.5	61.8	60.3
CV + rs2277940 + rs2286662	64.3	61.7	60.1
CV + rs7104333 + rs2286662	63.7	61	59.4
Progression ALL (N=76 events)			
CV + rs698079 ^d	78.1	74.9	72.2
CV + rs941405 °	78.1	75.1	72.6
Progression HiR (N=52 events)			
CV + rs2256023	71.6	65.5	62
CV + rs9901404	71.9	65.7	62.1
CV + rs2269217	72.2	67	63.9
CV + rs2269217 + rs9901404	74.3	68.8	65.6
CV + rs9901404 + rs2256023	73.8	63.8	67.5
CV + rs2269217 + rs2256023	73.6	68	64.8

^a TNIP1; ^b CD5; ^cJAK3; ^d MASP1; ^e AIRE; ^f CARD4; ^g MAP2K3; ^h TMEM189

Figure S1. Methodology used for statistical analyses. A Single Marker Method (SMM) consists of inclusion of each SNP individually while a Multi Marker Method (MMM) allows inclusion of all SNPs together in the model. Interest lies in mimicking the polygenic scenario that features bladder cancer prognosis.



Covariates considered in the models for *Time to First Recurrence* (area, gender, stage and grade, tumor size, multiplicity, treatment) and for *Time to Progression* (area, age, stage and grade, number of recurrence, multiplicity, treatment)

Figure S2. Survival curve for time to recurrence considering all of non-muscle invasive bladder cancer patients.



Figure S3. Survival curve for time to first progression considering all of non-muscle invasive bladder cancer patients.



ARTICLE 2: ASSOCIATION BETWEEN GERMLINE PDL1 VARIANTS AND BLADDER CANCER PROGNOSIS

1.1. Abstract

Targeting immune checkpoints has shown promising results in metastatic urothelial bladder cancer (UBC) treatment, particularly in patients whose tumours express PDL1 in immune cells. There is extensive evidence that immune responses are under genetic control but the role of *PDL1 (CD274)* inherited variation in the prognosis of cancer patients has not yet been investigated. We analyzed the association of *PDL1* variants with UBC patient prognosis. Genotypes of 25 SNPs tagging *PDL1* (gene-coverage of 63%) were available for 810 and 245 patients with non-muscle and muscle invasive UBC, respectively. Two approaches at the SNP (adjusted Cox regression) and gene (sequence kernel association test, SKAT) level were used to evaluate the associated with progression, disease-specific, and overall mortality in muscle invasive UBC. No significant results were observed among non-muscle invasive UBC. Data from 271 patients from TCGA did not replicate these results neither at the SNP nor at the gene level. This is the first report suggesting highly significant association between germline *PDL1* variants and muscle invasive UBC prognosis. The ability of *PDL1* variation to predict response to anti-PDL1 therapies warrants further investigation.

1.2. Manuscript

ASSOCIATION BETWEEN GERMLINE *PDL1* VARIANTS AND BLADDER CANCER PROGNOSIS

A Masson-Lecomte* (1,2), E Lopez de Maturana* (1), S Pineda (1), M Rava (1), A Carrato (3), A Tardón (4), DT Silverman (5), N Rothman (5), M García-Closas (5), SJ Chanock (5), M Kogevinas (6), Y Allory (7), Francisco X. Real (8,9), and N Malats (1) on behalf of the SBC/EPICURO Study Investigators (10).

 Genetic and Molecular Epidemiology Group, Spanish National Cancer Research Centre (CNIO), Madrid, and CIBERONC, Spain.

(2) Department of Urology, Henri Mondor Hospital, IMRB, Créteil, France.

(3) Department of Oncology, Hospital Ramon y Cajal, Madrid, and CIBERONC, Spain.

(4) Preventive Medicine and Public Health, University of Oviedo, and CIBERESP, Spain.

(5) Division of Cancer Epidemiology & Genetics, National Cancer Institute, USA.

(6) Centre de Recerca en Epidemiologia Ambiental (CREAL), Barcelona, and CIBERESP, Spain.

(7) Pathology Department, Henri Mondor Hospital, IMRB, Créteil, France

(8) Epithelial Carcinogenesis Group, Spanish National Cancer Research Centre (CNIO), Madrid, and CIBERONC, Spain.

(9) Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Barcelona, Spain.

(10) In Annex 1.

Alexandra Masson-Lecomte

* A. Masson-Lecomte and E.L. de Maturana contributed equally to this article.

Abstract

Targeting immune checkpoints has shown promising results in metastatic urothelial bladder cancer (UBC) treatment, particularly in patients whose tumours express PDL1 in immune cells. There is extensive evidence that immune responses are under genetic control but the role of *PDL1 (CD274)* inherited variation in the prognosis of cancer patients has not yet been investigated. We analyzed the association of *PDL1* variants with UBC patient prognosis. Genotypes of 25 SNPs tagging *PDL1* (gene-coverage of 63%) were available for 810 and 245 patients with non-muscle and muscle invasive UBC, respectively. Two approaches at the SNP (adjusted Cox regression) and gene (sequence kernel association test, SKAT) level were used to evaluate the associated with progression, disease-specific, and overall mortality in muscle invasive UBC. No significant results were observed among non-muscle invasive UBC. Data from 271 patients from TCGA did not replicate these results neither at the SNP nor at the gene level. This is the first report suggesting highly significant association between germline *PDL1* variants and muscle invasive UBC prognosis. The ability of *PDL1* variation to predict response to anti-PDL1 therapies warrants further investigation.

Patient summary

Anti PD1/PDL1 therapies are gaining importance in several tumours, among them muscleinvasive bladder cancer. This study shows, for the first time, that genetic variation in *PDL1* may predict the clinical outcomes of aggressive bladder cancer patients.

Manuscript

Tumour initiation and progression relies on close interactions with microenvironmental cells [32], immune cells infiltration displaying genetic variations. The role of those cells in tumour development and progression is already established. In the recent years, a major breakthrough in the management of metastatic urothelial bladder cancer (UBC) has been the use of drugs targeting the immune checkpoints [87]. Trials are being conducted worldwide assessing the efficacy of anti PD1/PDL1 therapies in diverse settings (i.e., metastatic disease, adjuvant chemotherapy, BCG resistance). The precise mechanism of action of these drugs is still unclear. The use of anti-PDL1 antibodies might reactivate antitumour immunity. Although best results are seen in patients showing strong expression of PDL1 in the stroma cells, this is not yet a reliable marker to predict treatment response [187]. There is extensive evidence that genetic variation shapes immune responses but this area is poorly explored in human cancer and the role of inherited *PDL1* variation in UBC patient management has not yet been explored. Therefore we aimed at assessing potential associations between germline *PDL1* variation and UBC prognosis.

We first considered 995 non muscle-invasive (NMIBC) and 296 muscle-invasive (MIBC) bladder cancer patients included in the Spanish Bladder Cancer (SBC)/EPICURO Study between 1997 and 2001 [188]. Clinico-pathological information at diagnosis and during follow-up was obtained from the clinical charts. *PDL1* variant genotyping was done with the Illumina Infinium HumanHap1M array [189]. After the quality control process (see **Supplementary Methods** for more detail), complete clinico-pathological information and genotypes of 25 SNPs (gene-coverage 63%) were available for 810 NMIBC and 245 MIBC patients. **Table S1** shows the characteristics of the patients included in the study. We used Cox proportional hazard regression adjusted for classical clinico-pathological prognosticators to assess the association between individual SNPs and time to disease recurrence,

progression, cancer-specific mortality, and overall mortality (Supplementary Methods). Among NMIBC patients, 268 (33.1%) presented tumour recurrence and 76 (9.4%), tumour progression. Among MIBC patients, 129 (52.6%) presented tumour progression, 112 (45.7%) died from UBC, and 169 (69%) died from other causes, with a mean follow-up for diseasefree patients of 82.7 (2.5-117.6) months (Figure S1). While no significant association was observed in NMIBC (Table S2), five variants were significantly associated with all endpoints in patients with MIBC after multiple testing correction (rs822339, rs860290, rs2282055, rs1411262, rs10125854, Table 1). SNP rs860290 was strongly associated with disease progression (HR=1.91, p-value =0.0001), cancer-specific mortality (HR=1.57, p-value = 0.03) and overall mortality (HR=1.77, *p*-value=0.0002). Five-year progression-free survival was 50% for «AA» patients vs 29% in «Aa» patients and 9% in «aa» patients (Log rank pvalue=0.0001, Figure 1). We performed a gene-based analysis using the SkatMeta R package (https://cran.r-project.org/web/packages/skatMeta/index.html, Supplementary Methods). At the gene level, PDL1 was significantly associated with the 3 MIBC outcomes (p-values= 0.002, 0.03 and 0.006 for tumour progression, cancer-specific survival, and overall survival, respectively, Table 1). We explored the GTEx portal (http://www.gtexportal.org) to identify eQTLs for the SNPs considered here. All significant variants, except rs10125854, correlated with PDL1 expression levels in different tissues, none of them in normal bladder. (Table **S3**).

We then sought independent replication of the results. The best genotyped patient series available was that of The Cancer Genome Atlas (TCGA) MIBC project [65]. Complete data were available for 161 patients regarding tumour progression and for 238 patients regarding overall survival. Cancer-specific survival data were not available. Table S1 shows a comparison of the clinical and pathological characteristics of the patients with MIBC included in both studies. Mean follow-up for patients in the TCGA study was 27.6 months,

during which 91 (49.2%) underwent tumour progression and 126 (46.5%) died (**Figure S2**). Genotypes of 11 out of the 25 SNPs used in the SBC/EPICURO Study were available in TCGA dataset. The variants that were associated with outcome in the SBC/EPICURO study were not replicated in the TCGA group. Age- and sex-adjusted Cox regression models let us to identify two new variants (rs7043593 and rs4742100) associated with risk of progression (*p*-value= 0.002 and 0.03) and with overall mortality (*p*-value= 0.001 and 0.03). This association did not stand after adjusting for stage suggesting a stage-related association in TCGA (*p*-value= 0.08 and 0.07 for progression, respectively, **Table S4**). At the gene level, *PDL1* was not significantly associated with either outcome in TCGA (*p*-values > 0.05)

We performed an eQTL analysis to assess the association between the germline (blood) and tumour SNP genotypes and *PDL1* expression levels in TCGA tumours. One hundred and nineteen patients had the SNPs genotyped in both tissues. We found that rs860290, the strongest significant SNP in SBC/EPICURO, was associated with *PDL1* expression in the tumour (*p*-value=0.03). Interestingly, the eQTL was observed only in patients whose tumour genotypes differed from those in germline DNA (**Figure S3**). *PDL1* gene is located on the short arm of chromosome 9 that is frequently lost in early stages of bladder carcinogenesis, suggesting that the association between rs860290 and *PDL1* expression in the tumour might be driven by infiltrating leukocytes.

Restoration of intra-tumour immune function has recently led to major advances in the treatment of urologic malignancies [190]. Four studies looked at the association between *PDL1* expression and MIBC prognosis with discordant results [120, 191-193]. Positive treatment responses are mainly seen in cases where PDL1 is overexpressed in immune cells rather than in tumour cells [87, 120]. However, regulation on PDL1 expression is still poorly understood [194]. In a well-designed study with a large number of patients and proper follow-up, we found that *PDL1* germline variation is associated with MIBC prognosis. The

lack of replication in the independent patient series of the TCGA study may well be due to differences in study design and patient characteristics, as well as shorter follow-up. Unfortunately treatment data was not available in TCGA. The eQTL with rs860290 found both in TCGA and GTEx suggests functionality with the most significant variant identified in the SBC/EPICURO Study. Our findings should stimulate further research on the contribution of germline variation in shaping the immune responses in patients with cancer and, more specifically, in patients with MIBC. In addition to PDL1 expression in tumors, *PDL1* variation could be accounted for in predicting response to anti-PDL1 therapies in UCB.

References

1. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer cell. 2012;21(3):309-22.

2. Powles T, Eder JP, Fine GD, Braiteh FS, Loriot Y, Cruz C, et al. MPDL3280A (anti-PD-L1) treatment leads to clinical activity in metastatic bladder cancer. Nature. 2014;515(7528):558-62.

3. Park JC, Hahn NM. Emerging role of immunotherapy in urothelial carcinoma-Future directions and novel therapies. Urologic oncology. 2016;34(12):566-76.

4. Garcia-Closas M, Malats N, Silverman D, Dosemeci M, Kogevinas M, Hein DW, et al. NAT2 slow acetylation, GSTM1 null genotype, and risk of bladder cancer: results from the Spanish Bladder Cancer Study and meta-analyses. Lancet. 2005;366:649-59.

5. Rothman N, Garcia-Closas M, Chatterjee N, Malats N, Wu X, Figueroa JD, et al. A multi-stage genome-wide association study of bladder cancer identifies multiple susceptibility loci. Nature genetics. 2010;42(11):978-84.

6. Cancer Genome Atlas Research N. Comprehensive molecular characterization of urothelial bladder carcinoma. Nature. 2014;507(7492):315-22.

7. Carosella ED, Ploussard G, LeMaoult J, Desgrandchamps F. A Systematic Review of Immunotherapy in Urologic Cancer: Evolving Roles for Targeting of CTLA-4, PD-1/PD-L1, and HLA-G. European urology. 2015;68(2):267-79.

8. Xylinas E, Robinson BD, Kluth LA, Volkmer BG, Hautmann R, Kufer R, et al. Association of T-cell co-regulatory protein expression with clinical outcomes following radical cystectomy for urothelial carcinoma of the bladder. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology. 2014;40(1):121-7.

9. Bellmunt J, Mullane SA, Werner L, Fay AP, Callea M, Leow JJ, et al. Association of PD-L1 expression on tumor-infiltrating mononuclear cells and overall survival in patients with urothelial carcinoma. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2015;26(4):812-7.

10. Faraj SF, Munari E, Guner G, Taube J, Anders R, Hicks J, et al. Assessment of tumoral PD-L1 expression and intratumoral CD8+ T cells in urothelial carcinoma. Urology. 2015;85(3):703 e1-6.

11. Huang Y, Zhang SD, McCrudden C, Chan KW, Lin Y, Kwok HF. The prognostic significance of PD-L1 in bladder cancer. Oncology reports. 2015;33(6):3075-84.

12. Ribas A, Hu-Lieskovan S. What does PD-L1 positive or negative mean? The Journal of experimental medicine. 2016.

Acknowledgments

The authors thank the coordinators, field and administrative workers, technicians, secretaries, and study participants of the Spanish Bladder Cancer (SBC)/EPICURO study.

Grant Support

The project was funded partially by Fondo de Investigaciones Sanitarias (FIS, #PI00-0745, #PI05-1436, and #PI06-1614) and Red Temática de Investigación Cooperativa en Cáncer (RTICC, #RD12/0036/0050 and #RD12/0036/0034), Instituto de Salud Carlos III; and Asociación Española Contra el Cáncer (AECC), Spain; and EU-FP7-HEALTH-F2-2008-201663-UROMOL and EU-7FP-HEALTH-TransBioBC #601933. D. Silverman, N. Rothman, and S.J. Chanock received funding from the Intramural Research Program of the Division of Cancer Epidemiology and Genetics, National Cancer Institute (contract NCI NO2-CP-11015). A. Masson-Lecomte was awarded with a fellowship of the European Urological Scholarship Program for Research (EUSP Scholarship S-01-2013) and E.L. de Maturana with a Sara Borrell fellowship, Instituto de Salud Carlos III, Spain.

ANNEX 1. Spanish Bladder Cancer (SBC)/EPICURO Investigators

Institut Municipal d'Investigació Mèdica, Universitat Pompeu Fabra, Barcelona-CoordinatingCenter (M. Kogevinas, N. Malats, F.X. Real, M. Sala, G. Castaño, M. Torà, D. Puente, C. Villanueva, C. Murta-Nascimento, J. Fortuny, E. López, S. Hernández, R. Jaramillo, G. Vellalta, L. Palencia, F. Fernández, A. Amorós, A. Alfaro, G. Carretero); Hospital del Mar, Universitat Autònoma de Barcelona, Barcelona (J. Lloreta, S. Serrano, L. Ferrer, A. Gelabert, J. Carles, O. Bielsa, K. Villadiego), Hospital Germans Trias i Pujol, Badalona, Barcelona (L. Cecchini, J.M. Saladié, L. Ibarz); Hospital de Sant Boi, Sant Boi de Llobregat, Barcelona (M. Céspedes); Consorci Hospitalari Parc Taulí, Sabadell (C. Serra, D. García, J. Pujadas, R. Hernando, A. Cabezuelo, C. Abad, A. Prera, J. Prat); Centre Hospitalari i Cardiològic, Manresa, Barcelona (M. Domènech, J. Badal, J. Malet); Hospital Universitario de Canarias, La Laguna, Tenerife (R. García-Closas, J. Rodríguez de Vera, A.I. Martín); HospitalUniversitarioNuestra Señora de la Candelaria, Tenerife (J. Taño, F. Càceres); Hospital General Universitario de Elche, Universidad Miguel Hernàndez, Elche, Alicante (A. Carrato, F. García-López, M. Ull, A. Teruel, E. Andrada, A. Bustos, A. Castillejo, J.L. Soto); Universidad de Oviedo, Oviedo, Asturias (A. Tardo n); Hospital San Agustín, Avilés, Asturias (J.L. Guate, J.M. Lanzas, J. Velasco); Hospital Central Covadonga, Oviedo, Asturias (J.M. Fernández, J.J. Rodríguez, A. Herrero), Hospital Central General, Oviedo, Asturias (R. Abascal, C. Manzano, T. Miralles); Hospital de Cabueñes, Gijón, Asturias (M. Rivas, M. Arguelles); Hospital de Jove, Gijón, Asturias (M. Díaz, J. Sánchez, O. González); Hospital de Cruz Roja, Gijón, Asturias (A.Mateos, V. Frade); Hospital Alvarez-Buylla (Mieres, Asturias): P. Muntañola, C. Pravia; Hospital Jarrio, Coaña, Asturias (A.M. Huescar, F. Huergo); Hospital Carmen y Severo Ochoa, Cangas, Asturias (J. Mosquera).

Table 1: PDL1 variants association with time to progression (TP), cancer specific (CSS) and overall survival (OS) in MIBC using Cox regression additive model and SKAT model in SBC/EPICURO data. SNPs are presented according to their position in the genome. Benjamini and Hochberg adjusted p-value<0.05 were deemed significant. Comparable results were obtained using a codominant model. SNPs associated with the 3 outcomes are bold-faced.

Reorder according to the location. Modify format and homogenize. Scientific value.

			Risk of P1	rogression		Risk of U	BC death		Risk o	of death
SNP	MAF	HR	p.value	p.value.adjust	HR	p.value	p.value.adjust	HR	p.value	p.value.adjust
rs4742095	0.11	0.86	5.05E-01	6.01E-01	0.82	4.33E-01	6.37E-01	0.72	1.25E-01	2.09E-01
rs866066	0.44	0.73	1.42E-02	4.81E-02	0.75	3.94E-02	1.10E-01	0.83	9.84E-02	1.76E-01
rs822339	0.24	1.91	2.00E-05	1.10E-04	1.57	7.21E-03	3.61E-02	1.77	2.00E-05	1.50E-04
rs860290	0.24	1.91	2.00E-05	1.10E-04	1.57	7.21E-03	3.61E-02	1.77	2.00E-05	1.50E-04
rs4742097	0.46	0.76	3.09E-02	6.44E-02	0.77	6.83E-02	1.71E-01	0.84	1.42E-01	2.21E-01
rs2282055	0.24	1.91	2.00E-05	1.10E-04	1.57	7.21E-03	3.61E-02	1.77	2.00E-05	1.50E-04
rs7023227	0.4	0.73	1.54E-02	4.81E-02	0.73	3.22E-02	1.01E-01	0.80	6.23E-02	1.42E-01
rs17804441	0.27	0.83	1.99E-01	3.31E-01	0.97	8.64E-01	8.66E-01	0.77	5.47E-02	1.42E-01
rs10481593	0.18	1.46	2.49E-02	5.66E-02	1.30	1.68E-01	3.30E-01	1.48	8.08E-03	2.88E-02
rs1411262	0.24	1.89	2.00E-05	1.10E-04	1.64	2.68E-03	3.61E-02	1.74	2.00E-05	1.50E-04
rs7048841	0.48	0.72	1.02E-02	4.25E-02	0.71	1.79E-02	6.94E-02	0.80	5.78E-02	1.42E-01
rs10975123	0.18	1.46	2.49E-02	5.66E-02	1.30	1.68E-01	3.30E-01	1.48	8.08E-03	2.88E-02
rs1536926	0.49	0.74	2.19E-02	5.66E-02	0.71	1.94E-02	6.94E-02	0.80	5.88E-02	1.42E-01
rs10125854	0.06	2.19	1.70E-04	8.30E-04	2.01	4.15E-03	3.61E-02	1.90	1.57E-03	7.85E-03
rs2890658	0.1	0.82	3.96E-01	5.50E-01	0.69	1.76E-01	3.30E-01	0.69	9.70E-02	1.76E-01
rs2297137	0.21	1.42	5.87E-02	1.13E-01	1.07	7.58E-01	8.24E-01	1.32	8.20E-02	1.71E-01
rs2297136	0.46	0.90	4.29E-01	5.63E-01	0.85	2.48E-01	4.13E-01	0.89	3.44E-01	4.78E-01
rs4742100	0.23	1.03	8.76E-01	9.04E-01	1.07	7.08E-01	8.24E-01	0.99	9.30E-01	9.30E-01

rs7043593	0.24	1.03	8.76E-01	9.04E-01	1.07	7.08E-01	8.24E-01	0.99	9.30E-01	9.30E-01
rs10815236	0.31	1.19	2.48E-01	3.65E-01	0.95	7.41E-01	8.24E-01	1.06	6.64E-01	8.30E-01
rs11790653	0.1	1.03	9.04E-01	9.04E-01	0.70	1.85E-01	3.30E-01	0.94	7.74E-01	9.21E-01
rs10815237	0.35	1.17	2.26E-01	3.53E-01	1.15	3.32E-01	5.19E-01	1.01	9.24E-01	9.30E-01
rs1556188	0.45	1.20	1.88E-01	3.31E-01	1.03	8.66E-01	8.66E-01	0.98	8.37E-01	9.30E-01
rs10435744	0.13	1.04	8.43E-01	9.04E-01	1.10	6.31E-01	8.24E-01	0.84	3.35E-01	4.78E-01
rs10975138	0.22	1.14	4.51E-01	5.63E-01	1.07	7.15E-01	8.24E-01	1.13	4.17E-01	5.48E-01

MAF: Minor Allele Frequency; HR : Hazard Ratio ; TP models were adjusted for geographical area, age, TSG, nodal status and treatment; CSS and OS models were adjusted for geographical area, age, TSG, nodal status, treatment, and metastatic status.

Figure 1 : Kaplan Meier curves for progression free survival according to the rs860290 genotypes in muscle invasive bladder cancer patients from the Spanish Bladder Cancer (SBC)/EPICURO Study.



Progression free survival

INDEX
Table S1: Patient and tumour characteristics in the SBC/EPICURO and TCGA cohorts.
Table S2: Hazard ratios (HR) and <i>P</i> -values for the association between <i>PDL1</i> variants and Time to Recurrence and Time to Progression in non-
muscle invasive bladder cancer using Cox regression additive model in SBC/EPICURO data. Benjamini and Hochberg adjusted P-value<0.05
were deemed significant. Comparable results were obtained using a codominant model.
Table S3: eQTL of the studied SNPs with CD274/PDL1 and the neighbouring genes (PLGEKT and PDCD1LG2/PDL2). Data from GTEx
(http://www.gtexportal.org/home).
Table S4: PDL1 variants and gene results from the association analysis with time to progression and overall survival in the TCGA cohort using
Cox regression additive and SKAT models. Adjusted <i>P</i> -values<0.05 were deemed significant. The SNPs found significant in the EPICURO
study are bold-faced.
Figure S1: Kaplan Meier curves for the studied outcomes in non-muscle invasive bladder cancer (NMIBC, panels A and B) and muscle invasive
bladder cancer (MIBC, panels C, D, and E) patients from the Spanish Bladder Cancer (SBC)/EPICURO Study.
Figure S2: Kaplan Meier curves for the TCGA outcomes in muscle invasive bladder cancer (MIBC) patients (red) in comparison to those from
the Spanish Bladder Cancer (SBC)/EPICURO Study (blue).

Supplementary Tables and Figures

Figure S3: eQTL for tumour PDL1 expression and the variant rs860290 in tumour-blood concordant (A) and discordant (B) genotypes in the TCGA cohort. Genotypes for *PDL1*- rs860290 in blood and tumour are shown in the table. In red, discordant genotypes.

	SBC/EPICURO NMIBC N (%)	SBC/EPICURO MIBC N (%)	TCGA MIBC N (%)
Total	810	245	271
<u>Age</u> Median [IQR] Mean (SD)	68 [60-73] 65.5 (10.2)	68 [62-74] 66.8 (8.8)	70 [61-77] 69 (9.92)
<u>Gender</u> Male Female	712 (87.9) 98 (12.1)	218 (88.9) 27 (11.1)	201 (74.2) 70 (25.8)
<u>Stage</u> ^a T0 Ta	- 684 (84.4)		1 (0.4) -
T1 T2 T2	126 (15.6)	- 134 (54.7) 50 (22 2)	2 (0.7) 60 (22.1)
15 T4 NA		53 (23.7) 53 (21.6) -	1.50 (50.2) 43 (15.9) 29 (10.3)
<u>Nodal status</u> N0 Nx NA	810 (100) - -	166 (67.7) 50 (20.5) 29 (11.8) -	141 (52.0) 99 (36.5) 26 (9.6) 5 (1.8)
<u>Metastatic status</u> M0 M1 Mx NA	810 (100) - -	194 (79.2) 30 (12.2) 21 (8.6) -	102 (37.6) 7 (2.6) 160 (59.0) 2 (0.7)

Table S1. Patient and tumour characteristics in the SBC/EPICURO and TCGA cohorts.

		91 (49.2) 126 $(\overline{46.5})$	variables.
	117 (47.7) 64 (26.1) 40 (16.2)	129 (52.6) 112 (45.7) 169 (69.0)	data for the adjustment
352 (43.5) 446 (55.1) 12 (1.4)		268 (33.1) 74 (9.1)	s, as well as the missing
Treatment TURB alone TURB + intravesical treatment Other	Cystectomy Systemic chemotherapy Radiotherapy	Disease outcome Patients with tumour recurrence Patients with tumour progression Patients dead of bladder cancer Patients dead of any cause	a T0 and T1 were excluded from the analyse

muscle invasive bladder cancer using Cox regression additive model in SBC/EPICURO data. Benjamini and Hochberg adjusted *P*-value<0.05 were deemed significant. Comparable results were obtained using a codominant model. Table S2. Hazard ratios (HR) and *P*-values for the association between *PDLI* variants and Time to Recurrence and Time to Progression in non-

			Risk of UCH	3 Recurrence		Risk of UBC	C Progression
SNP	MAF	HR	p.value	p.value.adjust	HR	p.value	p.value.adjust
rs4742095	0.11	0.83	2.12E-01	6.62E-01	0.83	5.46E-01	8.03E-01
rs866066	0.44	1.21	2.52E-02	1.57E-01	1.32	9.85E-02	6.83E-01
rs822339	0.24	0.96	6.66E-01	7.93E-01	0.81	3.09E-01	6.83E-01
rs860290	0.24	0.96	6.66E-01	7.93E-01	0.81	3.09E-01	6.83E-01
rs4742097	0.46	1.26	6.92E-03	1.42E-01	1.26	1.73E-01	6.83E-01
rs2282055	0.24	0.94	5.65E-01	7.93E-01	0.84	3.90E-01	6.83E-01
rs7023227	0.40	1.15	1.12E-01	4.65E-01	1.16	3.74E-01	6.83E-01
rs17804441	0.27	0.82	4.50E-02	2.25E-01	0.99	9.70E-01	9.96E-01
rs10481593	0.18	0.95	6.31E-01	7.93E-01	0.97	8.75E-01	9.51E-01
rs1411262	0.24	0.95	6.31E-01	7.93E-01	0.77	1.99E-01	6.83E-01
rs7048841	0.48	1.23	1.70E-02	1.42E-01	1.24	2.17E-01	6.83E-01
rs10975123	0.18	0.95	6.30E-01	7.93E-01	0.97	8.72E-01	9.51E-01
rs1536926	0.49	1.23	1.65E-02	1.42E-01	1.21	2.67E-01	6.83E-01
rs10125854	90.0	1.01	9.64E-01	9.64E-01	0.59	1.54E-01	6.83E-01
rs2890658	0.10	0.94	6.57E-01	7.93E-01	1.08	7.87E-01	9.51E-01
rs2297137	0.21	0.96	7.04E-01	8.00E-01	1	9.96E-01	9.96E-01
rs2297136	0.46	1.09	3.20E-01	7.27E-01	0.95	7.34E-01	9.51E-01
rs4742100	0.23	1.12	2.68E-01	7.27E-01	1.05	8.09E-01	9.51E-01
rs7043593	0.24	1.09	3.99E-01	7.32E-01	1.13	5.43E-01	8.03E-01
rs10815236	0.31	1.08	4.01E-01	7.32E-01	1.3	1.32E-01	6.83E-01
rs11790653	0.10	0.88	4.10E-01	7.32E-01	0.9	7.46E-01	9.51E-01

rs10815237	0.35	1.00	9.62E-01	9.64E-01	1.18	3.46E-01	6.83E-01
rs1556188	0.45	0.99	9.14E-01	9.64E-01	1.15	4.01E-01	6.83E-01
rs10435744	0.13	0.82	1.39E-01	4.96E-01	0.81	4.10E-01	6.83E-01
rs10975138	0.22	1.11	3.00E-01	7.27E-01	1.48	4.97E-02	6.83E-01

MAF: Minor Allele Frequency; HR: Hazard Ratio; Models were adjusted for geographical area, age, gender, and TG.

Table S3. eQTL of the studied SNPs with CD274/PDL1 and the neighbouring genes (PLGEKT and PDCD1LG2/PDL2). Data from GTEx (http://www.gtexportal.org/home).

		Id	LGRKT			CD	\274/PDL1		1	DCD1LG2/PDL2
		[535796	56 - 54384	76]		[54505	503 - 5470567]		[]	510438 - 5571282]
rs_id	Position	Effect Size	P.value	Tissue	Effect Size	P.value	Tissue	Effect Size	P.value	Tissue
rs4742095	5445990	1	ı	1	0.32	2.3e-8	Whole Blood		ı	-
rs866066	5450953	1			-0.95	1.0e-21	Pancreas	1	1	-
					0.78	2.4e-10	Brain - Cerebellar Hemisphere			
					-0.20	1.9e-8	Whole Blood			
					0.65	1.1e-7	Brain - Cerebellum			
rs822339	5453172				1.0	2.0e-16	Brain - Cerebellar Hemisphere			-
					0.92	4.5e-10	Brain - Cerebellum			
rs860290	5453198				1.0	1.7e-16	Brain - Cerebellar Hemisphere			-
					0.93	4.2e-10	Brain - Cerebellum			
rs4742097	5455632				0.88	3.5e-18	Pancreas			
					-0.74	1.4e-9	Brain - Cerebellar Hemisphere			
					0.19	9.2e-8	Whole Blood			
					-0.64	4.1e-7	Brain - Cerebellum			
rs2282055	5455732				-1.0	1.6e-15	Brain - Cerebellar Hemisphere			-
					-0.88	1.8e-9	Brain - Cerebellum			
rs7023227	5456550	-	,		86.0	3.4e-21	Pancreas		,	-
					-0.73	2.9e-8	Brain - Cerebellum			
					-0.73	7.1e-8	Brain - Cerebellar Hemisphere			
					0.19	4.9e-7	Whole Blood			
					-0.21	2.6e-6	Muscle - Skeletal			
rs17804441	5457733	I	ı	ı	0.21	4.0e-7	Whole Blood		I	-
rs10481593	5458095	1	,		-0.98	9.1e-11	Brain - Cerebellar Hemisphere	1	1	-
					-0.86	3.4e-8	Brain - Cerebellum			

								Esophagus - Muscularis	Esophagus - Muscularis	Adipose - Visceral (Omentum) Phyroid Adipose - Subcutaneous Ssophagus - Muscularis Artery - Aorta Verve - Tibial		Verve - Tibial Sophagus - Muscularis Adipose - Visceral (Omentum) Ssophagus - Gastroesophageal Junction Artery - Coronary Adrenal Gland Adipose - Subcutaneous Artery - Tibial	Adipose – Visceral (Omentum) Esophagus - Muscularis		Adipose - Visceral (Omentum) Verve - Tibial 5sophagus - Muscularis Heart - Left Ventricle Thyroid
								3.20e-5 I	1.60e-5 1	5.4e-12 / 1.6e-8 7 8.3e-8 / 9.3e-7 1 3.7e-6 1 3.7e-6 1		5.0e-9 1 1.2e-8 1 2.0e-8 2 3.6e-7 1 1.2e-6 2 3.4e-6 2 8.1e-6 2 1.2e-5 2 1.2e-5 2	1.9e-6 / 4.8e-5 1		3.6e-12 / 8.9e-11 1 8.9e-11 1 2.2e-10 1 9.6e-10 1 9.6e-10 1 5.4e-9
I	1	1	1		1		-	0.41	0.44	0.63 0.42 0.35 0.47 0.40 0.39		0.48 0.54 0.56 0.67 0.52 0.52 0.28 0.28	0.44 0.37		0.73 0.60 0.69 0.61 0.48
Brain - Cerebellar Hemisphere Brain - Cerebellum Pancreas	Pancreas Brain - Cerebellar Hemisphere Whole Blood Brain - Cerebellum		Pancreas Brain - Cerebellar Hemisphere Brain - Cerebellum Whole Blood		Whole Blood	Brain - Cerebellar Hemisphere Brain - Cerebellum	Whole Blood Pancreas	-		Cells - Transformed fibroblasts Adipose - Subcutaneous	-	1	1	Lung	Cells – Transf. fibroblasts Adipose - Subcutaneous
6.9e-15 1.6e-8 2.9e-5	4.8e-17 1.0e-11 3.4e-8 8.5e-8		1.1e-17 1.1e-10 1.3e-7 1.8e-7		8.6e-10	5.1e-7 1.6e-6	1.9e-9 3.6e-6			3.4e-6 1.9e-6	1	1	1	9.90e-06	3.1e-7 6.4 e-5
-0.95 -0.77 0.53	0.85 -0.83 0.20 -0.66		0.89 -0.81 -0.67 0.19		0.36	-0.84 -0.74	0.21 0.50	1		0.23 0.31	1	1		-0.36	0.28 0.33
	1	,		,		,	ı	,		1	,	,	ı	ı	
	1	,		,		1	ı	,		1		,		,	
						1	1			1					1
5459419	5460801	5461379	5463988	5464065	5465130	5465732	5467955	5470954	5479822	5483659	5484418	5485166	5486106	5486856	5489303
rs1411262	rs7048841	rs10975123	rs1536926	rs10125854	rs2890658	rs2297137	rs2297136	rs4742100	rs7043593	rs10815236	rs11790653	rs10815237	rs1556188	rs10435744	rs10975138

Adipose - Subcutaneous	Artery - Aorta	Esophagus - Gastroesoph Junc	Artery - Coronary	Colon - Sigmoid	Muscle - Skeletal
6.4e-8	6.4e-7	6.7e-7	1.3e-6	1.6e-5	2.4e-5
0.40	0.46	0.65	0.56	0.52	0.31

Table S4. *PDL1* variants and gene results from the association analysis with time to progression and overall survival in the TCGA cohort using Cox regression additive and SKAT models. Adjusted *P*-values<0.05 were deemed significant. The SNPs found significant in the EPICURO study are bold-faced.

		Risk of progression			Risk of death		
SNP	MAF	HR	p.value	p.value.adjust	HR	p.value	p.value.adjust
rs7043593	0.23	1.65		8.00E-02	1.36		4.00E-01
rs10815236	0.33	0.85		4.90E-01	0.92		4.90E-01
rs4742095	0.11	1.16		5.00E-01	1.13		3.20E-01
rs860290	0.27	1.09		5.40E-01	1.10		3.90E-01
rs7023227	0.42	1.10		7.20E-01	1.03		9.20E-01
rs17804441	0.22	1.00		8.50E-01	0.93		7.40E-01
rs10481593	0.22	1.17		5.70E-01	1.19		6.10E-01
rs1411262	0.29	1.05		6.90E-01	1.07		5.70E-01
rs2890658	0.42	1.18		4.30E-01	1.15		5.00E-01
rs2297137	0.22	0.74		2.00E-01	0.81		3.00E-01
rs4742100	0.23	1.66		7.00E-02	1.36		3.90E-01
PDL1 gene				2.80E-01			7.30E-01

MAF: Minor Allele Frequency; HR: Hazard Ratio; TP models were adjusted for age at diagnosis. nodal status and tumour stage; OS models were adjusted for age at diagnosis. nodal status, tumour stage, and metastatic status.

Figure S1. Kaplan Meier curves for the studied outcomes in non-muscle invasive bladder cancer (NMIBC, panels A and B) and muscle invasive bladder cancer (MIBC, panels C, D, and E) patients from the Spanish Bladder Cancer (SBC)/EPICURO Study.



A. Time to recurrence in NMIBC

Time to recurrence (months) SBC/EPICURO







D. Cancer specific survival in MIBC





Figure S2. Kaplan Meier curves for the TCGA outcomes in muscle invasive bladder cancer (MIBC) patients (red) in comparison to those from the Spanish Bladder Cancer (SBC)/EPICURO Study (blue). Same scale than Epicro



A. Time to progression





Overall survival time (months)

Figure S3. eQTL for tumour PDL1 expression and the variant rs860290 in tumour-blood concordant (A) and discordant (B) genotypes in the TCGA cohort. Genotypes for *PDL1*-rs860290 in blood and tumour are shown in the table. In red, discordant genotypes.



Supplementary Material and Methods

Spanish Bladder Cancer (SBC)/EPICURO study. Germline DNA extracted from blood or saliva, was used for genotyping. SNPs were genotyped with the GoldenGate Illumina Genotyping Assay (San Diego, CA, USA) platform. We excluded SNPs with a low genotyping rate (<95%) and minor allele frequency (MAF) <0.05. Missing genotypes were imputed with BEAGLE.(1) To reduce the number of statistical tests, pairwise linkage disequilibrium between SNPs was estimated using the *R*-package GENETICS (http://cran.rproject.org/web/packages/genetics/index.html). We retained the SNPs with the highest MAF of each pair when $r^2 > 0.5$. At the end of the quality control process, 810 non-muscle invasive bladder cancer (NMIBC) and 245 muscle invasive bladder cancer (MIBC) patients with genotypes for 25 SNPs tagging PDL1 gene and complete clinical and pathological information were available for the analysis. These patients were comparable to the whole series for age, gender, area, and tumor stage and grade. Cox proportional hazard regression (additive and co dominant modes of inheritance) was used to estimate the hazard ratio (HR) and 95% confidence intervals (95%CI) to assess the association between individual SNPs and NMIBC outcomes (time to tumour recurrence - time elapsed between TURB and tumour reappearance as NMIBC - and progression - time elapsed between TURB and tumour reappearance as MIBC), and MIBC outcomes (time to tumour progression - time elapsed between first MIBC treatment and occurrence of a new tumour event - and time to cancerspecific and overall mortality). Each SNP effect was adjusted for classical clinicopathological prognosticators (Table S1). For time to progression (TP) outcome, models were adjusted for "geographical area" (Barcelona, Vallès, Elche, Tenerife, and Asturias), "age" (continuous), "TSG" (T2, T3, and T4), "nodal status" (N0, N+, and Nx) and "treatment" (cystectomy, radiochemotherapy, and others including palliative or non reference treatment). Cancer specific survival (CSS) and overall survival (OS) models also included "metastatic status" (M0, M1, Mx). Analyses were run in R (<u>http://www.R-project.org</u>). Correction for multiple testing was performed using the Benjamini & Hochberg method. SNPs with *p*-values <0.05, two-sided test, after multiple testing correction were deemed significant.

The Cancer Genome Atlas (TCGA) MIBC population was used for replicative purposes (N=229) (2). TCGA cohort was also used to assess the association between the SNPs and gene expression, eQTL (Gene Expression (PDL1) = $\alpha + \beta$ *SNP (tumor), N=119). Linear regression model were used to assess significance.

Gene-based analysis. The association of *PDL1* at the gene level was assessed in both cohorts using the sequence kernel association test (SKAT) under flat weights in a survival setting [1]. Briefly, this approach tests for association between variants in a region while adjusting for covariates. The *p*-value for the gene-based association was calculated using the skatMeta R package (https://cran.r-project.org/web/packages/skatMeta/index.html).

References

 Browning SR, Browning BL. Rapid and accurate haplotype phasing and missing-data inference for whole-genome association studies by use of localized haplotype clustering. Am J Hum Genet. 2007;81(5):1084-97.

2. Cancer Genome Atlas Research N. Comprehensive molecular characterization of urothelial bladder carcinoma. Nature. 2014;507(7492):315-22.

ARTICLE 3: CD8+ CYTOTOXIC INFLAMMATORY INFILTRATE IN NON-MUSCLE INVASIVE BLADDER CANCER: A STANDARDIZED METHODOLOGY TO STUDY ASSOCIATION WITH PROGNOSIS AND CLINICO-PATHOLOGICAL FEATURES.

1.1. Abstract

Introduction and Objectives: Major interest lies in the evaluation of inflammatory infiltrate in bladder cancer. CD8+ cytotoxic lymphocytes are key effectors of adaptive immune response. The aims of the study were to set up a standardized methodology for CD8+ lymphocytes estimation in bladder tumors, investigate how intra-tumoral heterogeneity influences CD8+ immune infiltrate assessment and analyze the association between CD8+ lymphocytes and both urothelial differentiation markers and prognosis of NMIBC.

Materials and methods: We considered 995 NMIBC included in the Spanish Bladder Cancer (SBC)/EPICURO Study. Duplicate 0.6 mm TMA spots (all cases) and paired full sections (FS) for 50 selected cases were double stained with anti-pan cytokeratine AE1-AE3 antibody cocktail and anti-CD8 antibody. Slides were digitalized and CalopixTM software (Tribvn, France) was used for tissue recognition and automated CD8+ cells counting. Spatial heterogeneity was assessed and a resampling strategy was applied to estimate the proper number of 0.6 mm TMA spots providing an adequate CD8+ cell estimate in comparison with full sections. Association between CD8+ count and protein expression of urothelial differentiation markers was performed as well as evaluation of association between CD8+ count and recurrence and progression of NMIBC using adjusted Cox regression models.

Results: CD8+ cells were automatically identified and counted within each histological compartment separated by cytokeratin staining (tumor and stroma). Microscopic examination of full sections showed spatial heterogeneity for CD8+ infiltrates. Simulation analyses
demonstrated that 5 TMA regions provided a correct sampling of both tumor and stromal compartments in stage Ta while 2 and 6 TMA regions were necessary for tumor and stroma assessment in stage T1. CD8+ count was associated with both stage and histological compartment (median CD8+/mm² in tumor and stromal compartment were $25/mm^2$ and $129/mm^2$ in Ta and $111/mm^2$ and $344/mm^2$ in T1; *p*-value =0.006 for comparison between Ta and T1). CD8+ infiltration in tumor compartment was significantly associated with FGFR3 expression status. CD8+/mm² count in the tumor compartment was significantly associated with risk of recurrence in T1 (HR=0.39, 95%CI 0.15-0.99, *p*-value=0.04).

Conclusion: We propose a standardized methodological pipe-line for CD8+ cell assessment in non-muscle invasive bladder cancer which emphasizes thespatial heterogeneity in tumor and stromal compartments. These results provide a new framework to investigate microenvironment complexity in bladder cancer.

1.2. Manuscript

CD8+ CYTOTOXIC INFLAMMATORY INFILTRATE IN NON-MUSCLE INVASIVE BLADDER CANCER: A STANDARDIZED METHODOLOGY TO STUDY ASSOCIATION WITH PROGNOSIS AND CLINICO-PATHOLOGICAL FEATURES

Alexandra Masson-Lecomte (1,2,3), Pascale Maillé (1, 4), Silvia Pineda (3), Pascale Soyeux
(1), Ana Sagrera (5), Marta Rava (3), Evangelina Lopez de Maturana (3), Mirari Márquez
(3), Adonina Tardón (6), Alfredo Carrato (7), Manolis Kogevinas (8), Alexandre de la Taille
(1,9), Arndt Hartmann (10), Paco Real (5), Núria Malats (3), Yves Allory (1, 4)

 Translational research in uro-genital carcinogenesis, INSERMU955 team 7, IMRB, Créteil, France

(2) Urology Department, CHU Saint Louis, Paris, France

(3) Genetic and Molecular Epidemiology Group, Spanish National Cancer Research Centre (CNIO), Madrid, and CIBERONC, Spain.

(4) Pathology Department, Henri Mondor Hospital, Créteil, France

(5) Epithelial Carcinogenesis Group, Spanish National Cancer Research Centre (CNIO), Madrid, and CIBERONC, Spain.

(6) Preventive Medicine and Public Health, University of Oviedo, and CIBERESP, Spain.

(7) Department of Oncology, Hospital Ramon y Cajal, Madrid, and CIBERONC, Spain.

(8) Centre de Recerca en Epidemiologia Ambiental (CREAL), Barcelona, and CIBERESP,Spain.

Alexandra Masson-Lecomte

(9) Urology Department, CHU Henri Mondor, Creteil, France

(10) Pathology Department, Universitätsklinikum, Erlangen, Germany

Key words: urothelial carcinoma, CD8, methodology, prognosis, urothelial differentiation.

Abstract

Introduction and Objectives: Major interest lies in the evaluation of inflammatory infiltrate in bladder cancer. CD8+ cytotoxic lymphocytes are key effectors of adaptive immune response. The aims of the study were to set up a standardized methodology for CD8+ lymphocytes estimation in bladder tumors, investigate how intra-tumoral heterogeneity influences CD8+ immune infiltrate assessment and analyze the association between CD8+ lymphocytes and both urothelial differentiation markers and prognosis of NMIBC.

Materials and methods: We considered 995 NMIBC included in the Spanish Bladder Cancer (SBC)/EPICURO Study. Duplicate 0.6 mm TMA spots (all cases) and paired full sections (FS) for 50 selected cases were double stained with anti-pan cytokeratine AE1-AE3 antibody cocktail and anti-CD8 antibody. Slides were digitalized and CalopixTM software (Tribvn, France) was used for tissue recognition and automated CD8+ cells counting. Spatial heterogeneity was assessed and a resampling strategy was applied to estimate the proper number of 0.6 mm TMA spots providing an adequate CD8+ cell estimate in comparison with full sections. Association between CD8+ count and protein expression of urothelial differentiation markers was performed as well as evaluation of association between CD8+ count and recurrence and progression of NMIBC using adjusted Cox regression models.

Results: CD8+ cells were automatically identified and counted within each histological compartment separated by cytokeratin staining (tumor and stroma). Microscopic examination of full sections showed spatial heterogeneity for CD8+ infiltrates. Simulation analyses demonstrated that 5 TMA regions provided a correct sampling of both tumor and stromal compartments in stage Ta while 2 and 6 TMA regions were necessary for tumor and stroma assessment in stage T1. CD8+ count was associated with both stage and histological compartment (median CD8+/mm² in tumor and stromal compartment were 25/mm² and 129/mm² in Ta and 111/mm² and 344/mm² in T1; *p*-value =0.006 for comparison between Ta and T1). CD8+ infiltration in tumor compartment was significantly associated with FGFR3 expression status. CD8+/mm² count in the tumor compartment was significantly associated with risk of recurrence in T1 (HR=0.39, 95%CI 0.15-0.99, *p*-value=0.04).

Conclusion: We propose a standardized methodological pipe-line for CD8+ cell assessment in non-muscle invasive bladder cancer which emphasizes the spatial heterogeneity in tumor and stromal compartments. These results provide a new framework to investigate microenvironment complexity in bladder cancer.

INTRODUCTION

Recent successes in the usage of immune checkpoints inhibitors for cancer treatment have confirmed that restoration of antitumor immunity is a powerful anticancer strategy [87]. A key effector of adaptive cellular immune response are CD8+ lymphocytes that have the ability to exert cytotoxicity through different pathways [195, 196]. Consequently, major interest has emerged in assessing CD8+ inflammatory cells.Numerous studies in different cancer types have shown association between CD8+ tumor infiltration and cancer prognosis [197]. In bladder cancer, characterization of immune microenvironment and assessment of association with prognosis has initially been done independently from molecular subtyping. Sharma and colleagues investigated in 2007 the prognosis of 69 MIBC and demonstrated that patients with advanced tumors and strong CD8+ tumor infiltration had better prognosis than their "low-infiltrated" counterparts [123]. Other authors studied association between CD8+ cells and prognosis in both MIBC and non-muscle invasive bladder cancers (NMIBC) with mitigated results [131]. Meanwhile, independent groups have described a molecular taxonomy of muscle invasive bladder cancer (MIBC) [65, 66, 68]. Subgroups of MIBC have been identified and validated, with variable expression of urothelial differentiation markers but also variable association with inflammatory cells infiltration (CD3, CD8, CD68) [72]. These observations suggest possible associations between molecular subtyping and immune response triggering [73]. While the molecular taxonomy of NMIBC is much more heterogeneous, some analogies with MIBC have already been demonstrated [198, 199], enhancing the interest in assessing the association between molecular subtyping and inflammatory response also in NMIBC.

More recently, Patschan *et al.* demonstrated that usage of both molecular subtyping and T cells immune-histochemical markers provided finer prognosis classification than the use of molecular subtypes alone [199]. However, proper evaluation of inflammatory infiltrate is challenging. Methodological drawbacks limit the applicability of the study results and consensus is needed about how to best consider the inflammatory response as a component of tumor sub-classification [131]. For instance, crucial questions such as the relevance of tumor micro arrays (TMA) for microenvironment assessment, the importance of normalization, and the potential variations between tumor and stromal infiltration have not been investigated. Due to the strong molecular heterogeneity of NMIBC and disrupted architecture after to trans urethral resection of the bladder (TURB), those challenges are even more considerable in those tumors than in MIBC.

The aims of the study were to set up a standardized methodology for CD8+ lymphocytes estimation in bladder tumors, to investigate how intra-tumoral heterogeneity influences CD8 immune infiltrate assessment, and to explore the association of CD8+ lymphocytes with both urothelial differentiation markers and prognosis of NMIBC.

MATERIAL AND METHODS

Ethics statement

Informed consent was obtained from study participants in accordance with the Institutional Review Board of the U.S. National Cancer Institute and the Ethics Committees of each participating hospital.

Study population and tissue samples

Population and samples

We primarily considered 995 newly diagnosed patients with urothelial NMIBC included in the Spanish Bladder Cancer (SBC)/EPICURO Study, a multicentre hospital-based study conducted between 1997-2001 in 18 hospitals [10]. Tumors were reviewed and confirmed by trained uropathologists who classified their stage and grade homogeneously using TNM 1997 AJCC, and 1973 WHO 3-tier-grade-classifications. Clinical data and information on primary treatment was retrieved from the hospital charts by trained monitors using a structured questionnaire. Patients were followed up for >10 years both using the hospital charts and through direct telephone calls to patients/families. The follow-up rate for NMIBC patients was 94%. One FFPE block representative of each tumor was selected. It was used for TMA building using two 0.6 mm cores. Each TMA was representative of one stage and grade. Two cores from the same patient were geometrically opposed on the slide. For 50 cases representative of each tumor stages and grades (8 TaG1, 7 TaG2, 4 TaG3, 4 T1G2 and 25 T1G3), the same initial block was used for full section study.

Immunohistochemistry and automated morphometry

Duplicate 0.6 mm TMA spots (all cases) and paired full sections (FS) for the 50 selected cases were double stained with anti-pan cytokeratine AE1-AE3 antibody cocktail to identify tumor cells and anti-CD8 antibody to identify cytotoxic T-cells. Double staining was performed using a Bond automated immunohistochemistry stainer from Leica Biosystems[™], with monoclonal mouse anti- human CD8 antibody (clone C8/144B, 1/200, DAKO), and monoclonal mouse anti-human CKAE1/AE3 (clone AE1/AE3, 1/500 DAKO). Dewaxing, antigen retrieval and primary antibody dilution were performed using Bond – Dewax solution (ref. AR9222, Leica biosystem). Bond – Epitope retrieval solution (ref. AR9961, Leica biosystem) and Bond – Primary antibody diluent (Leica biosystem). Counter

coloration was performed using Haematoxylin to visualize cells nucleus. Bluing reagent was incubated for 4 minutes. TMA sections were also stained for urothelial differentiation markers including GATA3 (mouse monoclonal Ab L50-823, BiocareMedical), FGFR3 and CK5/6 (mouse monoclonal Ab D5/16B4, Dako).

All slides were digitalized using Aperio scanscope XT (Leica). CalopixTM software (Tribvn, France) was used for tissue recognition and automated CD8+ cells counting. Non suitable areas with holes were retrieved automatically, tumor cell and stroma areas were identified differentially using the AE1AE3 staining. CD8+ cells were identified using a combination of colour, shape and size recognition, and cell density was computed as number of CD8+ cells/mm2 in tumor or stroma compartment respectively. For comparison purpose, CD8+ cells counting was also performed through human eye examination for all TMA cases with at least one spot available. GATA3 nuclear staining was determined automatically using Calopix software, FGFR3 and CK5/6 were assessed manually. The Quick Score (percentage stained cells x intensity ranging from 0 to 3) was computed for these 3 differentiation markers.

Statistical analyses

Spearman correlation was computed between manual and automated counting. CD8+ count was computed as the mean value of 2 spots on TMA when 2 spots where available or absolute value of the unique available spot when only one spot was remaining after staining. On full section, CD8+ count was computed as the mean value for all the respective areas considered. A resampling strategy was applied to estimate the proper number of 0.6 mm TMA spots providing an adequate CD8+ cell estimate in comparison with full sections, as described before [200]. Briefly, number of CD8 was counted in up to 10 randomly selected

regions in full sections. Area of the regions was similar to the area of a 0.6 mmm TMA spot (0.28 mm²). 500 bootstrapped samples were generated to simulate estimation of CD8+ infiltrate according to the number of regions selected. The Inter Quartile Ranges (IQR) of the mean CD8 count according to the number of regions analyzed were compared with the reference value obtained for 10 regions using Wilcoxon test. Numbers of CD8+/mm2 were compared according to tumor stages (Ta/T1) and histological compartment (tumor/stroma) using Wilcoxon test and correlation between histological compartments was assessed through Spearman coefficient.

Association between CD8 count and protein expression of urothelial differentiation markers was performed using Kruskall-Wallis and logistic regression tests. Time to first recurrence (TFR) was defined as time elapsed between first TURB and histological diagnosis of a new NMIBC of any stage/grade. Time to progression (TP) was defined as time between first TURB and a subsequent histological diagnosis of a MIBC, occurrence of metastasis, or death due to bladder cancer. Survival curves were modeled using Kaplan Meier method. Log rank test was performed to assess univariate differences between survivals. Cox proportional hazard regression was used to estimate the hazard ratio (HR) and 95% confidence intervals (95%CI) of the association between CD8 count and the outcomes of interest. Cox regression models were adjusted for classical clinico-pathological prognosticators for TFR and TP (Table S1). All analyses were run in R-language (http://www.R-project.org).

RESULTS

Standardized methodology for CD8+ lymphocytes estimation in bladder tumors

An automated morphometric approach was set up to facilitate the CD8+ cell counting allowing distinction between lymphocytes in contact with tumor cells or scattered within stroma. The commercial CalopixTM software was used for tumor/stroma tracking using colour recognition provided by tumor cell cytokeratin staining. The full tissue compartment (tumor + stroma) was delimited first (**Figure 1A**) with background suppressed, and then the tumor compartment was delimited from the stroma compartment (**Figure 1B**). CD8+ cells were then automatically identified and counted within each histological compartment (tumor and stroma) (**Figure 1C**). The surface of the tumor and stromal compartments were provided along with the CD8+ count, allowing further normalization to mm^2 . Spearman correlation coefficient between manual and automated counting was 0.88 validating the use of the software.

Influence of tumor heterogeneity on CD8+ immune infiltrate assessment

Microscopic examination of full sections showed spatial heterogeneity for CD8+ infiltrates questioning the use of TMA for CD8+ cells number assessment. Consequently, simulations were performed using bootstrapping to estimate the proper number of 0.6 mm TMA cores necessary for accurate CD8+ estimate, considering separately Ta and T1 tumors. Ten areas were randomly selected on full sections, each one mimicking the area of a 0.6 mm TMA spot (**Figure 2**). CD8+ count / mm2 was estimated in each area. Using a 500 bootstrapping (resampling) strategy, a matrix simulating the CD8+ count according to the number of regions selected was constructed for each tumor (each row corresponding to a resampling strategy had been applied to each of the tumors, a second matrix was constructed for the full tumor cohort calculating the interquartile range of CD8+ count according to the number of areas selected). IQR was used as the best statistical value to estimate heterogeneity in CD8+ count.

Visualization of the IQR of CD8+ count in the tumor compartment was performed using boxplots (**Figure 2**) and comparison of IQR distribution using Wilcoxon test between different number of regions selected (**Table 1**) demonstrated that 5 regions provided a precise sampling in Ta while only 2 regions were enough in T1. The same procedure was performed to assess heterogeneity in the stoma compartment (**Table 1** and **Figure S2**). In Ta tumors, stroma heterogeneity was comparable to the one observed in the tumor compartment with 5 regions necessary to obtain a precise sampling. In T1, the heterogeneity observed in the stroma compartment was much higher than the one in the tumor compartment with 6 regions necessary for precise sampling (**Table 1**).

Moreover, examination of full sections demonstrated association both "stage-related" and "histological compartment-related" in CD8 count. We did not observe any invasive front effect at the invasive border of the tumors. Mean and median numbers of CD8+/mm² in tumor compartment were respectively $64/mm^2$ and $25/mm^2$ in Ta and $175/mm^2$ and $111/mm^2$ in T1 (*p*-value =0.006). In Ta mean and median number of CD8/mm² in stroma were 226 and $129/mm^2$ while mean and median number of CD8/mm² in stroma in T1 were 513 and $344/mm^2$. Correlation coefficients between tumor and stroma CD8 count were 0.51 in Ta and 0.62 in T1 (**Figure 3**).

Overall, TMA cores were available for analyses for 586 patients (467 Ta, 115 T1 and 4 Tis). Patients' characteristics are presented in **Table 2**. No significant differences were observed between those patients and the full SBC/EPICURO series except for grade (p= 0.0018, **Supplementary Table 1**). As observed on full section, the CD8+/mm² count in tumor compartment was significantly higher in T1 vs. Ta tumors (mean CD8/mm² = 174/mm² in T1 vs 61/mm² in Ta, *p*-value <0.0001, median CD8/mm² = 43/mm² in T1 vs 18/mm² in Ta). Correlation coefficient between paired TMA and full section CD8+ count were 0.26 in Ta

tumors vs 0.68 in T1 tumors, corroborating the inaccuracy of TMA for CD8+ cells count in Ta.

Association between CD8+infiltrate and urothelial differentiation markers

Distribution of FGFR3, GATA3 and CK5/6 expressions in the NMIBC series determined on TMA sections were plotted through histograms presented in **Supplementary Figure 1**. No association was found between CD8+ count (assessed on the same tissue cores included in the TMA blocks) and GATA3 or CK5/6 expression, but CD8+ infiltration in tumor compartment was significantly associated with FGFR3 status with stronger infiltration seen in patients with low FGFR3 expression in tumor (**Figure 4**). Logistic regression adjusted for stage demonstrated that the association between CD8+ count and FGFR3 expression was dependent from stage (*p*-value = 0.28).

Association between TMA CD8+ count and prognosis in T1 NMIBC

Based on results from the methodological part, association between CD8+ count in the tumor compartment and prognosis was assessed using TMAs in T1 tumors (N=115). Out of 115 T1 patients, 48 had only one spot available for analyses. Up to July 2007, mean follow-up period for patients "free of disease" was 82.7 months (2.5 - 117.6 months). According to the abovementioned definitions, 55 (47.8%) patients suffered, at least, one event in the T1 cohort (23 recurrences and 32 progressions). Number of CD8+/mm² was categorized in 2 groups according to the median value. Adjusted Cox regression analysis results for the full T1 cohort are presented in Table 2. Results for the reduced cohort are presented in Table S2. CD8+/mm² count in the tumor compartment was significantly associated with risk of

recurrence (HR=0.39, 95%CI 0.15-0.99, *p*-value=0.04, Figure 7). No association was found with risk of progression (HR=0.8, 95%CI 0.40-1.94, *p*-value= 0.76). Analyses were also performed adjusting for FGFR3 status (**Table S3**). FGFR3 IHC results were available for 75 out of 115 T1 patients. Adjustment for FGFR3 did not change the trend of the results although not significant due to reduced number of events in this cohort.

DISCUSSION

Activating anti-tumor immunity using attenuated BCG has been the baseline of high risk NMIBC treatment for decades [80]. With the approval of anti PDL1 therapies, metastatic MIBC treatment is being re-designed with possible usage of those drugs at earlier stages of the disease. Evading immune response is one of the hallmarks of cancer [32] and anti PD1/PDL1 therapies have the power of reactivating CD8+ T cells mediated anti-tumor activity with excellent response [201]. Consequently, assessing both the extent and the type of inflammatory infiltrate in tumors could have major prognosis implications.

This study is the first to describe a standardized methodological process for tumor- or stroma associated CD8+ cytotoxic lymphocytes evaluation. Using tissue recognition approaches based on tumor cell pan-cytokeratin immunostaining and automated counting, we were able to normalize results to mm^2 of tumor in TMA cores enabling easiest replication/validation. While the usage of TMAs allows sparing both time, resources and samples when studying large cohorts, no study ever demonstrated the applicability of TMA to inflammatory infiltrate assessment. Resampling strategies allowed the identification of spatial heterogeneity in Ta tumors demonstrating the inaccuracy of CD8+ cells infiltration assessment in Ta compared to T1 tumors using classical "full section" methods. Consequently, correlation between CD8+ count in the tumor compartment on full section and TMA in Ta was very low (r=0.26). We also demonstrated a moderate correlation between tumor and stroma CD8+ count on full

sections both in Ta and T1 tumors indicating that tumor CD8+ count stromal CD8+ count have to be performed separately.. Overall, it results that spatial heterogeneity in NMIBC and a potential core selection bias have to be considered when assessing inflammatory infiltrate on TMAs. While recommendation of systematic usage of full section is probably excessive, TMAs have to be designed keeping tissue heterogeneity in mind by increasing the number and the size of the spots. Tumor and stromal compartments should be assessed independently using full section or TMAs designed for micro environmental studies.

Many studies have already been published suggesting associations between CD3, CD8 and CD68 positive cells and bladder cancer prognosis [123, 139, 142, 202, 203]. Using a strong methodology we replicated some published results regarding CD8+ infiltrate. We confirmed previously observed association between CD8+ infiltration and stage. Pichler *et al.* recently described a highest CD3+, CD8+ and CD68+ T cells count in T1 tumors compared to Ta [204]. Area in which CD8+ cells were counted was unclear in their study (per high power fields/per tumor region) emphasizing the absolute need for standardization. The associations between tumor stage and intensity of infiltrate explains some of the prognosis associations found only in univariate analyses. Both Sharma *et al.* and Kitamura *et al.* found strong univariate association between CD8+ T cells infiltrate and prognosis of NMIBC (respectively overall survival and recurrence free survival)[121, 123]. Associations where not identified anymore after adjustment for stage.

Regarding prognosis, we herein report for the first time association between CD8+ infiltrate and NMIBC risk of recurrence. Faraj *et al.* described association between both cancerspecific and overall survival and CD8+ infiltrate in 56 cystectomy specimen [192]. Based on results from the methodological part, we only used TMAs for prognosis evaluation in T1. We demonstrated independent association between high infiltration of CD8+ cells and risk of recurrence. Those results need to be interpreted cautiously because of limited sample size and contradictory results published recently [204]. Moreover, considering only one cell subpopulation is clearly insufficient to get a complete insight into the processes of immune response. As suggested elsewhere, it is likely that the ratio between effector cells (CD8+), regulatory cells (FOXP3+) and tumor associated macrophages (CD68+) displays the better prognosis information [72, 204, 205]. Those three populations in the context of PD1/PDL1 expression all need to be assessed together using a well-defined methodology as described herein.

Finally we assessed association between CD8 infiltrate and urothelial differentiation markers. Stage and histological compartment heterogeneity did not impact potential association since they were measured in the same tissue core and tumor compartment than CD8+ cells. Several studies recently described molecular subgroups of MIBC, each defined by different biomarkers and potential actionable targets [70]. Although those subgroups have been described using RNA expression profiles, they are associated with different marker expression patterns in immunohistochemistry (« luminal TCGA I » presenting FGFR3 activation, «luminal TCGA II » strongly expressing urothelial differentiation markers as GATA3 or FOXA1, «basal TCGA III » showing strong expression of CK5/6 and the « infiltrated, TCGA IV» showing very strong inflammatory infiltrate and expressing genes implicated in immune response). Patchan et al. recently described some of the MIBC subgroups in a series of T1 NMIBC [199]. They observed on a series of 167 T1 tumors that CD3+ cells count was higher in Genomically unstable (TCGA II) or Squamous Cell Carcinoma Like (Basal, TCGA III) tumors. Still, its association with CD8+ cells infiltration was not explored. In our study, molecular sub-classification based on RNA expression was not available. Based on immunostaining as surrogate for molecular subgroups, we only identified reverse association between CD8 infiltration and FGFR3 expression. This association is dependent from stage as Ta tumors display a higher amount of FGFR3

mutations, but it is consistent with results published for FGFR3 activated MIBC that were reported to be not T cell inflamed in comparison with other molecular subtypes [74].

CONCLUSION

This study is the first to describe a robust and standardized methodological pipe-line for CD8+ cell assessment in bladder cancer. Differences identified between Ta and T1 tumors comforted the hypothesis that rigorous efforts should be placed in proper study design. The association identified between CD8+ infiltrate and both stage and risk of recurrence of T1 supports the usefulness of inflammatory biomarkers in the management of patients, both for improved assessment of prognosis and to guide therapy. Researchers are only at the dawn of molecular subtyping of NMIBC and even though no clear association was identified between urothelial differentiation markers and CD8 infiltrate, integrated approaches need to be promoted to get a comprehensive insight into the biology of urothelial carcinomas.

REFERENCES

1. Powles T, Eder JP, Fine GD et al. MPDL3280A (anti-PD-L1) treatment leads to clinical activity in metastatic bladder cancer. Nature 2014; 515: 558-562.

2. Antony PA, Piccirillo CA, Akpinarli A et al. CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells. J Immunol 2005; 174: 2591-2601.

3. Dobrzanski MJ, Reome JB, Dutton RW. Type 1 and type 2 CD8+ effector T cell subpopulations promote long-term tumor immunity and protection to progressively growing tumor. J Immunol 2000; 164: 916-925.

4. Cancer Genome Atlas Research N. Comprehensive molecular characterization of urothelial bladder carcinoma. Nature 2014; 507: 315-322.

5. Choi W, Porten S, Kim S et al. Identification of distinct basal and luminal subtypes of muscle-invasive bladder cancer with different sensitivities to frontline chemotherapy. Cancer Cell 2014; 25: 152-165.

6. Sjodahl G, Lauss M, Lovgren K et al. A molecular taxonomy for urothelial carcinoma. Clin Cancer Res 2012; 18: 3377-3386.

7. Sjodahl G, Lovgren K, Lauss M et al. Infiltration of CD3(+) and CD68(+) cells in bladder cancer is subtype specific and affects the outcome of patients with muscle-invasive tumors. Urol Oncol 2014; 32: 791-797.

8. Rosenberg JE, Hoffman-Censits J, Powles T et al. Atezolizumab in patients with locally advanced and metastatic urothelial carcinoma who have progressed following treatment with platinum-based chemotherapy: a single-arm, multicentre, phase 2 trial. Lancet 2016; 387: 1909-1920.

9. Hedegaard J, Lamy P, Nordentoft I et al. Comprehensive Transcriptional Analysis of Early-Stage Urothelial Carcinoma. Cancer Cell 2016; 30: 27-42.

10. Patschan O, Sjodahl G, Chebil G et al. A Molecular Pathologic Framework for Risk Stratification of Stage T1 Urothelial Carcinoma. Eur Urol 2015; 68: 824-832; discussion 835-826.

11. Gooden MJ, de Bock GH, Leffers N et al. The prognostic influence of tumourinfiltrating lymphocytes in cancer: a systematic review with meta-analysis. Br J Cancer 2011; 105: 93-103.

12. Sharma P, Shen Y, Wen S et al. CD8 tumor-infiltrating lymphocytes are predictive of survival in muscle-invasive urothelial carcinoma. Proc Natl Acad Sci U S A 2007; 104: 3967-3972.

13. Masson-Lecomte A, Rava M, Real FX et al. Inflammatory biomarkers and bladder cancer prognosis: a systematic review. Eur Urol 2014; 66: 1078-1091.

14. Garcia-Closas M, Malats N, Silverman D et al. NAT2 slow acetylation, GSTM1 null genotype, and risk of bladder cancer: results from the Spanish Bladder Cancer Study and meta-analyses. Lancet 2005; 366: 649-659.

15. Rubin MA, Dunn R, Strawderman M, Pienta KJ. Tissue microarray sampling strategy for prostate cancer biomarker analysis. Am J Surg Pathol 2002; 26: 312-319.

16. Lamm DL, Blumenstein BA, Crissman JD et al. Maintenance bacillus Calmette-Guerin immunotherapy for recurrent TA, T1 and carcinoma in situ transitional cell carcinoma of the bladder: a randomized Southwest Oncology Group Study. J Urol 2000; 163: 1124-1129.

17. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell 2012; 21: 309-322.

18. Romano E, Romero P. The therapeutic promise of disrupting the PD-1/PD-L1 immune checkpoint in cancer: unleashing the CD8 T cell mediated anti-tumor activity results in significant, unprecedented clinical efficacy in various solid tumors. J Immunother Cancer 2015; 3: 15.

Alexandra Masson-Lecomte

19. Ayari C, LaRue H, Hovington H et al. Bladder tumor infiltrating mature dendritic cells and macrophages as predictors of response to bacillus Calmette-Guerin immunotherapy. Eur Urol 2009; 55: 1386-1395.

20. Otto W, Denzinger S, Wieland WF, Hartmann A. First analysis of immune cell infiltration in stage pT1 urothelial bladder carcinoma: CD3 positivity as a prognostic marker for cancer-specific survival. World J Urol 2012; 30: 875-877.

21. Sjodahl G, Lovgren K, Lauss M et al. Infiltration of CD3 and CD68 cells in bladder cancer is subtype specific and affects the outcome of patients with muscle-invasive tumors. Urol Oncol 2014.

22. Takayama H, Nishimura K, Tsujimura A et al. Increased infiltration of tumor associated macrophages is associated with poor prognosis of bladder carcinoma in situ after intravesical bacillus Calmette-Guerin instillation. J Urol 2009; 181: 1894-1900.

23. Pichler R, Fritz J, Zavadil C et al. Tumor-infiltrating immune cell subpopulations influence the oncologic outcome after intravesical bacillus calmette-guerin therapy in bladder cancer. Oncotarget 2016.

24. Kitamura H, Torigoe T, Honma I et al. Effect of human leukocyte antigen class I expression of tumor cells on outcome of intravesical instillation of bacillus calmette-guerin immunotherapy for bladder cancer. Clin Cancer Res 2006; 12: 4641-4644.

25. Faraj SF, Munari E, Guner G et al. Assessment of tumoral PD-L1 expression and intratumoral CD8+ T cells in urothelial carcinoma. Urology 2015; 85: 703 e701-706.

26. Horn T, Laus J, Seitz AK et al. The prognostic effect of tumour-infiltrating lymphocytic subpopulations in bladder cancer. World J Urol 2016; 34: 181-187.

27. Aine M, Eriksson P, Liedberg F et al. On Molecular Classification of Bladder Cancer: Out of One, Many. Eur Urol 2015; 68: 921-923.

28. Sweis RF, Spranger S, Bao R et al. Molecular Drivers of the Non-T-cell-Inflamed Tumor Microenvironment in Urothelial Bladder Cancer. Cancer Immunol Res 2016; 4: 563-568.

Table 1: Results of the Wilcoxon test for comparison of the CD8+ count inter quartile rangeaccording to the number of regions selected on the FS slide.

		1 vs 10 areas	2 vs 10 areas	3 vs 10 areas	4 vs 10 areas	5 vs 10 areas	6 vs 10 areas	7 vs 10 areas	8 vs 10 areas	9 vs 10 areas
10r	p- value in Ta	0.008	0.05	0.02	<u>0.02</u>	0.10	0.19	0.47	0.77	0.77
Tur	p- value in T1	<u>0.03</u>	0.17	0.07	0.14	0.29	0.44	0.69	0.83	0.83
ma	p- value in Ta	0.019	0.04	0.03	0.02	0.10	0.15	0.34	0.61	0.64
Stre	p- value in T1	<0.0001	0.0004	0.0004	0.001	<u>0.02</u>	0.05	0.21	0.49	0.89

	N=586
Age	
Mean	65.8
Median	68
Min	22
Max	80
Gender	
Male	517 (88%)
Female	69 (12%)
Area	
1 = Barcelona	116 (20%)
2 = Vallès	97 (17%)
3 = Elx	56 (10%)
4 = Tenerife	117 (20%)
5 = Astúries	199 (34%)
Stage and Grade	
PUNLMP/TaG1	179 (31%)
TaG2	214 (37%)
TaG3	74 (13%)
T1G2	13 (2%)
T1G3	102 (17%)
Tis	4 (1%)
EORTC Risk	
High	250 (43%)
Low	336 (57%)
Multiplicity	
1 tumor	364 (62.1%)
\geq 2 tumors	187 (31.9%)
Missing	35 (0.06%)

Table 2: Reduced cohort of patients characteristics

	Size	
	< 3cm	307 (52.4%)
	\geq 3cm	114 (19.5%)
	Missing	165 (28.2%)
	Traitement	
	TURB alone	222 (37.9%)
	TURB + IC	132 (22.5%)
	TURB + BCG	206 (35.2%)
	Others	26 (4.4%)
ľ	Number of CD8 / mm2	
	Mean +/- SD	83.8 +/- 191.02
	Median [IQR]	20.08 [0-346.48]
	Min	0
	Max	2029
1		

Table 3: Cox regression analysis results displaying association of CD8 infiltration with risk

 of recurrence in T1.

	HR	<i>p</i> -value		lower 95	upper 95
Number of CD8					
<43/mm ²	Ref				
\geq 43 /mm ²	0.39	0.049	*	0.16	0.99
Area					
Barcelona	Ref				
Valles	1.22	0.809		0.25	5.88
Elx	2.12	0.348		0.44	10.18
Tenerife	0.62	0.564		0.12	3.21
Asturies	1.78	0.423		0.44	7.26
Gender					
Male	Ref				
Female	0.41	0.280		0.08	2.06
Multiplicity					
1 tumor	Ref				
\geq 2 tumors	4.34	0.003	**	1.62	11.63
Missing	1.30	0.712		0.33	5.15
Size					
< 3cm	Ref				
\geq 3cm	4.70	0.006	**	1.57	14.08
Missing	1.60	0.397		0.54	4.73
Traitement					
TURB alone	Ref				
TURB + IC	0.30	0.125		0.06	1.40
TURB + BCG	0.71	0.532		0.24	2.08
Others	0.25	0.110		0.04	1.37

FIGURES LEGENDS

Figure 1: After digitalization of the slides using the Aperio scanscope XT, Leica scanner, CalopixTM software (Tribvn, France) allowed background suppression (A) and separate recognition of tumor (pink) and stroma (white) (Figure 1A and B). CD8+ cells were automatically identified using a combination of colour, shape and size recognition (see CD8+ cells circled in green, Figure 1C).

Figure 2: Determining the optimal number of 0.6 mm TMA cores to sample. Each box plot is the distribution of the Interquartile range (IQR) of mean CD8+ infiltration calculated from 500 random observations of a specified number of regions. The variability decreases as the number of regions (figurating TMA cores) increases. By statistical comparison of IQR distributions, the minimal number of regions for an accurate estimate of CD8+ expression as compared with the standard was reached at 5 cores per tumor region in Ta and 2 cores in T1.

Figure 3: Correlation between tumor and stroma CD8+ count according to tumor stage (Ta vs T1). Correlation was moderate, demonstrating heterogeneity between tumor and stroma inflammatory infiltrate.

Figure 4: Boxplots figuring CD8+/mm2 count in the EPICURO cohort according to FGFR3 expression and stage. CD8+ infiltration is lower in patients with high FGFR3 expression.

Figure 5: Kaplan Meier curves for time to recurrence according to CD8+ infiltration. 5-year recurrence free survival was 80% in the strong infiltration group (\geq 43 CD8/mm²) versus 69% in the low infiltration group (<43 CD8/mm²).

Alexandra Masson-Lecomte

Figure 1: After digitalization of the slides using the Aperio scanscope XT, Leica scanner, CalopixTM software (Tribvn, France) allowed background suppression (A) and separate recognition of tumor (pink) and stroma (white) (Figure 1A and B). CD8+ cells were automatically identified using a combination of colour, shape and size recognition (see CD8+ cells circled in green, Figure 1C).

Figure 1A



Figure 1B



Figure 1C



Figure 2: Determining the optimal number of 0.6 mm TMA cores to sample. Each box plot is the distribution of the Interquartile range (IQR) of mean CD8+ infiltration calculated from 500 random observations of a specified number of regions. The variability decreases as the number of regions (figurating TMA cores) increases. By statistical comparison of IQR distributions, the minimal number of regions for an accurate estimate of CD8+ expression as compared with the standard was reached at 5 cores per tumor region in Ta and 2 cores in T1.



Figure 3: Correlation between tumor and stroma CD8+ count according to tumor stage (Ta vs T1). Correlation was moderate, demonstrating heterogeneity between tumor and stroma inflammatory infiltrate.



Figure 4: Boxplots figuring CD8+/mm2 count in the EPICURO cohort according to FGFR3 expression and stage. CD8+ infiltration is lower in patients with high FGFR3 expression.



Ta + T1

FGFR3 Quick Score values (<=0, 1-30, 30-100, >100)



Figure 5: Kaplan Meier curves for time to recurrence according to CD8+ infiltration. 5-year recurrence free survival was 80% in the strong infiltration group (\geq 43 CD8/mm²) versus 69% in the low infiltration group (<43 CD8/mm²).



Time in months

RFS in the full T1 cohort

RFS in the reduced T1 cohort



Time in months

SUPPLEMENTARY TABLES

		Full cohort (981)	Reduced cohort
			(386)
Age			
	Mean	65.6	65.8
	Median	68	68
	Min	22	22
	Max	80	80
Gender			
	Male	857 (87%)	517 (88%)
	Female	124 (13%)	69 (12%)
Area			
	1	222 (23%)	116 (20%)
	2	161 (16%)	97 (17%)
	3	83 (8%)	56 (10%)
	4	150 (15%)	117 (20%)
	5	363 (37%)	199 (34%)
TG			
	1 (PUNLMP/TaG1)	424 (43%)	179 (31%)
	2 (TaG2)	292 (30%)	214 (37%)
	3 (TaG3)	102 (10%)	74 (13%)
	4 (T1G2)	28 (3%)	13 (2%)
	5 (T1G3)	129 (13%)	102 (17%)
	6 (Tis)	6 (1%)	4 (1%)
Risk			

 Table S1: Characteristics of the full and reduced cohorts of patients

Alexandra Masson-Lecomte

High	340 (35%)	250 (43%)
Low	641 (65%)	336 (57%)

Table S2: Cox regression analysis results displaying association of CD8 infiltration with risk of recurrence in the T1 cohort with 2 spots available (N=67). Reduced number of events (11 recurrences) only allowed adjustment for 3 variables.

	HR	<i>p</i> -value	lower 95	upper 95
Number of CD8				
<35/mm ²	Ref			
\geq 35 /mm ²	0.36	0.13	0.07	1.40
Multiplicity				
1 tumor	Ref			
\geq 2 tumors	3.70	0.06	0.95	14.47
Missing	1.15	0.92	0.11	11.79
Size				
< 3cm	Ref			
\geq 3cm	4.40	0.06	0.90	21.51
Missing	1.71	0.52	0.33	8.95

Table S3: Cox regression analysis results displaying association of CD8 infiltration with riskof recurrence in the T1 cohort adjusted for FGFR3 status (number of patients = 75, number ofrecurrences = 18).

	HR	<i>p</i> -value		lower 95	upper 95
Number of CD8					
<38/mm ²	Ref				
\geq 38 /mm ²	0.48	0.14		0.1801	1.295
FGFR3 QS	1.00	0.62		0.9936	1.011
Multiplicity					
1 tumor	Ref				
\geq 2 tumors	4.39	0.007	**	1.4839	13.018
Missing	3.28	0.11		0.7438	14.502
Size					
< 3cm	Ref				
\geq 3cm	2.51	0.13		0.7505	8.412
Missing	1.67	0.40		0.4949	5.692

SUPPLEMENTARY FIGURES

Figure S1 : Histograms showing the distribution of GATA3, FGFR3 and CK5/6 expression in the EPICURO cohort.



Distribution of CK5/6 expression



EFGR3 Quick Score

Distribution of FGFR3 expression

	Quick Score value					
	Mean	Median	Low IQR (0.25)	High IQR (0.75)		
FGFR3	60	30	0	100		
GATA3	149	167	81	218		
CK5/6	31	3	0	40		

Alexandra Masson-Lecomte
Figure S2 : Boxplots figuring CD8+ count Interquartile range in the stroma compartment in Ta and T1 tumors.



Number of regions analyzed in T1

IV DISCUSSION

1. INFLAMMATION RELATED GENETIC POLYMORPHISMS AND BLADDER CANCER PROGNOSIS

1.1. Different output of candidate-gene or a genome-wide approaches

First objective of the present work was to assess association between germline polymorphisms in inflammatory genes and bladder cancer prognosis. Two studies have been conducted in parallel, one genome-based approach in NMIBC assessing the association between SNPs in inflammatory genes and recurrence or progression and one gene-based approach in MIBC assessing the association between *PDL1* SNPs and survival.

In the field of NMIBC, according to literature, we first considered that no inflammatory biomarker had reached sufficient evidence of biological/clinical implication to apply a candidate-gene study. On the contrary, we hypothesized that NMIBC was a complex trait where the joint effect of multiple SNPs was more likely to drive the disease prognosis rather than a single gene. Consequently, we applied an inflammatory genome wide study mapping in order to detect inflammatory variants association with prognosis, some of which possibly located in or near candidate genes. This methodology not only allows identification of a large number of SNPs with small effect but also considers the relationships between each other's, adjusting for the statistical effect of one another. We identified a large number of inflammatory variants associated with recurrence and progression, some of which already identified as associated with bladder cancer risk. Three SNPs in TNIP1, CD5, and JAK3 showed very strong association with posterior probabilities >90% using multi-marker methods. Regarding risk-of-progression, one SNP in CD3G was significantly associated using Cox regression and two SNPs in MASP1 and AIRE, showed a posterior probability of association ≥80% with multi-marker methods. Variants identified herein should only be

considered as biomarkers as long as functional studies have not been performed. However, most significant SNPs are placed in genes implicated in immune tolerance. Biological relevance of those genes is particularly enlightened by the recent breakthrough made in BC treatment using drugs restoring immune function.

Contrary to NMIBC, known biological effect of checkpoint inhibitors in MIBC led us to consider that applying a candidate-gene approach to *PDL1* gene was of strong functional relevance. This work is the first to report highly significant association between germline *PDL1* variants and MIBC prognosis. Five variants were significantly associated with all endpoints (tumour progression, cancer-specific survival, and overall survival) in patients with MIBC after multiple testing correction (rs822339, rs860290, rs2282055, rs1411262, rs10125854). At the gene level, *PDL1* was significantly associated with the 3 endpoints. At the functional level, all significant variants, except one (rs10125854), correlated with PDL1 expression levels in different tissues. Main limitation was the absence of formal replication in the TGCA dataset. However, using a candidate gene approach in MIBC does not preclude applying a genome-based approach that will likely also allow identification of a large number of inflammatory variants associated with survival. The two approaches have to be applied jointly in order to comprehensively understand the genetic background underlying bladder cancer prognosis.

1.2. Biological relevance as the cornerstone of genetic studies

At the end, biological relevance should be one of the cornerstones of genetic studies, either being at the initiation of the process in candidate-gene studies or the final grail of genome wide exploration. Otherwise, variants remain biomarkers, which is insufficient in an era where the need for personalized medicine is imperious. While computational approaches provide researchers a very broad range of new information they need to enlarge the biological comprehension of the diseases, biology also needs to be at the initiation of large scale bioinformatical genomic analyses in order to hierarchize the interpretation of the results and avoid drowning into big data with no functional hypotheses.

1.3. The need for integration with the other -omics

As the field of genomics daily expands, genetic variations will have to be integrated along with epigenomics, transcriptomics and metagenomics. The influence of the host genetic background depends on the way it is differentially expressed based on epigenetic alterations but also on its interplay with 1) the millions of microorganisms that colonize each living individual 2) with the environment. Integration of the different –omics is huge challenge but is necessary to investigate the genomic mechanisms involved in complex diseases [206]. Application of single –omics strategies will irremediably lead to limited insights in the understanding of diseases.

2. TISSUE INFLAMMATION ASSESMENT AND ASSOCIATION WITH BLADDER CANCER PROGNOSIS

2.1. Strengths of the results

Study of association between tissue inflammation and bladder cancer prognosis has been a major challenge of the past decades. Literature suggesting association is overabundant, but methodological challenges preclude applicability of the results to daily clinical practice. For that reason, we figured that studying association between inflammation and UCB prognosis could only begun after a proper evaluation of the best methodological process had been made.

The work presented herein is the first to propose a standardized methodological pipeline for CD8+ cell assessment in bladder cancer. An automated morphometric approach combined to color-based tissue recognition allowed separated tumor and stroma count and

normalization of the results to mm2 of tumor or stroma. Spatial heterogeneity of CD8+ infiltrate in both tumor and stroma was demonstrated using resampling strategies. In the tumor compartment, two TMA-sized regions provided precise estimation of CD8+ infiltrate in T1 while 5 regions were necessary in Ta. In the stromal compartment, 5 and 6 regions were necessary for Ta and T1 respectively. CD8+ infiltrate was significantly associated with stage since CD8+ count was much higher in T1 than in Ta both in the tumor and in the stromal compartment. Correlation between tumor and stromal count were moderate in both Ta and T1. Finally, we explored prognosis in T1 and observed that CD8+ infiltrate in the tumor compartment was significantly and independently associated with risk of recurrence. Those results have to be considered cautiously taking into account the limitations associated with CD8+ estimates on TMA.

Altogether, those results support the need of separated approaches of tumor and stroma compartment and usage of material adapted to inflammatory environment studies (TMA specially designed or full sections). In general, the behaviour and function of tumor-associated inflammatory cells still remains largely unknown and progresses in biological understanding of tumor – inflammation interactions will go faster if rigorous approaches are favoured, considering tissue compartment and stages separately before looking at the whole. The association identified between CD8+ infiltrate and both stage and risk of recurrence of T1 supports the usefulness of inflammatory biomarkers in the management of patients, both for improved assessment of prognosis and to guide therapy.

2.2. <u>Remaining challenges</u>

2.2.1. Compartments

Although some challenges have been addressed, many remain. We demonstrated heterogeneity between tumor and stroma lymphocyte count. Herein, we based our preliminary prognostic analyses on the tumor compartment. However, in prognosis analyses, the more relevant compartment has never been investigated: Tumor/ Invasive front/ Stroma/ Ratio between tumor and stroma? The Immunoscore developed in colon cancer is based on the infiltrate at the invasive front of the tumor. In NMIBC, the tumor architecture is disrupted by the morcellation induced by TURB. Assessment of the invasive front is therefore compromised. Consequently, en bloc resection would have to be used to address this issue in NMIBC. However, this would create selection bias in tumor selection since en-bloc resection can only be offered to small tumors that can be extracted through the endoscope.

2.2.2. Impact of UCB natural history on tumor inflammation

The impact of treatment has also to be taken into consideration. In the EPICURO cohort we only considered incident tumors (first tumor to occur in a patient). UBC natural history is made of multiples recurrences and some progressions leading to repeated TURBT and intra vesical instillations. The impact of different treatment courses on the bladder wall inflammatory infiltrate has never been studied although intuitively obvious. The results obtained in an incident cohort of bladder tumors cannot be extrapolated to a prevalent cohort as long as treatment impact on tissue inflammation has not been studied. Many published studies in the field "mixed" incident and prevalent cohorts without any rational to do so.

2.2.3. Consideration of multiples inflammatory cell types

We focused on CD8+ cells to establish the methodological process. However, the tumor-immunity cycle is made of multiple cell types each acting jointly with the others to control/eradicated tumor cells (Figure 1, [207]). Comprehensive insight into the anti-tumor effect of the immune system along with the study of its association with tumor prognosis

cannot be conceived without exploring the impact of each tumor cell type individually but also together with the others. The challenge is huge again as limited knowledge is available regarding the relevant information: absolute number of each cell type, ratio between types? Moreover, cell types count should be integrated along with their location in the tissue: tumor compartment, stroma compartment. The development of multiplexed in situ analysis based on mRNA and protein expression of different key player genes will help to decipher the role of immune architecture in cancer biology.

Finally, presence of an inflammatory cell in the tissue does not mean biological effectiveness. Markers of cells functional state should also be considered together with the sole IHC staining [208, 209]. As seen in renal cell carcinoma, CD8+ anergy leads to irresponsiveness to tumor antigens. Consequently, identification of a cell type using IHC does not necessarily imply anti tumor effect, and this might explain some of the false negative results where strong CD8+ infiltrate was not associated with better prognosis. To overcome this limitation, authors have suggested usage of transcriptional immune signature as a tool for assessing the immune response [210, 211]. Enrichment in different immune cells subset is associated with differential expression of gene signatures that vary according to cells activation states. This methodology will allow much more precise investigation of cellular functions than sole IHC staining.



Figure 1: Multiple inflammatory cells (CD4+ and CD8+ T cells, Dendritic cells, NK cells, macrophages) act jointly to control/eliminate transformed malignant cells. (from [207])

3. SERUM INFLAMMATION IN THE CONTEXT OF BLADDER CANCER

3.1. Serum inflammation in the EPICURO cohort

Systemic inflammation is widely known to be associated with both cancer risk and prognosis. Based on the literature review, we identified CRP as the most extensively studied serum inflammatory marker in bladder cancer. High CRP levels have been consistently reported to be associated with MIBC adverse prognosis. Studies in NMIBC were scarce.

Consequently we initially planned to evaluate association between CRP and NMIBC risk of recurrence and progression.

This part of the project remained a blind spot since we encountered some obstacles. After reviewing the dates the serum was withdrawn from the patients, it occurred that out of 997 NMIBC patients, only 88 had the blood retrieved before the day of surgery or the day of surgery in the operating room before the surgeon started the TURB procedure. In all other cases blood was retrieved after surgery. In 278 cases blood was withdrawn at day 1. We hypothesised that CRP levels would be very much modified by TURBT since CRP is an early marker of inflammatory response (level rises between 6 and 8 hours after initiation of inflammation). To confirm the impossibility to use EPICURO blood sample for this study we decided to perform a pilot study in the department of Urology of Henri Mondor Hospital in order to evaluate how CRP levels at day 1 after surgery were correlated to the same markers before surgery despite the intervention. After obtaining approval of the hospital and patients, 5 patients were included. Results for pre and post operative CRP are presented in Table 1.

Table 1: Pre and post TURB CRP levels in 5 consecutive incident UCB hospitalized in Henri

 Mondor hospital department of Urology.

Date of TURB	CRP J-1	CRP J+1	ratio
18/02/14	0.6	2.5	4.17
18/02/14	0.5	1.4	2.8
18/02/14	12.2	23.7	1.94
19/02/14	3.7	6.4	1.73
20/02/14	0.6	2.4	4

After the 5 first patients it was clear that CRP retrieved at day 1 after TURB was not usable.

Facing the limited number of blood samples usable, we did not move on with the study of association between CRP levels and prognosis in NMIBC.

3.2. Future directions

Studies focusing on serum inflammation will not be feasible using the EPICURO cohort based on previously detailed limitations. This emphasizes the need to design prospective cohorts with both pre and post operative withdrawal of blood samples depending of the type of analyses planned. Future studies would have to explore both serum cytokines and presence/absence of inflammatory cells. Multi markers assessment technique would probably be more effective than exploring one marker at a time.

More generally, all the assessments performed for inflammatory cells in the tumor can also be performed in blood using flow cytometry methods. As shown in the "Tumor Immunity cycle" (Figure 2, [212]), exposure of tumor antigens to dendritic cells and antigen presenting cells leads to activation of the antitumor immunity in the peripheral lymph nodes. Inflammatory cells further traffic into the blood flow to reach cancer cells in the affected organ. Cells can therefore be identified in the blood before they reach the tumors. This technique would limit the need for tumor samples and could allow a follow up of anti tumor immune response along time and treatment courses.



Figure 2 : « The cancer-immunity cycle ». From [212].

4. INTEGRATIVE APROACH BETWEEN INFLAMMATION AND MOLECULAR SUBTYPES.

4.1. Integration between the different matrices

Inflammation is a systemic process that affects together the blood, the tissue, and the urine of the patients. Consequently, Pro/anti tumor inflammatory response can be explored together in each of the above-mentioned matrices.

As displayed in the introduction, assessment of the prognostic effect of the immune system has to be performed taking into consideration the host genetic context (Germline variants), the systemic inflammatory response (blood cytokines and inflammatory cells) and the tumoral response.



Figure 3: an integrative view of the inflammatory response to NMIBC

Each can be analysed independently but secondary integration (considering all the matrices jointly) would be more comprehensive. In the *PDL1* paper we suggested some functionality of the SNPs. Variation in *PDL1* gene could lead to differential expression of PDL1 in the tumor with potential interactions with the cytotoxic lymphocytes activation pathways. This single example emphasizes the need for integration in order to completely embrace the full scope of anti tumor immunity.

4.2. Integration with molecular taxonomy

The work performed in the past years to uncover the molecular background of UCB has revealed bladder cancer as a heterogeneous disease. Each subtype is associated with different immune profiles suggesting variable interaction with the microenvironment [68]. It is likely that the stromal compartment of different molecular subtypes is as much different as the tumor cells themselves. As tumor progression deeply depends on the interactions between the tumor cells and the tumor microenvironment in the stromal compartment [213], study of the anti tumor immunity will necessarily need in the future to be put into the perspective of the molecular landscape. While some authors have already begun [72] generalization is needed for better prediction of prognosis and immunotherapy response.

5. PREDICTION OF BLADDER CANCER PROGNOSIS AND RESPONSE TO IMMUNOTHERAPY TREATMENT

In many studies, authors confound identification of prognosis association and prediction of an outcome. Identifying an association between a marker and an outcome is the first step of building prediction models. However, multiple steps are to be applied for prediction tools to be usable in daily practice. After multivariable regression analyses are performed, a model needs to be developed and evaluated using defined criteria such as calibration and discrimination [214]. After the best model has been built in a population, it needs to be validated in an independent cohort as performances are commonly poorer in the new set of patients [215]. In the work presented here, only the first steps of the prediction process have been applied. We demonstrated strong associations; particularly at the germline level but usage of germline inflammatory variants in daily practice are far from being a reality. Calculation of the predictive ability of the models was low (c-index), validation was not performed in the NMIBC study and we lacked replication in the *PDL1* study. In the CD8 study, multiple retrospective studies suggested associations, which are a kind of an external validation, but again no predictive model was built and even less validated.

In the field of prediction of response to immunotherapy, usage of inflammatory biomarkers both at the germline and tumor level is very attractive. However, proven association between PDL1 and prognosis does not mean that PDL1 variation or expression will be predictive of response to anti PDL1 treatment [216]. Predictive ability of PDL1 expression in the tumors as a marker of response to anti PDL1 drugs was suggested by sub-groups analyses of randomized trials assessing Atezolizumab, Pembrolizumab and Nivolumab in metastatic bladder cancer [73, 216-218]. To explore potential similar predictive ability of germline variation, analyses of randomized control trials would also be needed. This emphasizes the tremendous difficulty to bring biomarkers to daily practice. Most studies limit themselves to identification of associations, misleadingly using the word prediction in the discussions. Bringing markers to the next steps is mandatory but very time, money and resources consuming.

Using inflammatory biomarkers in the field of bladder cancer prognosis and treatment prediction will need multi steps processes with strong/standardized methodology applied to association's identification in different molecular subtypes, matrices integration and model building, external validation and prediction testing in randomized trials. Pipelines need to be described and followed by researchers for our patients to ever see the clinical application of our works.

6. FUTURE PROJECTS

The work presented here has opened multiple questions and possible directions. In the field of germline variation we would pursue the work conducted in MIBC. The strong

association identified between *PDL1* SNPs and MIBC prognosis warrants further attempts of validation, and we should explore other optional cohorts. We have found association with gene expression in the public data sets explored but we should also explore biological validation using cellular models. In NMIBC, the genetic background was strongly associated with prognosis but at a multiple SNPs scale. Addition of SNPs to predictive models did not add enough prediction to consider genotyping patients in daily practice.

Regarding tumor inflammation the work to be done seems limitless. First we should confirm the independent association between CD8+ infiltrate in the tumor compartment and NMIBC risk of recurrence using full section on an independent cohort. We would also explore association between the stromal compartment and prognosis. The study should be enlarged also to the other markers initially planned: CD3 and CD68 at first based on the results from Lund [72]. For those markers the same methodological process separating tumor and stromal count and using full section in order to assess spatial heterogeneity would be used. Based on the new insights in BC molecular taxonomy, it would be of interest to conduct the new investigation of tumor inflammatory infiltrate considering the molecular background, at least using IHC urothelial differentiation markers but preferentially using expression signature for molecular subtypes.

Finally regarding serum, extensive literature has now shown the independent association between serum inflammation and BC prognosis. Most studied markers are CRP and neutrophil to lymphocyte ratio that both showed consistent association with prognosis mainly in MIBC but also NMIBC. We still need to investigate how to use those markers in daily practice to guide patients' therapy. Drivers of systemic inflammation are still unknown. We need to investigate their associations with molecular subtyping and tumor inflammatory infiltrate in prospective cohorts. To integrate the work performed in tumor, there is a need to explore inflammatory cells in blood using flow cytometry. CD8+ cells can be identified in the blood as easily as in the tumor. Association between CD8+ count in the blood and CD8+ infiltrate in the tumor is unknown. Moreover, expression of checkpoints markers such as PDL1, CTLA4 but also others can be identified in circulating immune cells as much as in infiltrated tumor cells. Investigations conducted in the tumors regarding infiltration with inflammatory cells but also expression of immune checkpoint molecules could be investigated in the blood with identification of similar associations and easiest access to patient samples.

V CONCLUSION

The work presented herein allowed the use of multiple and innovative methodological methods to explore association between constitutional genetics in the field of inflammation and bladder cancer prognosis. We identified multiple variants with small effects strongly associated to non-muscle invasive bladder cancer prognosis but also a strong association in a large prospective cohort between *PLD1* variation and muscle invasive bladder cancer survival. While external validation is lacking, those findings raise the question of the future place of genetics in the management of bladder cancer.

Molecular characterisation of bladder cancer is on going and interactions with the inflammatory microenvironment are merely uncovered. In that context, solving the question of the best methodology to estimate tumor-related inflammation is impelling. We proposed here a standardized counting approach of CD8+ cells after double immuno-staining and digitalization that can be applied to any inflammatory cellular subtype. Stage and histological compartment related heterogeneity led to restrictive estimation of the inflammatory infiltrate using TMAs. Future studies assessing environmental cellular features should start with a proper methodological evaluation of the environment to prevent false positive or negative results. Regarding prognosis, our results have to be considered cautiously but suggest possible association with recurrence. Exploration of muscle invasive bladder cancer and other inflammatory cellular subtypes remains to be done in the light of proper methodological approaches in order to enlarge our understanding of the tumor-immunity interactions.

VI REFERENCES

Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin 2016; 66:
 7-30.

2. Uhry Z, Remontet L, Colonna M et al. Cancer incidence estimation at a district level without a national registry: a validation study for 24 cancer sites using French health insurance and registry data. Cancer Epidemiol 2013; 37: 99-114.

3. Christoforidou EP, Riza E, Kales SN et al. Bladder cancer and arsenic through drinking water: a systematic review of epidemiologic evidence. J Environ Sci Health A Tox Hazard Subst Environ Eng 2013; 48: 1764-1775.

4. Rota M, Bosetti C, Boccia S et al. Occupational exposures to polycyclic aromatic hydrocarbons and respiratory and urinary tract cancers: an updated systematic review and a meta-analysis to 2014. Arch Toxicol 2014; 88: 1479-1490.

5. Underwood JM, Richards TB, Henley SJ et al. Decreasing trend in tobacco-related cancer incidence, United States 2005-2009. J Community Health 2015; 40: 414-418.

6. Lee PN, Thornton AJ, Hamling JS. Epidemiological evidence on environmental tobacco smoke and cancers other than lung or breast. Regul Toxicol Pharmacol 2016; 80: 134-163.

7. Clin B, RecoCancerProf' Working G, Pairon JC. Medical follow-up for workers exposed to bladder carcinogens: the French evidence-based and pragmatic statement. BMC Public Health 2014; 14: 1155.

 Beukers W, Hercegovac A, Zwarthoff EC. HRAS mutations in bladder cancer at an early age and the possible association with the Costello Syndrome. Eur J Hum Genet 2014; 22: 837-839.

9. Hafner C, Toll A, Real FX. HRAS mutation mosaicism causing urothelial cancer and epidermal nevus. N Engl J Med 2011; 365: 1940-1942.

10. Garcia-Closas M, Malats N, Silverman D et al. NAT2 slow acetylation, GSTM1 null genotype, and risk of bladder cancer: results from the Spanish Bladder Cancer Study and meta-analyses. Lancet 2005; 366: 649-659.

11. An Y, Li H, Wang KJ et al. Meta-analysis of the relationship between slow acetylation of N-acetyl transferase 2 and the risk of bladder cancer. Genet Mol Res 2015; 14: 16896-16904.

12. Figueroa JD, Han SS, Garcia-Closas M et al. Genome-wide interaction study of smoking and bladder cancer risk. Carcinogenesis 2014; 35: 1737-1744.

13. Garcia-Closas M, Rothman N, Figueroa JD et al. Common genetic polymorphisms modify the effect of smoking on absolute risk of bladder cancer. Cancer Res 2013; 73: 2211-2220.

14. Cuzick J, Otto F, Baron JA et al. Aspirin and non-steroidal anti-inflammatory drugs for cancer prevention: an international consensus statement. Lancet Oncol 2009; 10: 501-507.

15. Rothwell PM, Fowkes FG, Belch JF et al. Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomised trials. Lancet 2011; 377: 31-41.

16. Castelao JE, Yuan JM, Gago-Dominguez M et al. Non-steroidal anti-inflammatory drugs and bladder cancer prevention. Br J Cancer 2000; 82: 1364-1369.

17. Grubbs CJ, Juliana MM, Eto I et al. Chemoprevention by indomethacin of N-butyl-N-(4-hydroxybutyl)-nitrosamine-induced urinary bladder tumors. Anticancer Res 1993; 13: 33-36.

18. Pommergaard HC, Burcharth J, Rosenberg J, Raskov H. Oral chemoprevention with acetyl salicylic Acid, vitamin D and calcium reduces the risk of tobacco carcinogen-induced bladder tumors in mice. Cancer Invest 2013; 31: 490-493.

 Leppert JT, Shvarts O, Kawaoka K et al. Prevention of bladder cancer: a review. Eur Urol 2006; 49: 226-234.

20. Coghill AE, Newcomb PA, Poole EM et al. Genetic variation in inflammatory pathways is related to colorectal cancer survival. Clin Cancer Res 2011; 17: 7139-7147.

21. Wang SS, Davis S, Cerhan JR et al. Polymorphisms in oxidative stress genes and risk for non-Hodgkin lymphoma. Carcinogenesis 2006; 27: 1828-1834.

22. Lin MT, Storer B, Martin PJ et al. Relation of an interleukin-10 promoter polymorphism to graft-versus-host disease and survival after hematopoietic-cell transplantation. N Engl J Med 2003; 349: 2201-2210.

23. Andrew AS, Gui J, Sanderson AC et al. Bladder cancer SNP panel predicts susceptibility and survival. Hum Genet 2009; 125: 527-539.

24. Ahirwar D, Kesarwani P, Manchanda PK et al. Anti- and proinflammatory cytokine gene polymorphism and genetic predisposition: association with smoking, tumor stage and grade, and bacillus Calmette-Guerin immunotherapy in bladder cancer. Cancer Genet Cytogenet 2008; 184: 1-8.

25. Kucukgergin C, Isman FK, Dasdemir S et al. The role of chemokine and chemokine receptor gene variants on the susceptibility and clinicopathological characteristics of bladder cancer. Gene 2012; 511: 7-11.

26. Yang H, Gu J, Lin X et al. Profiling of genetic variations in inflammation pathway genes in relation to bladder cancer predisposition. Clin Cancer Res 2008; 14: 2236-2244.

27. Parada B, Sereno J, Reis F et al. Anti-inflammatory, anti-proliferative and antioxidant profiles of selective cyclooxygenase-2 inhibition as chemoprevention for rat bladder carcinogenesis. Cancer Biol Ther 2009; 8: 1615-1622.

Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature
 2008; 454: 436-444.

29. Bernardo C, Cunha MC, Santos JH et al. Insight into the molecular basis of Schistosoma haematobium-induced bladder cancer through urine proteomics. Tumour Biol 2016; 37: 11279-11287.

30. Kalisvaart JF, Katsumi HK, Ronningen LD, Hovey RM. Bladder cancer in spinal cord injury patients. Spinal Cord 2010; 48: 257-261.

31. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell 2010;140: 883-899.

32. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell 2012; 21: 309-322.

33. Terzic J, Grivennikov S, Karin E, Karin M. Inflammation and colon cancer. Gastroenterology 2010; 138: 2101-2114 e2105.

34. Naugler WE, Karin M. The wolf in sheep's clothing: the role of interleukin-6 in immunity, inflammation and cancer. Trends Mol Med 2008; 14: 109-119.

35. Okamoto M, Hattori K, Oyasu R. Interleukin-6 functions as an autocrine growth factor in human bladder carcinoma cell lines in vitro. Int J Cancer 1997; 72: 149-154.

36. Ho PL, Lay EJ, Jian W et al. Stat3 activation in urothelial stem cells leads to direct progression to invasive bladder cancer. Cancer Res 2012; 72: 3135-3142.

37. Judd LM, Bredin K, Kalantzis A et al. STAT3 activation regulates growth, inflammation, and vascularization in a mouse model of gastric tumorigenesis. Gastroenterology 2006; 131: 1073-1085.

38. Grivennikov S, Karin E, Terzic J et al. IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. Cancer Cell 2009; 15: 103-113.

39. Shain KH, Yarde DN, Meads MB et al. Beta1 integrin adhesion enhances IL-6mediated STAT3 signaling in myeloma cells: implications for microenvironment influence on tumor survival and proliferation. Cancer Res 2009; 69: 1009-1015.

40. Sylvester RJ, van der Meijden AP, Oosterlinck W et al. Predicting recurrence and progression in individual patients with stage Ta T1 bladder cancer using EORTC risk tables: a combined analysis of 2596 patients from seven EORTC trials. Eur Urol 2006; 49: 466-465; discussion 475-467.

41. Babjuk M, Oosterlinck W, Sylvester R et al. EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder, the 2011 update. Eur Urol 2011; 59: 997-1008.

42. Shariat SF, Karakiewicz PI, Palapattu GS et al. Outcomes of radical cystectomy for transitional cell carcinoma of the bladder: a contemporary series from the Bladder Cancer Research Consortium. J Urol 2006; 176: 2414-2422; discussion 2422.

43. Nuhn P, May M, Sun M et al. External validation of postoperative nomograms for prediction of all-cause mortality, cancer-specific mortality, and recurrence in patients with urothelial carcinoma of the bladder. Eur Urol 2012; 61: 58-64.

44. Witjes JA, Comperat E, Cowan NC et al. EAU guidelines on muscle-invasive and metastatic bladder cancer: summary of the 2013 guidelines. Eur Urol 2014; 65: 778-792.

45. Schrier BP, Hollander MP, van Rhijn BW et al. Prognosis of muscle-invasive bladder cancer: difference between primary and progressive tumours and implications for therapy. Eur Urol 2004; 45: 292-296.

46. Neoadjuvant cisplatin, methotrexate, and vinblastine chemotherapy for muscleinvasive bladder cancer: a randomised controlled trial. International collaboration of trialists. Lancet 1999; 354: 533-540. 47. Dash A, Pettus JAt, Herr HW et al. A role for neoadjuvant gemcitabine plus cisplatin in muscle-invasive urothelial carcinoma of the bladder: a retrospective experience. Cancer 2008; 113: 2471-2477.

48. Ghoneim MA, Abdel-Latif M, el-Mekresh M et al. Radical cystectomy for carcinoma of the bladder: 2,720 consecutive cases 5 years later. J Urol 2008; 180: 121-127.

49. Madersbacher S, Hochreiter W, Burkhard F et al. Radical cystectomy for bladder cancer today--a homogeneous series without neoadjuvant therapy. J Clin Oncol 2003; 21: 690-696.

50. Stein JP, Lieskovsky G, Cote R et al. Radical cystectomy in the treatment of invasive bladder cancer: long-term results in 1,054 patients. J Clin Oncol 2001; 19: 666-675.

51. Mertens LS, Meijer RP, Meinhardt W et al. Occult lymph node metastases in patients with carcinoma invading bladder muscle: incidence after neoadjuvant chemotherapy and cystectomy vs after cystectomy alone. BJU Int 2014; 114: 67-74.

52. Choudhury A, Swindell R, Logue JP et al. Phase II study of conformal hypofractionated radiotherapy with concurrent gemcitabine in muscle-invasive bladder cancer. J Clin Oncol 2011; 29: 733-738.

53. Leow JJ, Martin-Doyle W, Rajagopal PS et al. Adjuvant chemotherapy for invasive bladder cancer: a 2013 updated systematic review and meta-analysis of randomized trials. Eur Urol 2014; 66: 42-54.

54. Balar AV, Galsky MD, Rosenberg JE et al. Atezolizumab as first-line treatment in cisplatin-ineligible patients with locally advanced and metastatic urothelial carcinoma: a single-arm, multicentre, phase 2 trial. Lancet 2017; 389: 67-76.

55. Fernandez-Gomez J, Madero R, Solsona E et al. Predicting nonmuscle invasive bladder cancer recurrence and progression in patients treated with bacillus Calmette-Guerin: the CUETO scoring model. J Urol 2009; 182: 2195-2203.

56. Humphrey PA, Moch H, Cubilla AL et al. The 2016 WHO Classification of Tumours of the Urinary System and Male Genital Organs-Part B: Prostate and Bladder Tumours. Eur Urol 2016; 70: 106-119.

57. Fletcher A, Choudhury A, Alam N. Metastatic bladder cancer: a review of current management. ISRN Urol 2011; 2011: 545241.

58. Rink M, Lee DJ, Kent M et al. Predictors of cancer-specific mortality after disease recurrence following radical cystectomy. BJU Int 2013; 111: E30-36.

59. Lavery HJ, Stensland KD, Niegisch G et al. Pathological T0 following radical cystectomy with or without neoadjuvant chemotherapy: a useful surrogate. J Urol 2014; 191: 898-906.

60. Kikuchi E, Margulis V, Karakiewicz PI et al. Lymphovascular invasion predicts clinical outcomes in patients with node-negative upper tract urothelial carcinoma. J Clin Oncol 2009; 27: 612-618.

61. Tarin TV, Power NE, Ehdaie B et al. Lymph node-positive bladder cancer treated with radical cystectomy and lymphadenectomy: effect of the level of node positivity. Eur Urol 2012; 61: 1025-1030.

62. Fleischmann A, Thalmann GN, Markwalder R, Studer UE. Extracapsular extension of pelvic lymph node metastases from urothelial carcinoma of the bladder is an independent prognostic factor. J Clin Oncol 2005; 23: 2358-2365.

63. Rink M, Robinson BD, Green DA et al. Impact of histological variants on clinical outcomes of patients with upper urinary tract urothelial carcinoma. J Urol 2012; 188: 398-404.

64. Xylinas E, Rink M, Robinson BD et al. Impact of histological variants on oncological outcomes of patients with urothelial carcinoma of the bladder treated with radical cystectomy. Eur J Cancer 2013; 49: 1889-1897.

65. Cancer Genome Atlas Research N. Comprehensive molecular characterization of urothelial bladder carcinoma. Nature 2014; 507: 315-322.

66. Choi W, Porten S, Kim S et al. Identification of distinct basal and luminal subtypes of muscle-invasive bladder cancer with different sensitivities to frontline chemotherapy. Cancer Cell 2014; 25: 152-165.

67. Damrauer JS, Hoadley KA, Chism DD et al. Intrinsic subtypes of high-grade bladder cancer reflect the hallmarks of breast cancer biology. Proc Natl Acad Sci U S A 2014; 111: 3110-3115.

68. Sjodahl G, Lauss M, Lovgren K et al. A molecular taxonomy for urothelial carcinoma. Clin Cancer Res 2012; 18: 3377-3386.

69. Volkmer JP, Sahoo D, Chin RK et al. Three differentiation states risk-stratify bladder cancer into distinct subtypes. Proc Natl Acad Sci U S A 2012; 109: 2078-2083.

70. Aine M, Eriksson P, Liedberg F et al. On Molecular Classification of Bladder Cancer: Out of One, Many. Eur Urol 2015; 68: 921-923.

71. Lerner SP, McConkey DJ, Hoadley KA et al. Bladder Cancer Molecular Taxonomy: Summary from a Consensus Meeting. Bladder Cancer 2016; 2: 37-47.

72. Sjodahl G, Lovgren K, Lauss M et al. Infiltration of CD3(+) and CD68(+) cells in bladder cancer is subtype specific and affects the outcome of patients with muscle-invasive tumors. Urol Oncol 2014; 32: 791-797.

73. Rosenberg JE, Hoffman-Censits J, Powles T et al. Atezolizumab in patients with locally advanced and metastatic urothelial carcinoma who have progressed following treatment with platinum-based chemotherapy: a single-arm, multicentre, phase 2 trial. Lancet 2016; 387: 1909-1920.

74. Sweis RF, Spranger S, Bao R et al. Molecular Drivers of the Non-T-cell-Inflamed Tumor Microenvironment in Urothelial Bladder Cancer. Cancer Immunol Res 2016; 4: 563-568.

75. Morales A, Eidinger D, Bruce AW. Intracavitary Bacillus Calmette-Guerin in the treatment of superficial bladder tumors. J Urol 1976; 116: 180-183.

76. Fuge O, Vasdev N, Allchorne P, Green JS. Immunotherapy for bladder cancer. Res Rep Urol 2015; 7: 65-79.

77. Simons MP, O'Donnell MA, Griffith TS. Role of neutrophils in BCG immunotherapy for bladder cancer. Urol Oncol 2008; 26: 341-345.

78. Zuiverloon TC, Nieuweboer AJ, Vekony H et al. Markers predicting response to bacillus Calmette-Guerin immunotherapy in high-risk bladder cancer patients: a systematic review. Eur Urol 2012; 61: 128-145.

79. Bohle A, Bock PR. Intravesical bacille Calmette-Guerin versus mitomycin C in superficial bladder cancer: formal meta-analysis of comparative studies on tumor progression. Urology 2004; 63: 682-686; discussion 686-687.

80. Lamm DL, Blumenstein BA, Crissman JD et al. Maintenance bacillus Calmette-Guerin immunotherapy for recurrent TA, T1 and carcinoma in situ transitional cell carcinoma of the bladder: a randomized Southwest Oncology Group Study. J Urol 2000; 163: 1124-1129.

81. Nakamura T, Fukiage M, Higuchi M et al. Nanoparticulation of BCG-CWS for application to bladder cancer therapy. J Control Release 2014; 176: 44-53.

82. Luo Y, Henning J, O'Donnell MA. Th1 cytokine-secreting recombinant Mycobacterium bovis bacillus Calmette-Guerin and prospective use in immunotherapy of bladder cancer. Clin Dev Immunol 2011; 2011: 728930.

83. Burke J. Virus therapy for bladder cancer. Cytokine Growth Factor Rev 2010; 21: 99-102.

84. Pagliaro LC, Keyhani A, Williams D et al. Repeated intravesical instillations of an adenoviral vector in patients with locally advanced bladder cancer: a phase I study of p53 gene therapy. J Clin Oncol 2003; 21: 2247-2253.

85. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer 2012; 12: 252-264.

86. Plimack ER GS, Bellmunt J, Berger R, Montgomery B, Gonzales EJ. A phase 1b study of pembrolizumab (Pembro; MK-3475) in patients (Pts) with advanced urothelial tract cancer. Annals of Oncology (ESMO 2014 meeting abstract) 2014; 25: LBA23A.

87. Powles T, Eder JP, Fine GD et al. MPDL3280A (anti-PD-L1) treatment leads to clinical activity in metastatic bladder cancer. Nature 2014; 515: 558-562.

88. Antonia S, Goldberg SB, Balmanoukian A et al. Safety and antitumour activity of durvalumab plus tremelimumab in non-small cell lung cancer: a multicentre, phase 1b study. Lancet Oncol 2016; 17: 299-308.

89. Shariat SF, Lotan Y, Vickers A et al. Statistical consideration for clinical biomarker research in bladder cancer. Urol Oncol 2010; 28: 389-400.

90. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell 21: 309-322.

91. Finn OJ. Cancer immunology. N Engl J Med 2008; 358: 2704-2715.

92. Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer 12: 298-306.

93. Galon J, Pages F, Marincola FM et al. Cancer classification using the Immunoscore: a worldwide task force. J Transl Med 10: 205.

94. Denkert C, Loibl S, Noske A et al. Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. J Clin Oncol 28: 105-113.

95. Galon J, Costes A, Sanchez-Cabo F et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 2006; 313: 1960-1964.

96. Wang E, Miller LD, Ohnmacht GA et al. Prospective molecular profiling of melanoma metastases suggests classifiers of immune responsiveness. Cancer Res 2002; 62: 3581-3586.

97. Kunath F, Krause SF, Wullich B et al. Bladder cancer - the neglected tumor: a descriptive analysis of publications referenced in MEDLINE and data from the register clinicaltrials.gov. BMC Urol 13: 56.

98. Catalona WJ, Smolev JK, Harty JI. Prognostic value of host immunocompetence in urologic cancer patients. J Urol 1975; 114: 922-926.

99. Romics I, Feher J, Horvath J. Immunological studies of patients with tumours of the prostate and bladder (a retrospective analysis). Int Urol Nephrol 1983; 15: 339-345.

100. Sadoughi N, Mlsna J, Guinan P, Rubenstone A. Prognostic value of cell surface antigens using immunoperoxidase methods in bladder carcinoma. Urology 1982; 20: 143-146.

101. McShane LM, Altman DG, Sauerbrei W et al. Reporting recommendations for tumor marker prognostic studies. J Clin Oncol 2005; 23: 9067-9072.

102. Pouessel D, Neuzillet Y, Mertens LS et al. Tumor heterogeneity of fibroblast growth factor receptor 3 (FGFR3) mutations in invasive bladder cancer: implications for perioperative anti-FGFR3 treatment. Ann Oncol 2016; 27: 1311-1316.

Alexandra Masson-Lecomte

103. Leibovici D, Grossman HB, Dinney CP et al. Polymorphisms in inflammation genes and bladder cancer: from initiation to recurrence, progression, and survival. J Clin Oncol 2005; 23: 5746-5756.

104. Ahirwar DK, Agrahari A, Mandhani A, Mittal RD. Cytokine gene polymorphisms are associated with risk of urinary bladder cancer and recurrence after BCG immunotherapy. Biomarkers 2009; 14: 213-218.

105. Riemann K, Becker L, Struwe H et al. Insertion/deletion polymorphism in the promoter of NFKB1 as a potential molecular marker for the risk of recurrence in superficial bladder cancer. Int J Clin Pharmacol Ther 2007; 45: 423-430.

106. Lima L, Oliveira D, Ferreira JA et al. The role of functional polymorphisms in immune response genes as biomarkers of bacille Calmette-Guerin (BCG) immunotherapy outcome in bladder cancer: establishment of a predictive profile in a Southern Europe population. BJU Int 2015; 116: 753-763.

107. Gallagher DJ, Vijai J, Hamilton RJ et al. Germline single nucleotide polymorphisms associated with response of urothelial carcinoma to platinum-based therapy: the role of the host. Ann Oncol 2013; 24: 2414-2421.

108. Grotenhuis AJ, Dudek AM, Verhaegh GW et al. Independent Replication of Published Germline Polymorphisms Associated with Urinary Bladder Cancer Prognosis and Treatment Response. Bladder Cancer 2016; 2: 77-89.

109. Seike M, Yanaihara N, Bowman ED et al. Use of a cytokine gene expression signature in lung adenocarcinoma and the surrounding tissue as a prognostic classifier. J Natl Cancer Inst 2007; 99: 1257-1269.

110. Puvvada SD, Funkhouser WK, Greene K et al. NF-kB and Bcl-3 activation are prognostic in metastatic colorectal cancer. Oncology 2010; 78: 181-188.

111. Mo M, Zhou M, Wang L et al. CCL21/CCR7 enhances the proliferation, migration, and invasion of human bladder cancer T24 cells. PLoS One 2015; 10: e0119506.

112. Zheng J, Zhu X, Zhang J. CXCL5 knockdown expression inhibits human bladder cancer T24 cells proliferation and migration. Biochem Biophys Res Commun 2014; 446: 18-24.

113. Mukherjee N, Houston TJ, Cardenas E, Ghosh R. To be an ally or an adversary in bladder cancer: the NF-kappaB story has not unfolded. Carcinogenesis 2015; 36: 299-306.

114. Czachorowski MJ, Amaral AF, Montes-Moreno S et al. Cyclooxygenase-2 expression in bladder cancer and patient prognosis: results from a large clinical cohort and metaanalysis. PLoS One 2012; 7: e45025.

115. Hilmy M, Campbell R, Bartlett JM et al. The relationship between the systemic inflammatory response, tumour proliferative activity, T-lymphocytic infiltration and COX-2 expression and survival in patients with transitional cell carcinoma of the urinary bladder. Br J Cancer 2006; 95: 1234-1238.

116. Dovedi SJ, Kirby JA, Davies BR et al. Celecoxib has potent antitumour effects as a single agent and in combination with BCG immunotherapy in a model of urothelial cell carcinoma. Eur Urol 2008; 54: 621-630.

117. Boorjian SA, Sheinin Y, Crispen PL et al. T-cell coregulatory molecule expression in urothelial cell carcinoma: clinicopathologic correlations and association with survival. Clin Cancer Res 2008; 14: 4800-4808.

118. Nakanishi J, Wada Y, Matsumoto K et al. Overexpression of B7-H1 (PD-L1) significantly associates with tumor grade and postoperative prognosis in human urothelial cancers. Cancer Immunol Immunother 2007; 56: 1173-1182.

119. Wang Y, Zhuang Q, Zhou S et al. Costimulatory molecule B7-H1 on the immune escape of bladder cancer and its clinical significance. J Huazhong Univ Sci Technolog Med Sci 2009; 29: 77-79.

120. Bellmunt J, Mullane SA, Werner L et al. Association of PD-L1 expression on tumorinfiltrating mononuclear cells and overall survival in patients with urothelial carcinoma. Ann Oncol 2015; 26: 812-817.

121. Kitamura H, Torigoe T, Honma I et al. Effect of human leukocyte antigen class I expression of tumor cells on outcome of intravesical instillation of bacillus calmette-guerin immunotherapy for bladder cancer. Clin Cancer Res 2006; 12: 4641-4644.

122. Levin I, Klein T, Goldstein J et al. Expression of class I histocompatibility antigens in transitional cell carcinoma of the urinary bladder in relation to survival. Cancer 1991; 68: 2591-2594.

 Sharma P, Shen Y, Wen S et al. CD8 tumor-infiltrating lymphocytes are predictive of survival in muscle-invasive urothelial carcinoma. Proc Natl Acad Sci U S A 2007; 104: 3967-3972.

124. Homma I, Kitamura H, Torigoe T et al. Human leukocyte antigen class I downregulation in muscle-invasive bladder cancer: its association with clinical characteristics and survival after cystectomy. Cancer Sci 2009; 100: 2331-2334.

125. Calderwood SK, Murshid A, Gong J. Heat shock proteins: conditional mediators of inflammation in tumor immunity. Front Immunol 3: 75.

126. Syrigos KN, Harrington KJ, Karayiannakis AJ et al. Clinical significance of heat shock protein-70 expression in bladder cancer. Urology 2003; 61: 677-680.

127. Yu HJ, Chang YH, Pan CC. Prognostic significance of heat shock proteins in urothelial carcinoma of the urinary bladder. Histopathology 62: 788-798.

128. Pages F, Kirilovsky A, Mlecnik B et al. In situ cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer. J Clin Oncol 2009; 27: 5944-5951.

129. Galon J, Pages F, Marincola FM et al. Cancer classification using the Immunoscore: a worldwide task force. J Transl Med 2012; 10: 205.

130. Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer 2012; 12: 298-306.

131. Masson-Lecomte A, Rava M, Real FX et al. Inflammatory biomarkers and bladder cancer prognosis: a systematic review. Eur Urol 2014; 66: 1078-1091.

132. Winerdal ME, Marits P, Winerdal M et al. FOXP3 and survival in urinary bladder cancer. BJU Int 108: 1672-1678.

133. Cai T, Nesi G, Boddi V et al. Prognostic role of the tumor-associated tissue inflammatory reaction in transitional bladder cell carcinoma. Oncol Rep 2006; 16: 329-334.

134. Flamm J, Havelec L. Factors affecting survival in primary superficial bladder cancer.Eur Urol 1990; 17: 113-118.

135. Offersen BV, Knap MM, Marcussen N et al. Intense inflammation in bladder carcinoma is associated with angiogenesis and indicates good prognosis. Br J Cancer 2002;87: 1422-1430.

136. Samaratunga H, Fairweather P, Purdie D. Significance of stromal reaction patterns in invasive urothelial carcinoma. Am J Clin Pathol 2005; 123: 851-857.

137. Sanchez de la Muela P, Rosell D, Aguera L et al. Superficial bladder cancer: survival and prognostic factors. Eur Urol 1991; 20: 184-191.

138. Flamm J. The value of tumor-associated tissue inflammatory reaction in primary superficial bladder cancer. Urol Res 1990; 18: 113-117.

139. Ayari C, LaRue H, Hovington H et al. Bladder tumor infiltrating mature dendritic cells and macrophages as predictors of response to bacillus Calmette-Guerin immunotherapy. Eur Urol 2009; 55: 1386-1395.

140. Maniecki MB, Etzerodt A, Ulhoi BP et al. Tumor-promoting macrophages induce the expression of the macrophage-specific receptor CD163 in malignant cells. Int J Cancer 2012; 131: 2320-2331.

141. Ayari C, LaRue H, Hovington H et al. High level of mature tumor-infiltrating dendritic cells predicts progression to muscle invasion in bladder cancer. Hum Pathol 2013;44: 1630-1637.

142. Takayama H, Nishimura K, Tsujimura A et al. Increased infiltration of tumor associated macrophages is associated with poor prognosis of bladder carcinoma in situ after intravesical bacillus Calmette-Guerin instillation. J Urol 2009; 181: 1894-1900.

143. Hanada T, Nakagawa M, Emoto A et al. Prognostic value of tumor-associated macrophage count in human bladder cancer. Int J Urol 2000; 7: 263-269.

144. Ayari C, LaRue H, Hovington H et al. High level of mature tumor-infiltrating dendritic cells predicts progression to muscle invasion in bladder cancer. Hum Pathol 44: 1630-1637.

145. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003; 299: 1057-1061.

146. deLeeuw RJ, Kost SE, Kakal JA, Nelson BH. The prognostic value of FoxP3+ tumor-infiltrating lymphocytes in cancer: a critical review of the literature. Clin Cancer Res 2012;
18: 3022-3029.

147. Winerdal ME, Marits P, Winerdal M et al. FOXP3 and survival in urinary bladder cancer. BJU Int 2011; 108: 1672-1678.

148. Allin KH, Bojesen SE, Nordestgaard BG. Baseline C-reactive protein is associated with incident cancer and survival in patients with cancer. J Clin Oncol 2009; 27: 2217-2224.

149. Andrews B, Shariat SF, Kim JH et al. Preoperative plasma levels of interleukin-6 and its soluble receptor predict disease recurrence and survival of patients with bladder cancer. J Urol 2002; 167: 1475-1481.

150. Jee SH, Shen SC, Chiu HC et al. Overexpression of interleukin-6 in human basal cell carcinoma cell lines increases anti-apoptotic activity and tumorigenic potency. Oncogene 2001; 20: 198-208.

151. Kinoshita T, Ito H, Miki C. Serum interleukin-6 level reflects the tumor proliferative activity in patients with colorectal carcinoma. Cancer 1999; 85: 2526-2531.

152. Tanaka N, Kikuchi E, Shirotake S et al. The Predictive Value of C-reactive Protein for Prognosis in Patients with Upper Tract Urothelial Carcinoma Treated with Radical Nephroureterectomy: A Multi-institutional Study. Eur Urol 2012.

153. Scambia G, Testa U, Benedetti Panici P et al. Prognostic significance of interleukin 6 serum levels in patients with ovarian cancer. Br J Cancer 1995; 71: 354-356.

154. Stark JR, Li H, Kraft P et al. Circulating prediagnostic interleukin-6 and C-reactive protein and prostate cancer incidence and mortality. Int J Cancer 2009; 124: 2683-2689.

155. Nakagawa T, Hara T, Kawahara T et al. Prognostic Risk Stratification of Patients with Urothelial Carcinoma of the Urinary Bladder who Developed Recurrence after Radical Cystectomy. J Urol 2012.

156. Saito K, Kihara K. C-reactive protein as a biomarker for urological cancers. Nat Rev Urol 2011; 8: 659-666.

157. Yoshida S, Saito K, Koga F et al. C-reactive protein level predicts prognosis in patients with muscle-invasive bladder cancer treated with chemoradiotherapy. BJU Int 2008; 101: 978-981.

158. Trichopoulos D, Psaltopoulou T, Orfanos P et al. Plasma C-reactive protein and risk of cancer: a prospective study from Greece. Cancer Epidemiol Biomarkers Prev 2006; 15: 381-384.

159. Gakis G, Todenhofer T, Renninger M et al. Development of a new outcome prediction model in carcinoma invading the bladder based on preoperative serum C-reactive protein and standard pathological risk factors: the TNR-C score. BJU Int 2011; 108: 1800-1805.

160. Gakis G, Todenhofer T, Renninger M et al. Development of a new outcome prediction model in carcinoma invading the bladder based on preoperative serum C-reactive protein and standard pathological risk factors: the TNR-C score. BJU Int 108: 1800-1805.

161. Gondo T, Nakashima J, Ohno Y et al. Prognostic value of neutrophil-to-lymphocyte ratio and establishment of novel preoperative risk stratification model in bladder cancer patients treated with radical cystectomy. Urology 79: 1085-1091.

162. Hwang EC, Hwang IS, Yu HS et al. Utility of inflammation-based prognostic scoring in patients given systemic chemotherapy first-line for advanced inoperable bladder cancer. Jpn J Clin Oncol 42: 955-960.

163. Ishioka J, Saito K, Sakura M et al. Development of a nomogram incorporating serum C-reactive protein level to predict overall survival of patients with advanced urothelial carcinoma and its evaluation by decision curve analysis. Br J Cancer 107: 1031-1036.

164. Nakagawa T, Hara T, Kawahara T et al. Prognostic risk stratification of patients with urothelial carcinoma of the bladder with recurrence after radical cystectomy. J Urol 189: 1275-1281.

165. Saito K, Kawakami S, Ohtsuka Y et al. The impact of preoperative serum C-reactive protein on the prognosis of patients with upper urinary tract urothelial carcinoma treated surgically. BJU Int 2007; 100: 269-273.

166. Lin CT, Tung CL, Tsai YS et al. Prognostic relevance of preoperative circulating CD8-positive lymphocytes in the urinary bladder recurrence of urothelial carcinoma. Urol Oncol 30: 680-687.

167. Gondo T, Nakashima J, Ohno Y et al. Prognostic value of neutrophil-to-lymphocyte ratio and establishment of novel preoperative risk stratification model in bladder cancer patients treated with radical cystectomy. Urology 2012; 79: 1085-1091.

168. Hermanns T, Bhindi B, Wei Y et al. Pre-treatment neutrophil-to-lymphocyte ratio as predictor of adverse outcomes in patients undergoing radical cystectomy for urothelial carcinoma of the bladder. Br J Cancer 2014; 111: 444-451.

169. van Kessel KE, de Haan LM, Fransen van de Putte EE et al. Elevated Derived Neutrophil-to-Lymphocyte Ratio Corresponds With Poor Outcome in Patients Undergoing Pre-Operative Chemotherapy in Muscle-Invasive Bladder Cancer. Bladder Cancer 2016; 2: 351-360.

170. Viers BR, Boorjian SA, Frank I et al. Pretreatment Neutrophil-to-Lymphocyte Ratio Is Associated with Advanced Pathologic Tumor Stage and Increased Cancer-specific Mortality Among Patients with Urothelial Carcinoma of the Bladder Undergoing Radical Cystectomy. Eur Urol 2014.

171. Dalpiaz O, Pichler M, Mannweiler S et al. Validation of the pretreatment derived neutrophil-lymphocyte ratio as a prognostic factor in a European cohort of patients with upper tract urothelial carcinoma. Br J Cancer 2014; 110: 2531-2536.

172. Sung HH, Jeon HG, Jeong BC et al. Clinical significance of prognosis of the neutrophil-lymphocyte ratio and erythrocyte sedimentation rate in patients undergoing radical nephroureterectomy for upper urinary tract urothelial carcinoma. BJU Int 2014.
173. Tanaka N, Kikuchi E, Kanao K et al. A Multi-Institutional Validation of the Prognostic Value of the Neutrophil-to-Lymphocyte Ratio for Upper Tract Urothelial Carcinoma Treated with Radical Nephroureterectomy. Ann Surg Oncol 2014; 21: 4041-4048.
174. Mano R, Baniel J, Shoshany O et al. Neutrophil-to-lymphocyte ratio predicts progression and recurrence of non-muscle-invasive bladder cancer. Urol Oncol 2015; 33: 67 e61-67.

175. Mbeutcha A, Shariat SF, Rieken M et al. Prognostic significance of markers of systemic inflammatory response in patients with non-muscle-invasive bladder cancer. Urol Oncol 2016.

176. Wei Y, Jiang YZ, Qian WH. Prognostic role of NLR in urinary cancers: a metaanalysis. PLoS One 2014; 9: e92079.

177. Taniguchi K, Koga S, Nishikido M et al. Systemic immune response after intravesical instillation of bacille Calmette-Guerin (BCG) for superficial bladder cancer. Clin Exp Immunol 1999; 115: 131-135.

178. Saint F, Patard JJ, Maille P et al. Prognostic value of a T helper 1 urinary cytokine response after intravesical bacillus Calmette-Guerin treatment for superficial bladder cancer. J Urol 2002; 167: 364-367.

179. Saint F, Patard JJ, Maille P et al. T helper 1/2 lymphocyte urinary cytokine profiles in responding and nonresponding patients after 1 and 2 courses of bacillus Calmette-Guerin for superficial bladder cancer. J Urol 2001; 166: 2142-2147.

180. Rigaud J, Leger A, Devilder MC et al. Development of Predictive Value of Urinary Cytokine Profile Induced During Intravesical Bacillus Calmette-Guerin Instillations for Bladder Cancer. Clin Genitourin Cancer 2015; 13: e209-215. 181. Masson-Lecomte A, Vordos D, Yiou R et al. [Oncological outcome of radical cystectomy for BCG failure compared to primary invasive disease]. Prog Urol 2013; 23: 456-463.

182. Albert JH, Chib S. Sequential ordinal modeling with applications to survival data. Biometrics 2001; 57: 829-836.

183. Sorensen D, Gianola D. Likelihood, Bayesian, and MCMC Methods in Quantitative Genetics. New York: Springer Science & Business Media,2002.

184. Lopez de Maturana E, Ibanez-Escriche N, Gonzalez-Recio O et al. Next generation modeling in GWAS: comparing different genetic architectures. Hum Genet 2014; 133: 1235-1253.

185. Geweke J. Bayesian Statistics. In. 1992; 169-193.

186. Che X, Xu S. Significance test and genome selection in bayesian shrinkage analysis.Int J Plant Genomics 2010; 2010: 893206.

187. Park JC, Hahn NM. Emerging role of immunotherapy in urothelial carcinoma-Future directions and novel therapies. Urol Oncol 2016; 34: 566-576.

188. Garcia-Closas M, Malats N, Silverman D et al. NAT2 slow acetylation, GSTM1 null genotype, and risk of bladder cancer: results from the Spanish Bladder Cancer Study and meta-analyses. Lancet 2005; 366: 649-659.

189. Rothman N, Garcia-Closas M, Chatterjee N et al. A multi-stage genome-wide association study of bladder cancer identifies multiple susceptibility loci. Nat Genet 2010; 42: 978-984.

190. Carosella ED, Ploussard G, LeMaoult J, Desgrandchamps F. A Systematic Review of Immunotherapy in Urologic Cancer: Evolving Roles for Targeting of CTLA-4, PD-1/PD-L1, and HLA-G. Eur Urol 2015; 68: 267-279.

191. Xylinas E, Robinson BD, Kluth LA et al. Association of T-cell co-regulatory protein expression with clinical outcomes following radical cystectomy for urothelial carcinoma of the bladder. Eur J Surg Oncol 2014; 40: 121-127.

192. Faraj SF, Munari E, Guner G et al. Assessment of tumoral PD-L1 expression and intratumoral CD8+ T cells in urothelial carcinoma. Urology 2015; 85: 703 e701-706.

193. Huang Y, Zhang SD, McCrudden C et al. The prognostic significance of PD-L1 in bladder cancer. Oncol Rep 2015; 33: 3075-3084.

194. Ribas A, Hu-Lieskovan S. What does PD-L1 positive or negative mean? The Journal of Experimental Medicine 2016.

195. Antony PA, Piccirillo CA, Akpinarli A et al. CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells. J Immunol 2005; 174: 2591-2601.

196. Dobrzanski MJ, Reome JB, Dutton RW. Type 1 and type 2 CD8+ effector T cell subpopulations promote long-term tumor immunity and protection to progressively growing tumor. J Immunol 2000; 164: 916-925.

197. Gooden MJ, de Bock GH, Leffers N et al. The prognostic influence of tumourinfiltrating lymphocytes in cancer: a systematic review with meta-analysis. Br J Cancer 2011; 105: 93-103.

198. Hedegaard J, Lamy P, Nordentoft I et al. Comprehensive Transcriptional Analysis of Early-Stage Urothelial Carcinoma. Cancer Cell 2016; 30: 27-42.

199. Patschan O, Sjodahl G, Chebil G et al. A Molecular Pathologic Framework for Risk
Stratification of Stage T1 Urothelial Carcinoma. Eur Urol 2015; 68: 824-832; discussion 835826.

200. Rubin MA, Dunn R, Strawderman M, Pienta KJ. Tissue microarray sampling strategy for prostate cancer biomarker analysis. Am J Surg Pathol 2002; 26: 312-319.

201. Romano E, Romero P. The therapeutic promise of disrupting the PD-1/PD-L1 immune checkpoint in cancer: unleashing the CD8 T cell mediated anti-tumor activity results in significant, unprecedented clinical efficacy in various solid tumors. J Immunother Cancer 2015; 3: 15.

202. Otto W, Denzinger S, Wieland WF, Hartmann A. First analysis of immune cell infiltration in stage pT1 urothelial bladder carcinoma: CD3 positivity as a prognostic marker for cancer-specific survival. World J Urol 2012; 30: 875-877.

203. Sjodahl G, Lovgren K, Lauss M et al. Infiltration of CD3 and CD68 cells in bladder cancer is subtype specific and affects the outcome of patients with muscle-invasive tumors. Urol Oncol 2014.

204. Pichler R, Fritz J, Zavadil C et al. Tumor-infiltrating immune cell subpopulations influence the oncologic outcome after intravesical bacillus calmette-guerin therapy in bladder cancer. Oncotarget 2016.

205. Horn T, Laus J, Seitz AK et al. The prognostic effect of tumour-infiltrating lymphocytic subpopulations in bladder cancer. World J Urol 2016; 34: 181-187.

206. Pineda S, Real FX, Kogevinas M et al. Integration Analysis of Three Omics Data Using Penalized Regression Methods: An Application to Bladder Cancer. PLoS Genet 2015; 11: e1005689.

207. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science 2011; 331: 1565-1570.

208. Mescher MF, Popescu FE, Gerner M et al. Activation-induced non-responsiveness (anergy) limits CD8 T cell responses to tumors. Semin Cancer Biol 2007; 17: 299-308.

209. Noessner E, Brech D, Mendler AN et al. Intratumoral alterations of dendritic-cell differentiation and CD8(+) T-cell anergy are immune escape mechanisms of clear cell renal cell carcinoma. Oncoimmunology 2012; 1: 1451-1453.

210. Liu H, Liu J, Toups M et al. Gene signature-based mapping of immunological systems and diseases. BMC Bioinformatics 2016; 17: 171.

211. Touzot M, Dahirel A, Cappuccio A et al. Using Transcriptional Signatures to Assess Immune Cell Function: From Basic Mechanisms to Immune-Related Disease. J Mol Biol 2015; 427: 3356-3367.

212. Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. Immunity 2013; 39: 1-10.

213. van der Horst G, Bos L, van der Pluijm G. Epithelial plasticity, cancer stem cells, and the tumor-supportive stroma in bladder carcinoma. Mol Cancer Res 2012; 10: 995-1009.

214. Steyerberg EW, Vickers AJ, Cook NR et al. Assessing the performance of prediction models: a framework for traditional and novel measures. Epidemiology 2010; 21: 128-138.

215. Hendriksen JM, Geersing GJ, Moons KG, de Groot JA. Diagnostic and prognostic prediction models. J Thromb Haemost 2013; 11 Suppl 1: 129-141.

216. Italiano A. Prognostic or predictive? It's time to get back to definitions! J Clin Oncol2011; 29: 4718; author reply 4718-4719.

217. Plimack ER, Bellmunt J, Gupta S et al. Safety and activity of pembrolizumab in patients with locally advanced or metastatic urothelial cancer (KEYNOTE-012): a non-randomised, open-label, phase 1b study. Lancet Oncol 2017; 18: 212-220.

218. Sharma P, Callahan MK, Bono P et al. Nivolumab monotherapy in recurrent metastatic urothelial carcinoma (CheckMate 032): a multicentre, open-label, two-stage, multi-arm, phase 1/2 trial. Lancet Oncol 2016; 17: 1590-1598.

VII APPENDIX: Inflammatory Biomarkers and Bladder Cancer Prognosis: a systematic review. *A Masson-Lecomte et al. Eur Urol 2014*

available at www.sciencedirect.com journal homepage: www.europeanurology.com



Review – Bladder Cancer

Inflammatory Biomarkers and Bladder Cancer Prognosis: A Systematic Review

Alexandra Masson-Lecomte^{*a,b*}, Marta Rava^{*a*}, Francisco X. Real^{*c,d*}, Arndt Hartmann^{*e*}, Yves Allory^{*a,f*}, Núria Malats^{*a,**}

^a Genetic and Molecular Epidemiology Group, Spanish National Cancer Research Centre (CNIO), Madrid, Spain; ^b Urology Department, Henri Mondor Academic Hospital, INSERM U955Eq7, Créteil, France; ^c Epithelial Carcinogenesis Group, Spanish National Cancer Research Centre (CNIO), Madrid, Spain; ^d Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Barcelona, Spain; ^e Department of Pathology, University Erlangen-Nürnberg, Erlangen, Germany; ^f Pathology Department, Henri Mondor Academic Hospital, INSERM U955Eq7, Créteil, France

Article info

Article history: Accepted July 25, 2014

Keywords:

Bladder cancer Inflammation Biomarkers Progression Survival

Abstract

Context: Host immune response has an impact on tumour development and progression. There is interest in the use of inflammatory biomarkers (InfBMs) in cancer care. Although several studies assessing the potential prognostic value of InfBMs in cancer have been published in the past decades, they have had no impact on the management of patients with urothelial bladder carcinoma (UBC). *Objective:* To review and summarise the scientific literature on the prognostic value of

Objective: To review and summarise the scientific literature on the prognostic value of tumour, serum, urine, and germline DNA InfBMs on UBC.

Evidence acquisition: A systematic review of the literature was performed searching the Medline and Embase databases for original articles published between January 1975 and November 2013. The main inclusion criterion was the provision of a survival analysis (Kaplan-Meier and/or Cox) according to the Reporting Recommendations for Tumor Marker Prognostic Studies guidelines for the assessment of prognostic markers. We focused on markers assessed at least twice in the literature. Findings are reported following Preferred Reporting Items for Systematic Reviews and Meta-analysis guidelines.

Evidence synthesis: Overall, 34 publications, mostly retrospective, fulfilled the main inclusion criterion. Main limitations of these studies were missing relevant information about design or analysis and heterogeneous methodology used. Inflammatory cells, costimulatory molecules in tumour cells, and serum cytokines showed prognostic significance, mainly in univariable analyses. High C-reactive protein values were consistently reported as an independent prognostic factor for mortality in invasive UBC. *Conclusions:* There is a dearth of studies on InfBMs in UBC compared with other tumour types. Evidence suggests that InfBMs may have an impact on the management of patients with UBC. Currently, methodological drawbacks of the studies limit the translational potential of results.

Patient summary: In this review, we analysed studies evaluating the impact of inflammatory response on bladder cancer progression. Despite methodological limitations, some inflammatory biomarkers should be further analysed because they hold promise to improve patient care.

© 2014 European Association of Urology. Published by Elsevier B.V. All rights reserved.

* Corresponding author. Genetic and Molecular Epidemiology Group, Spanish National Cancer Research Centre (CNIO), C/Melchor Fernández Almagro, 3, 28029 Madrid, Spain. Tel. +34 912 246 900 ext. 3330; Fax: +34 912 246 911. E-mail address: nmalats@cnio.es (N. Malats).





1. Introduction

Evading the immune system is one of the emerging hallmarks of cancer [1]. It is well established that the inflammatory microenvironment has an impact on tumour prognosis, either positively or negatively [2]. The proper assessment of the composition and function of the microenvironment is challenging, and consensus is needed in the field about how to best consider the inflammatory response as a component of tumour subclassification [3]. In this regard, melanoma, colon, and breast cancer have taken the lead [4–6].

The definition of inflammatory biomarkers (InfBMs) is in itself challenging. Any molecule involved in innate or adaptive immune response could be considered; this makes the list of candidates very long, and it is difficult to establish a definition of InfBMs due to the interaction between inflammatory pathways and other cellular functions. In this review, we focus on markers with the primary known function in the immune response.

Urothelial bladder carcinoma (UBC) is highly prevalent. It represents an important economic burden, affects patient quality of life, and is life threatening when it invades muscle. However, in many ways, UBC remains a neglected disease [7]. The dearth of information on InfBMs and UBC is paradoxical, considering that UBC is one of the few tumours for which there is long-standing evidence of the efficacy of immunotherapy.

Studies on the prognostic value of InfBMs in UBC have been published since the 1970s [8–10]. The infiltration of the tumour by inflammatory cells and their association with prognosis has been explored more extensively than blood or urine cytokine levels and germline DNA polymorphisms in inflammatory genes. Unfortunately, none of these markers has proven to be sufficiently useful for clinical application. Methodological flaws, technical heterogeneity, and lack of appropriately designed validation studies have been the most important limitations. Guidelines were published in 2005 to improve the reporting of prognostic markers, but



Fig. 1 – Integrative scope of inflammatory biomarkers to support urothelial bladder cancer patient prognostication and treatment response prediction. unfortunately they are rarely followed [11]. We report a systematic review of the published results and methods applied in studies that assessed tumour, blood, urine, and germline DNA InfBMs related to the prognosis of patients with UBC. The review was conducted following the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guidelines criteria. The ultimate goals of this effort were to provide a rationale for promoting research in the promising field of immunity and UBC, to identify the main limitations of the studies performed, and to select the most promising markers for prospective studies and clinical trials through an integrative scope (Fig. 1).

2. Evidence acquisition

2.1. Material and methods

2.1.1. Information sources and eligibility criteria

The Medline, Medline In-Process, and Embase databases were searched for all original articles published from January 1975 to November 2013 on the topic of interest. Medline was searched through PubMed. Reporting followed the Preferred Reporting Items for Systematic Reviews and Meta-analysis guidelines.

The inclusion criteria were (1) original article, (2) human research, (3) English language, (4) accessibility to the full manuscript, and (5) availability of Kaplan-Meier/Cox regression-derived results about the prognostic value of the InfBMs on UBC outcomes according to the REMARKS guidelines for assessment of a prognostic marker [12]. Studies using association tests instead of survival analysis, with or without adjustment for other relevant variables, were not included, but they are listed in Supplementary Table 1. The outcomes considered were recurrence and progression for non-muscle-invasive bladder cancer (NMIBC) and local progression, metastasis, and cancerspecific mortality and overall mortality for NMIBC and muscle-invasive bladder cancer (MIBC). We report on studies assessing an InfBM twice or more in the literature. All other studies are listed in Supplementary Table 1.

2.1.2. Search strategy

We searched PubMed using the controlled vocabulary of the Medical Subject Heading database along with open text. The algorithm applied was (bladder OR urothelium OR transitional cell) AND (cancer OR tumour OR tumour OR carcinoma OR neoplasm) AND (inflammation OR inflammatory OR immune OR immunity) AND (prognosis OR survival OR recurrence OR progression). The search in Embase used the Emtree vocabulary 'bladder cancer' AND ('inflammation' OR 'immunity') AND 'prognosis'. All selected articles were further hand-searched to identify additional relevant articles.

2.1.3. Study selection and the data collection process

The first stage of the search in Medline and Embase was performed by A.M.L. to screen and exclude studies unrelated to UBC or InfBMs. Second-stage selection was performed by four investigators (A.M.L., Y.A., F.X.R., and N.M.).

Information was retrieved according to the REMARK guidelines for reporting prognostic markers including author, country, journal, publication year, marker examined, study design, study population (sample size, recruitment period, and follow-up), patient characteristics (age, gender, stage, comorbidities), treatment received, biologic material/matrix used (urine, tumour, serum, saliva), methodology (for urine and blood markers: dosage kit for the marker, period of retrieval, and cut-off for positivity; for immunohistochemistry (IHC) on tumour tissue: antibody, area of interest on the slide, magnification, scoring, cut-off for positivity, and percentage of positive tumours; for germline DNA variants/single-nucleotide polymorphism (SNP): gene name, and genotyping technique), statistical method applied (with variables used for adjustment), and reported impact of examined markers on UCB outcome using univariable or multivariable survival analyses.

2.1.4. Meta-analysis

We conducted a meta-analysis to summarise quantitatively the overall prognostic value of serum C-reactive protein (CRP) because this was the most frequently studied marker in association with UBC outcome (eight studies). One author (Pr. Saito [24]) was directly contacted to obtain data required for the meta-analysis. Two studies could not be included due to the lack of important information; the results of Ishioka et al. [22] could not be included because the variable was not log-transformed. Finally, we were obliged to stratify the meta-analysis according to whether the studies used a dichotomous variable (two studies) or a continuous variable (three studies) to assess CRP. Random effect meta-analysis was performed as a sensitivity analysis. We quantified heterogeneity using the I² statistic [12] that describes the proportion of heterogeneity across studies that is not due to chance. The analysis showed no heterogeneity between the studies (I² = 0% for both type of studies). Consequently, a fixed effect model was applied. Risk of publication/reporting bias across studies is likely, although we could not test it appropriately because of the small number of studies included [13]. Analyses were done using R v.3.0.1 software.

3. Evidence synthesis

A total of 1045 original articles were identified using Medline and Medline In-Process and 1651 using Embase. Figure 2 shows a flow diagram of the study selection strategy. At the end of the process, there were 87 articles assessing the association between germline DNA, blood, urinary, or tumour InfBMs, and UBC outcome (77 from the online search abstract screening and 10 added from reference list screening). From those, 23 articles lacked a



Fig. 2 – Flow diagram showing the study selection process. BCG = bacillus Calmette-Guérin; COX = cyclooxygenase; IL = interleukin.

survival analysis, as defined in the REMARKS criteria, and 24 investigated a marker that had only been reported once in the UBC domain (see Supplementary Table 1). Six studies evaluated the association of cyclooxygenase-2 tumour expression and prognosis. Our group recently published a meta-analysis on this topic [14]; therefore, these studies were not considered in this review. Finally, 34 original articles assessing 13 InfBMs (germline DNA variants in interleukin [IL]-6 and tumour necrosis factor [TNF]- α ; serum CRP, IL-6, and CD8 levels, and neutrophil-to-lymphocyte ratio (NLR); and expression of CD3, CD8, CD68, CD83, FOXP3, B7-H1 (PD-L1), HLA class I molecules, HSP70, as well as "inflammatory infiltrate" in tumour samples) were included. None of the urinary InfBMs fulfilled the main study criteria.

3.1. Study methodology

There were 8 and 18 prospective and retrospective studies, respectively. This information was missing in eight studies. Only one article provided a rationale for sample size and statistical power. Studies included in the main tables were published between 1990 and 2013. Patient recruitment period ranged from 1971 to 2010. Median number of patients included was 69 (range: 30–530). Median age of the patients was 67 yr (range: 23–93 yr). Median follow-up was 29 mo (range: 1–240 mo) (Table 1).

Missing information about study or patient characteristics was common: 11 (33%), 10 (30%), 5 (15%), 10 (30%), and 28 (84%) studies lacked information on age, gender, stage, follow-up, and comorbidities, respectively. Treatment information was provided by 31 studies (91%). When assessing germline DNA variants, all studies included NMIBC patients treated with bacillus Calmette-Guérin (BCG). Two studies also included a second cohort of cystectomised patients. For blood biomarkers, CRP was mainly investigated in patients undergoing radical cystectomy (RC) or among those with locally advanced/recurrent UBC treated by chemotherapy. NMIBCs were considered together with MIBC in two studies. Note that, in the study by Himly et al., pT1G3 patients were considered as having muscle-invasive UBC. For tumour markers, cohorts included both NMIBC and MIBC, separately or merged (Table 1).

3.2. Assay methodology

The three studies considering germline DNA biomarkers analysed candidate SNPs in blood-extracted DNA. Genotyping was performed using methods based on polymerase chain reaction methods. Regarding blood biomarkers (11 studies), the dosage technique/kit was specified in four of eight studies on CRP. The threshold chosen to define positivity of CRP ranged from 0.5 to 1 mg/dl. In all studies, CRP was assessed before treatment (transurethral resection of the bladder [TURB] for NIMBC; RC or chemotherapy for MIBC). Two of eight studies considered CRP on a continuous scale. One study assessed serum IL-6 and categorised the levels using a threshold of 4.80 pg/ml (median value). NLR was assessed in two studies; both applied the same threshold (2.5). One study assessed the percentage of blood CD8 lymphocytes using flow cytometry; no further specification was given about the technique or threshold chosen for the analysis (Table 2).

As for tumour InfBMs (20 studies), all markers were assessed by IHC. Overall, reporting of technique and interpretation of results were heterogeneous. Full sections and tissue microarrays were used in 12 and 2 studies, respectively. This information was missing in five studies. Only seven studies reported how the region and compartment (tumoural/stroma) of interest were selected. Definition of a high-power field varied from \times 200 to \times 1000 magnification. Quantification of staining was highly variable. In six studies, no information was provided about the method of quantification. Similarly, six studies lacked information regarding the cut-off applied in survival analysis or the reference value chosen for Cox regression analysis. Five studies reported "inflammatory infiltrate" as a marker without further characterisation. The definition of "strong inflammatory infiltrate" varied widely (Table 3).

3.3. Study findings

InfBMs identified from the literature could be grouped in three large categories according to their biologic significance: inflammatory cells, inflammatory costimulatory molecules in tumour cells, and serum cytokines. Table 4 provides a detailed list of the biomarkers according to these three categories.

3.3.1. Germline DNA inflammatory biomarkers

Two studies assessed the association between germline IL-6 rs1800795 variant and UBC outcome [15,16] (Table 5). Ahirwar et al. found a decreased risk of NMIBC recurrence among carriers (hazard ratio [HR]: 0.41; 95% confidence interval [CI], 0.17–0.94) [15]; Leibovici et al. described a 4.6-fold higher risk of recurrence among high-risk NMIBC carriers receiving maintenance BCG therapy (n = 38) [16]. As for TNF- α , two polymorphisms were studied (rs1800629 and rs1799964) [16,17]. Both studies found that the SNP considered was associated with a decreased risk of recurrence among patients with NMIBC. However, the findings among patients with MIBC were discordant.

3.3.2. Blood inflammatory biomarkers

CRP was the most widely studied serum marker. Eight studies assessed the association of high CRP levels with overall or cancer-specific survival [18–25]. All of them reported consistent results showing that high CRP levels are associated with adverse outcome. In one study, the association was only significant in univariable analysis [21]. Another study lacked multivariable analysis [19]. In the six remaining studies, three using dichotomised CRP levels and three using a continuous variable, CRP was an independent prognostic factor for both cancer-specific and overall mortality, although variables for adjustment varied among studies (Table 5; Supplementary Fig. 1 and 2). CD8 cell count was assessed as a serum marker using flow cytometry [26]. Authors observed that serum CD8 was

Table 1 – Study and patients	characteristics of the 34 studies
------------------------------	-----------------------------------

Study	Marker	Country	No. of patients	NMIBC/ MIBC	BCG	Recruitment period	Age, yr (range)	Male/ female, no.	Follow-up (range)	Treatment								
						Germline												
Ahirwar et al. [15]	IL-6 (rs1800795)	India	136	136/0	69	2004-2007	62 (NA)	119/17	13 (3-60)	TURB \pm iBCG								
Leibovici et al. [16]	IL-6 (rs1800795)	USA	353	204/149	123	1995-2003	NA	NA	21 (1-74)	TURB \pm iBCG or mBCG or RC								
Leibovici et al. [16]	TNF-α (rs1800629)	USA	465	204/146	123	1995-2003	NA	NA	21 (1-74)	$\text{TURB}\pm\text{iBCG or mBCG or RC}$								
Ahirwar et al. [17]	TNF-α (rs1799964)	India	73	73/0	73	2003-2007	61 (NA)	NA	14 (3-60)	TURB and iBCG								
						Serum												
Hwang et al [21]	CRP	lanan	67	0/67	0	2004-2010	71(45-86)	53/14	11 (2 5-46 5)	Chemotherany ±								
Yoshida et al. [25]	CRP	Japan	88	0/88	0	1997-2006	70 (63-75)	63/25	33 (3-117)	Radiochemotherapy								
Hilmy et al. [20]	CRP	UK	103	61/42	6	1992-2001	NA	70/33	60 (NA)	NA								
Gakis et al. [18]	CRP	Germany	246	0/246	26	1999-2009	67 (43-84)	191/55	30 (6-116)	RC								
Ishioka et al. [22]	CRP	lapan	232	0/232	NA	1995-2010	71 (66-77)	162/70	11 (NA)	Radiochemotherapy or BSC [†]								
Nakagawa et al. [23]	CRP	lapan	114	NA	16	1990-2010	67 (32-84)	92/22	11 (0.2-206)	RC [‡]								
Saito et al. [24]	CRP	Japan	80	NA	NA	2000-2009	NA	57/23	12 (2-99)	Second-line chemo after RC								
Gondo et al. [19]	CRP	Japan	189	62/127	NA	2000-2009	68 (38-85)	158/31	25 (2-128)	RC								
Gondo et al. [19]	NLR	Japan	189	62/127	NA	2000-2009	68 (38-85)	158/31	25 (2-128)	RC								
Krane et al. [27]	NLR	USA	68	NA	NA	2005-2011	67.4 (NA)	55/13	25 (13-61)	RC								
Andrews et al. [66]	IL-6	USA	51	15/36	24	1995-2000	65 (41-76)	47/4	46 (4-61)	RC								
Lin et al. [26]	CD8	Taiwan	68	NA	NA	2007-2008	67 (26-90)	NA	NA	TURB								
						Tumour												
Otto et al. [67]	CD3	Germany	61	61/0	NA	1995-1998	NA	NA	74 (11-179)	TURB								
Winerdal et al. [29]	CD3	Sweden	37	4/33	NA	1999-2002	67 (46-81)	NA	NA	RC								
Sharma et al. [28]	CD8	USA	69	38/31	NA	1996-2001	NA	51/18	NA	TURB or RC								
Kitamura et al. [41]	CD8	Japan	30	30/0	30	NA	NA	NA	NA	TURB and iBCG								
Hanada et al. [39]	CD68	Japan	63	40/23	NA	NA	65 (34-84)	51/12	65 (3-153)	TURB or RC								
Kitamura et al. [41]	CD68	Japan	30	30/0	30	NA	NA	NA	NA	TURB and iBCG								
Takayama et al. [38]	CD68	Japan	41	41 CIS/0	41	1995-2005	70 (47–91)	36/5	53 (3-120)	TURB and iBCG								
Ayari et al. [36]	CD68	Canada	46	46/0	46	1997-2002	68 (NA)	NA	31 (NA)	TURB and mBCG								
Ayari et al. [37]	CD68	Canada	93	93/0	8	1990-1992	NA	NA	66 (NA)	TURB								
Ayari et al. [36]	CD83	Canada	53	53/0	53	1997-2002	68 (NA)	NA	31 (NA)	TURB and mBCG								
Ayarı et al. [37]	CD83	Canada	93	93/0	8	1990-1992	NA CT (4C 01)	NA	66 (NA)	TURB								
Winerdal et al. [29]	FOXP3	Sweden	37	4/33	NA	1999-2002	67 (46-81)	NA	NA	RC								
Kitamura et al. [41]	FUXP3	Japan	30	30/0	30	NA 2000 2002	NA 62 (42, 78)	INA 40/10	NA 28 (C 52)	IURB and IBCG								
Wallg et al. [44]	D/-FT I	Japan	30	19/51	INA	2000-2002	62 (42-78)	40/10	28 (0-52)	NA BC								
Booljiali et al. [42]	D7-F11	USA	510	96/216	NA NA	1990-1994	09 (57-90)	230/00	NA 2C (1 110)	TUPP as PC								
Nakaliisii et al. [45]	D/-FII	Japan	60	20/29	INA	1996-2005	IN/A NIA	4//18	20 (1-116)	TURB of RC								
Sildi IIId et di. [26]	FILA CIdSS I	USA	09	0/22	INA	1990-2001	N/A N/A	51/16	NA NA	IURB OF RC								
Levill et al. [45]	FILA CIdSS I	Isidei	20	20/0	INA 20	NA NA	IN/A NIA	INA NA	INA NA	TURP and iPCC								
Kitdilluld et al. [41]	FILA CIdSS I	Japan	50	50/0	50	1001 2002	INA (55 (28 70)	NA 51/14	NA 25 (2, 172)	IURB allu IBCG								
Nu at al [40]	HLA CIdSS I	Japan	520	520/0	INA NA	1991-2002	72 (22 02)	31/14	35 (3-172) 86 (1, 340)	TIDD								
Surigos et al [49]	HSP70	Crooco	530	15/52	NA	1991-2005 NA	72 (25-92) NA	452/76 NA	80 (1-240) NA	IURD								
Cai at al [20]	Infl inflants	Greece	410	13/32	201	Inn Inn Den 1005	CR (22, 02)	200/104	NA NA	TUPP as PC								
Car et al. [30]	Infl infiltrate	Australia	410	521/96 NA	201	Jan-Dec 1995	72 (28, 01)	500/104	57 (5 169)	TIDD								
Offersen et al [32]	Infl infiltrate	Denmark	107	16/01	29	1007_1008	NA (28-91)	91/16	NA	TURB								
Sanchez et al [34]	Infl infiltrate	Spain	10/	10/51	NA	1952-1998	62 (18-03)	174/20	NΔ	TURB + instillations								
Flamm and Haveker	Infl infiltrate	Austria	345	345/0	NA	1970-1990	52 (18-55) 69 (NA)	235/110	NA	TURB + instillations								
[31]	nni, inintiate	Ausula	140	010+5	11/1	15/1=1502	05 (INA)	233/110	in	TOND T INSUNATIONS								
± = with or without; BSC = I Guérin; MIBC = muscle-inva	best supportive care; CIS sive bladder cancer; NA	= carcinoma in situ; CR = not available; NLR = r	P = C-reactive protein neutrophil-to-lympho	i; iBCG = induction bac cyte ratio; NMIBC = no	illus Calmette-Gué n-muscle-invasive	rin; IL = interleukin; Infl. bladder cancer; RC = rad	. = inflammatory; Jan dical cystectomy; TN	-Dec = January throug F = tumour necrosis f	gh December; mBCG = actor; TURB = transur	maintenance bacillus Calmette- ethral resection of the bladder.								
T1G3 tumours were cons	sidered MIBC.								TIG3 tumours were considered MIBC.									

[†] Series of advanced urothelial
 [‡] Series of recurrence after RC.

1082

Study	Marker	Threshold in analysis	Assessment period				
Hwang et al. [21]	CRP	1 mg/dl	1 d before first chemotherapy cycle				
Yoshida et al. [25]	CRP	0.5 mg/dl	Before radiochemotherapy				
Hilmy et al. [20]	CRP	1 mg/dl	Before transurethral resection				
Gakis et al. [18]	CRP	0.5 mg/dl or continuous	1-3 d before radical cystectomy				
Ishioka et al. [22]	Log CRP	Continuous, mg/l	Before chemo/radiation or best supportive care				
Nakagawa et al. [23]	CRP	0.5 mg/dl	At recurrence				
Saito et al. [24]	CRP	Continuous, mg/l	Before second-line treatment				
Gondo et al. [19]	CRP	0.5 mg/dl	Before radical cystectomy				
Gondo et al. [19]	NLR	2.5	Before radical cystectomy				
Krane et al. [27]	NLR	2.5	Before radical cystectomy				
Andrews et al. [66]	IL-6	4.80 pg/ml	Morning of radical cystectomy				
Lin et al. [26]	CD8	NA	Immediately before surgery				
CRP = C-reactive protein: IL = interleukin: NA = not applicable: NLR = neutrophil-to-lymphocyte ratio.							

Table 2 – Threshold and time of blood retrieval for serum markers

inversely correlated to tumour infiltration with CD8 cells ($r^2 = 0.63$; p < 0.0001). Low levels of CD8 cells in blood were associated with lower intravesical recurrence after TURB applying a multivariable analysis (HR: 0.4; 95% Cl, 0.17–0.94). Two studies reported prognostic value for NLR after RC for UCB [19,27]. With a threshold of 2.5, both studies observed that high NLR was an independent adverse prognostic factor for survival after RC (HR: 1.94; 95% Cl, 1.03–3.66, and HR: 2.49; 95% Cl, 1.14–6.09).

3.3.2.1. Tumour inflammatory biomarkers: inflammatory cells in the tumour environment. Tumour infiltration by CD3⁺ and CD8⁺ cells was associated with a better outcome (Table 6). All studies showed significant results in univariable analysis. Results were confirmed after adjustment for stage and other prognosticators only in the two studies assessing MIBC [28,29]. Winerdal et al. considered the impact of CD3 lymphocyte infiltration in the tumour of 37 patients treated by RC [29]. Strong infiltration was associated with increased overall survival (HR: 0.24; 95% CI, 0.08–0.71; *p* = 0.01). Sharma et al. studied CD8 infiltration in a series of 69 patients treated with TURB or RC [28]. HR for overall survival among MIBC patients with strong CD8 cell infiltration in the tumour on RC specimen was 0.3 (95% CI, 0.09–0.96). The presence of an "inflammatory infiltrate" without further characterisation of the cells has also been widely studied [30-34]. The main limitations of those studies were the heterogeneity in the definition of the studied populations and the term inflammatory infiltrate. Three studies including NMIBC and MIBC patients showed that strong inflammatory infiltrate was an independent good prognosticator for survival after adjustment for stage and other clinicopathologic factors [30,32,35]. Despite some caveats, the available evidence supports the notion that strong inflammatory infiltration in the tumour might be associated with a better prognosis, in concordance with the studies of CD3 and CD8. A precise characterisation of the inflammatory infiltrate in the tumour is required to draw further conclusions and establish mechanistic hypotheses.

It is difficult to come to a conclusion on the value of CD68 because of the heterogeneity of patient characteristics, tissue location, and methods of quantification. Ayari et al. combined the count in the tumour and in the stroma, whereas Takayama et al. did it separately [36–38]. A total of

three studies showed statistically significant results in multivariate analysis, with strong infiltration by CD68 macrophages associated with adverse outcome [36,38,39]. Ayari et al. described that CD68 infiltration in the tumour was associated with a 3.8-fold (95% CI, 1.32-11; p = 0.013) higher risk of recurrence after TURB and maintenance BCG in a series of patients with NMIBC [36]. This result was not validated by a subsequent publication of the same author in a series of patients not treated with BCG therapy [37]. Hanada et al. combined NMIBC and MIBC patients and showed that those presenting strong CD68 infiltration in the tumour had a fivefold higher risk of mortality [39]. However, the study lacked details about patient management and definition of survival end points. Finally, Takayama et al. published a study with 41 patients with carcinoma in situ (CIS) treated with TURB and induction BCG [38]. The end point was time to recurrence defined by positive cytology. In multivariate analysis adjusted for age and gender, high CD68 count in CIS regions (four or more CD68⁺ cells) was independently associated with recurrence (HR: 1.7). These authors also analysed CD68 counts in the lamina propria but found no association with recurrence. Together with CD68, Ayari et al. explored the impact of dendritic cell infiltration defined by CD83, and high levels of infiltration were associated with adverse outcome-recurrence after maintenance BCG [36]-and progression to muscle invasion [39]. Results were statistically significant, but the reliability of the risk estimates by Cox was questionable because of the small number of events (only one tumour recurred in the low CD83 group yielding an upper 95% CI of 85). Altogether these papers pinpoint a possible adverse effect of macrophage infiltration in tumours, but evidence remains weak.

The transcription factor FOXP3 is a master regulator of regulatory T cells (Treg) [40]. Two studies analysed tumour infiltration by FOXP3⁺ lymphocytes [29,41]. Both showed better survival in association with strong infiltration, although the findings were statistically significant, after adjustment, in only one of them (HR: 0.17; 95% CI, 0.05–0.6; p = 0.006, for overall survival) [29].

3.3.2.2. Tumour inflammatory biomarkers: costimulatory molecules in *tumour cells*. B7-H1 (PD-L1) is a T-cell coregulatory molecule. All three studies evaluating this marker showed that high

Study	Marker	Ab	FS/TMA	Region selection	Area of interest	Magnification	Quantification of staining	Cut-off for positivity/ Reference in survival analysis	% of PM
Otto et al. [67]	CD3	Rabbit mono.	FS	10 HPF in high CD3 + area	Tumour nest	×1000	No. of CD3 ⁺ cells	\leq 4 vs >4 CD3 + cells/HPF	NA
Winerdal et al. [29]	CD3	Mouse mono.	FS	Random selection of 3 HPF	Tumour nest	×400	No. of CD3 ⁺ cells	<3 vs≥3 CD3 + cells/field (mean)	NA
Sharma et al. [28]	CD8	Mouse mono.	FS	3 fields of 0.0625 mm ² in the high CD8 + area	Tumour nest	NA	No. of CD8 ⁺ cells	$<\!\!8 \text{ vs} \geq \!\!8 \text{ CD8} + \text{cells/field} \text{ (mean)}$	NA
Kitamura et al. [41]	CD8	Mono.	FS	NA	NA	×400	0-10 CD8 ⁺ cells/HPF = -; 10-50 = +; >50 = + +	NA	NA
Hanada et al. [39]	CD68	Mono.	FS	3 areas with the highest CD68 density	Tumour nest	×200	NA	$<\!67 \text{ vs} \geq\!\!67$	NA
Kitamura et al. [41]	CD68	Mono.	FS	NA	NA	$\times 400$	-	NA	NA
Takayama et al. [38]	CD68	Mono.	FS	6 random fields of 0.0625 mm ²	Stroma and TN	×400	No. of CD68 ⁺ cells in S and TN	${<}4~vs \geq \!\!4~TN/{<}24~vs \geq \!\!24~S$	61
Ayari et al. [36]	CD68	Mono.	FS	NA	Stroma and TN	×200	0 (no CD68 ⁺ cells),1 (1–5), 2 (6–10),3 (>10)	0-1 vs 2-3 (mean of S and TN)	46
Ayari et al. [37]	CD68	Mono.	FS	NA	Stroma and TN	×200	Same as 2009	0-1 vs 2-3 (mean of S and TN)	78
Ayari et al. [36]	CD83	Mono.	FS	NA	Stroma and TN	×200	0 (no CD68 ⁺ cells),1 (1–5), 2 (6–10), 3 (>10)	0-1 vs 2-3 (mean of S and TN)	54
Ayari et al. [37]	CD83	Mono.	FS	NA	Stroma and TN	×200	Same as 2009	0-1 vs 2-3 (mean of S and TN)	29
Winerdal et al. [29]	FOXP3	Mono. mouse	FS	Random selection of 3 HPF	Tumour nest	×400	No. of FOXP3 + cells	<3 vs \geq 3 FOXP3 + cells/field	NA
Kitamura et al. [41]	FOXP3	Mono.	FS	NA	NA	×400	Same as CD8 and CD68	NA	NA
Wang et al. [44]	B7-H1	Poly.	TMA	NA	NA	×400	% positive cells	>10%	NA
Boorjian et al. [42]	B7-H1	NA	FS	NA	NA	NA	% positive cells	>5%	12
Nakanishi et al. [43]	B7-H1	Mouse mono.	FS	Random selection of 3 HPF in high B7H1 + area	Tumour nest	×400	% positive cells	NA	NA
Sharma et al. [28]	HLA 1	Mono.	NA	NA	NA	NA	% positive cells	10%	NA
Levin et al. [45]	HLA 1	Rabbit	FS	NA	NA	NA	% positive cells	20%	57
Kitamura et al. [41]	HLA 1	Mono.	FS	NA	NA	×400	0,1-2 (low mb/cytoplasm),	0, 1, 2 vs 3	NA
							3 (mb and cytoplasm >80% cells)		
Homma et al. [46]	HLA 1	Mono.	FS	5 areas; no more precision	Tumour cells	×400	0-1 = no/incomplete mb/cytoplasm staining, 2 = >80% mb staining	0-1 vs 2	66.2
Yu et al. [49]	HSP70	NA	TMA	NA	NA	NA	Intensity $(0-3) \times \%$ stained cells	NA	NA
Sirigos et al. [48]	HSP70	Rabbit poly.	FS	NA	NA	NA	Intensity $(0-3) \times \%$ stained cells	$3 \times 25\%$	66
Cai et al. [30]	Infl. inf	NA	NA	NA	NA	×400	NA	>20 lymphocytes	29
Samaratunga et al. [33]	Infl. inf	NA	NA	NA	NA	NA	NA	Inflammation vs oedema	36
Offersen et al. [32]	Infl. inf	NA	NA	NA	NA	×200	NA	Stroma not visible	30
Sanchez et al. [34]	Infl. inf	NA	NA	NA	NA	NA	NA	NA	NA
Flamm and Havekec [31]	Infl. inf	NA	NA	Selection of 10 HPF	NA	NA	NA	>100 cells/HPF	NA

AD = antibody; mD = membrane microarray; TN = tumour nest.

Table 4 – Categories of inflammatory biomarkers considered in the review

Inflammatory cells	Costimulatory molecules in tumour cells	Serum cytokines
CD3 (cytotoxic TL)	PDL1	CRP
CD8 (cytotoxic TL)	HLA	IL-6
FOXP3 (regulatory TL)	HSP	TNF-α
CD68 (macrophages)		
CD83 (dendritic cells)		
Inflammatory infiltrate		
Neutrophil-to-lymphocyte ratio		
CRP = C-reactive protein; IL = intended in the	rleukin; TL = T lymphocyte; TN	NF = tumour

B7-H1 expression was associated with decreased cancerspecific or overall survival (two studies after adjustment for classical prognosticators and one in univariable analysis only) [42–44]. In a series of cystectomised patients, Boorjian et al. demonstrated that B7-H1 expression (>5% cells) was associated with a 3.18-fold higher risk of overall mortality compared with those lacking the marker (95% CI, 1.74–5.79; p < 0.001) [42].

HLA class I molecules are required for the cytotoxic activity of T cells. Loss of HLA class 1 molecules was associated with adverse outcome. Four studies evaluated the impact of HLA class I expression on survival [28,41,45,46]. Strong HLA class I expression was associated with decreased recurrence in a series of patients with NMIBC treated with TURB and induction BCG (HR: 0.06; 95% CI, 0.01-0.40; p = 0.003) [41]. The main limitation of the study was the small sample size (n = 30 patients) and number of events. Homma et al. presented a series of 65 patients treated with RC with similar results [46]. Patients whose tumour lost HLA class I expression had a 2.39-fold higher risk of recurrence after RC than those who did not, after adjustment for stage and histologic variant. HSP70 participates in pro- or antitumour immunity through its secretion by tumour cells or membrane expression through which it can present antigens to the immune system [47]. HSP70 has been evaluated twice in UBC [48,49]. Yu et al. assessed its association with recurrence and progression in a series of 530 patients with NMIBC treated by TURB [49]. Strong expression of HSP70 in tumour cells was independently associated with an increased risk of recurrence (HR: 1.52; 95% CI, 1.15–2; p < 0.001), but there was no independent association with progression in a multivariable analysis. By contrast, HSP27 expression was independently inversely associated with progression (HR: 0.49; 95% CI, 0.33–0.73; *p* < 0.0001).

3.4. Discussion

In spite of their limitations, the major prognosticators for both NMIBC and MIBC at present remain clinical and pathologic factors. The usefulness of several promising molecular prognostic markers, such as Ki-67 overexpression or fibroblast growth factor receptor 3 (*FGFR3*) mutations, has not been conclusively established. Other markers, such as tumour protein p53 (*TP53*) mutations or p53 overexpression, have failed to demonstrate clinical usefulness when combined with standard clinical and pathologic parameters [50]. Molecular profiling has identified new subgroups of UBC, but independent replication is required [51,52]. In addition to their prognostic value, such profiles aim at improving patient stratification and outcome prediction through the use of targeted therapies. However, none of these strategies is ready to be used in the clinical setting.

Overall, there is a need to improve methodology to assess prognostic markers in UBC, as in other tumour types [53]. The field of InfBM discovery is no exception to that rule. The REMARKS guidelines, published in 2005, provide a quality control framework to improve research quality for prognosis biomarker assessment [11]. From the 34 studies analysed in this review in detail, 28 were published in or after 2005. Most of them did not fulfil the REMARKS criteria. Study and patient characteristics were often poorly described, assay methodology was very heterogeneous, important information was missing, and analysis and presentation of results were irregular. Unsuitable methodology prevents reproducibility and diminishes the impact of the work.

The lack of rigor in conducting and reporting studies renders validation of the results less likely. In this review, none of the studies included provided internal or external validation. Statistical significance from Cox multivariable analysis does not mean that a marker is worth translation into clinics. Studies should provide evidence of the marker's analytical validity (robustness of the test method) and discriminative ability (ie, c-index) [54]. Finally, the ideal biomarker should allow identification of patients at risk of a certain outcome with acceptable cost [4]. None of the studies included in this review provided any of those estimates. Consequently, translation into the clinics is inefficient, and much effort is wasted in the replication of poor quality studies. Despite these methodological limitations, we provide evidence that some InfBMs merit additional study as markers of UBC prognosis (Fig. 3).

SNPs in inflammatory pathways possibly play a role in UBC prognosis, although their individual value will probably be too small to be useful in the clinical setting. It is, however, likely that the combined effect of multiple polymorphisms may be more important and more robust as a marker. The multi-inflammatory SNP approach has already been applied to UBC risk studies [55], and similar approaches should be applied to prognosis.

The prognostic significance of IL-6 and CRP has been demonstrated in other cancers including lung, breast, ovary, colon, prostate, and upper urinary tract. Our review suggests that CRP may be the first InfBM approaching translation into clinics. All studies reviewed consistently provide evidence that patients with a high CRP level have a poorer prognosis in MIBC, independently of standard clinical or pathologic factors. However, these results need to be interpreted cautiously because of the heterogeneous methodology applied. The prognostic significance of CRP in patients with NMIBC is unknown and also merits study.

NLR has recently attracted interest and is a promising marker for patients undergoing radical surgery. Since we

Table 5 - Impact of high levels of germline variant and serum markers on bladder cancer survival

Study	Marker	Outcome	Log rank	:	Сох	regression		Adjusted for	Note
			p value	U	Inivariate p value	Multiva (95% CI)	riate HR p value		
					Germlir	ie			
Ahirwar et al. [15]	IL-6	Recurrence				0.41 (0.17-0.94) 0.03	Age, gender	All NMIBC
Leibovici et al. [16]	IL-6	Recurrence				0.73 (0.38-1.39)) NS	Age, gender, smoking status, grade	iBCG
-		Recurrence				4.6 (1.24 -17.1)	NA	Age, gender, smoking status, grade	mBCG
-		Progression				0.88 (0.80-4.41)) NA	Age, gender, smoking status, grade	All NMIBC
-		CSS	0.018			0.39 (0.15-1.00)) NA	Age, gender, smoking status, grade	Only for MIBC
-		OS				0.43 (0.19-0.94)) NA	Age, gender, smoking status, grade	Only for MIBC
Leibovici et al. [16]	TNF-α	Recurrence				1.69 (0.86-3.32) NA	Age, gender, smoking status, grade	iBCG
-		Recurrence				0.43 (0.12-0.49)) NA	Age, gender, smoking status, grade	mBCG
-		Progression				0.71 (0.27-1.85) NA	Age, gender, smoking status, grade	All NMIBC
-		CSS				1.54 (0.58-4.12)) NA	Age, gender, smoking status, grade	Only for MIBC
-		OS				2.35 (1.07-5.16) NA	Age, gender, smoking status, grade	Only for MIBC
Ahirwar et al. [17]	TNF-α	Recurrence	0.024			0.38 (0.14-0.98) 0.048	Age, gender, smoking status	iBCG
					Serum				
Hwang et al. [21]	CRP	OS	0.001	0.001	NS	NS	Age, gender, meta	stasis by location, albumin level, ECOG	
Yoshida et al. [25]	CRP	CSS	0.0003	0.003	1.80 (1.01-2.97)	0.046	stage		
Himly et al. [20]	CRP	CSS	NA	0.016	2.89 (1.42-5.91)	0.004	Ki-67/COX2 expres	ssion, adjuvant therapy	Stratified by stage
Gakis et al. [18]	CRP	CSS	<0.001	0.0012	1.18 (1.09–1.27)	<0.001	Stage, LN density,	margins	CRP = continuous variable
Ishioka et al. [22]	Log CRP	OS	< 0.0001	< 0.001	1.6 (1.19-2.15)	< 0.01	Age, gender, PS, H	o, LDH, visceral metastasis [°] , LN metastasis	
Nakagawa et al. [23]	CRP	OS	< 0.0001	< 0.0001	2.62 (1.6-4.4)	0.0002	Time to recurrence metastatic organs.	e, symptoms at recurrence, no. of LDH, chemo, metastasectomy	
Saito et al. [24]	CRP	OS	NA	< 0.01	1.02 (1.01-1.02)	0.001	ECOG, number of a	netastatic sites and nadir CRP	CRP = continuous variable
Gondo et al. [19]	CRP	CSS	0.025	0.02	NA	NA	NA		
Gondo et al. [19]	NLR	DSS	0.0015	0.0015	1.94 (1.03-3.66)	0.038	Tumour size, hvdr	onephrosis, hemoglobin	
Krane et al. [27]	NLR	OS	0.04	NA	2.49 (1.14-6.09)	NA	pT, pN, albuminen	nia, creatininemia, refraction to BCG	
Andrews et al. [66]	IL-6	MFS	0.024	NA	1.41 (0.95-2.10)	0.08	Stage, grade, LVL r	nodal status	
-	-	CSS	0.015	NA	2.17 (1.29-3.65)	0.05	Stage, grade, LVI, r	nodal status	
Lin et al. [26]	CD8	Recurrence	0.018	0.044	0.40 (0.17-0.94)	0.036	NA		Ref. = high CD8

BCG = bacillus Calmette-Guérin; Cl = confidence interval; COX = cyclooxygenase; CRP = C-reactive protein; CSS = cancer-specific survival; ECOG = Eastern Cooperative Oncology Group; Hb = hemoglobin; HR = hazard ratio; iBCG = induction bacillus Calmette-Guérin; IL = interleukin; LDH = lactate dehydrogenase; LN = lymph node; LVI = lymphovascular invasion; BCG = maintenance bacillus Calmette-Guérin; MFS = metastasis-free survival; NA = not available; NLR = neutrophil-to-lymphocyte ratio; NMIBC = non-muscle-invasive bladder cancer; NS = nonsignificant; OS = overall survival; PS = performance status; TNF = tumor necrosis factor.

Table 6 – Impact of tumour markers overexpression on bladder cancer survival

Study	Marker	Outcome	Log rank	Cox regression			Adjusted for	Notes
			p tutue	Univariate p value	Multivariate HR (95% CI) p value			
				Tumour				
Otto et al. 2012	CD3	CSS	0.045	NA	0.40 (0.05-3.24)	0.39	Age, gender, grade, tumour size, CIS, BCG, early or deferred cystectomy	
Winerdal et al. [29]	CD3	OS	0.04	0.013	0.24 (0.08-0.71)	0.01	Age, gender, T stage, metastasis, chemotherapy, FOXP3 expression	
Sharma et al. [28]	CD8	OS	0.001	0.15	0.3 (0.09-0.96)	0.04	Stage	Only for MIBC
Kitamura et al. [41]	CD8	Recurrence	0.0001	0.33	0.89 (0.12-6.59)	0.9	Stage, grade, HLA, CD4, CD20, CD68, TIA1, S100, FOXP3	
Hanada et al. [39]	CD68	Survival	< 0.0001	0.0005	5 (1.9-12.6)	0.0005	Age, grade, microvessel count, LVI, distant metastasis	
Kitamura et al. [41]	CD68	Recurrence	0.0039	0.32	0.31 (0.03-2.86)	0.29	Stage, grade, HLA, CD4, CD20, CD68, TIA1, S100, FOXP3	
Takayama et al. [38]	CD68 T	Recurrence	0.0002	NA	1.73 (1.47-5.03)	0.0012	Age, gender	T = in CIS
-	CD68 S	Recurrence	0.77	NA	1.01 (0.91-1.09)	0.87	Age, gender	S = lamina propria
Ayari et al. [36]	CD68	Recurrence	0.093	0.101	3.81 (1.32-11)	0.013	Age (continuous), gender, T stage, number of BCG maintenance	
Ayari et al. [37]	CD68	Recurrence	NA	NA	1.19 (0.5-2.5)	0.65	Age (continuous), T stage, grade, number of tumours	Stratified by sex and SS
	-	Progression	0.09	NA	NA	NA	Age (continuous), T stage, grade, number of tumours	Stratified by sex and SS
Ayari et al. [36]	CD83	Recurrence	NA	0.045	9.81 (1.1-85.7)	0.039	Age (continuous), gender, T stage	Only if >1 mBCG
Ayari et al. [37]	CD83	Recurrence	NA	NA	0.93 (0.5-1.8)	0.84	Age (continuous), T stage, grade, no. of tumours	Stratified by sex and SS
	-	Progression	0.04	NA	8.25 (1.4-47.3)	0.018	Age (continuous), T stage, grade, no. of tumours	Stratified by sex and SS
Winerdal et al. [29]	FOXP3	OS	0.037	0.019	0.17 (0.05-0.6)	0.006	Age, gender, T stage, metastasis, chemotherapy, CD3 expression	
Kitamura et al. [41]	FOXP3	Recurrence	NA	0.11	0.19 (0.02-1.41)	0.106	Stage, grade, HLA, CD4, CD20, CD68, TIA1, S100, FOXP3	
Wang et al. [44]	B7-H1	CSS	0.02	NA	2.24 (1.16-4.38)	0.01	Age, gender, stage, grade	
Boorjian et al. [42]	B7-H1	OS	0.005	0.005	3.18 (1.74-5.79)	< 0.001	Age, stage, ECOG, smoking	
Nakanishi et al. [43]	B7-H1	OS	0.021	NA	NA	NA		OE = worse survival
Sharma et al. [28]	HLA 1	MFS	NA	0.09*	NA	NA		* (HR: 0.57)
Levin et al. [45]	HLA 1	CSS	< 0.005	NA	NA	NA		OE = better CSS
Kitamura et al. [41]	HLA 1	Recurrence	0.019	0.04	0.06 (0.01-0.40)	0.003	Stage, grade, HLA, CD4, CD20, CD68, TIA1, S100, FOXP3	Ref. = HLA low
Homma et al. [46]	HLA 1	Rec. after RC	0.03	0.03	2.39 (1.2-4.8)	0.01	pT, pN, and histologic variant	Ref. = HLA high
Yu et al. [49]	HSP70	Recurrence	NA	0.001	1.52 (1.15-2)	< 0.001	Multiplicity	OE = more recurrence
Sirigos et al. [48]	HSP70	OS	< 0.05	NA	NA	NS	Stage and grade	OE = poor survival
Cai et al. [30]	Infl. Inf	OS	0.0098	NA	NA	0.027	Stage and grade	OE = better survival
Samaratunga et al. [33]	Infl. Inf	MFS	0.56	NA	NA	NS	Age, gender, grade, size, treatments, multifocality	OE = better survival
Offersen et al. [32]	Infl. Inf	CSS	0.004	NA	0.48 (0.24-0.96)	0.04	Stage, nodal status, grade, and vessel density	
Sanchez et al. [34]	Infl. Inf	CSS	< 0.01	NA	NA	NS	Grade	OE = poor survival
Flamm and Havekec [31]	Infl. Inf	CSS	0.053	NA	NA	0.021	Stage, grade, multiplicity, location, CIS, and adjuvant treatment	OE = better survival
BCG = bacillus Calmette-Guérin; CI = confidence interval; CIS = carcinoma in situ; CSS = cancer-specific survival; ECOG = Eastern Cooperative Oncology Group; HR = hazard ratio; iBCG = induction bacillus Calmette-Guérin; Infl. inf = Inflammatory infiltrate; LVI = lymphovascular invasion; mBCG = maintenance bacillus Calmette-Guérin; MFS = metastasis-free survival; NA = not applicable; NMIBC = non-muscle-invasive bladder cancer; NS = not significant; OE = overexpression; OS = overall survival; RC = radical cystectomy; Rec = recurrence; S = count in stroma; SS = smoking status; T = count in tumour.								



Fig. 3 – Role of inflammatory biomarkers in the development and progression of urothelial bladder cancer. BCG = bacillus Calmette-Guérin; CRP = C-reactive protein; IL = interleukin; NLR = neutrophil-to-lymphocyte ratio; NSAID = nonsteroidal antiinflammatory drug; UTI = urinary tract infection.

performed this search, two additional studies have shown that high NLR is associated with adverse outcome in UBC and in upper tract urothelial carcinoma [56,57]. A recent meta-analysis exploring the impact of NLR in all urologic malignancies confirmed its significance in urothelial carcinoma and showed comparable results for renal cell carcinoma [58].

There is also an increasing interest in inflammatory infiltrates of tumours. Galon et al. reported that the type, location, and density of inflammatory infiltrating cells in colorectal carcinoma were better predictors of survival than the commonly used clinical and histopathologic factors [5]. An Immunoscore based on the assessment of CD3⁺ and CD8⁺ cells in the centre of the tumour and at the invasion front was established for clinical translation [59], and a worldwide task force has been organised to "initiate the incorporation of Immunoscore as a component of cancer classification" [3]. We show that CD3 and CD8 infiltration in the tumour are directly associated with a better UBC prognosis, as in colon cancer. One of the main limitations, however, is the lack of standardised methods of immunostaining and scoring: Variation of the cut-off chosen for positivity and lack of normalisation of the results (ie, per square millimetre) reduces the robustness of the results. The relevance of these markers is also supported by gene expression profiling of UBC showing the existence of an "infiltrated" subtype characterised by a strong "immune signature." Sjodahl et al. reported high levels of CD3d molecule, delta (CD3-TCR complex) (*CD3D*), CD3 g molecule, gamma (CD3-TCR complex) (*CD3G*), and CD8a molecule (*CD8A*) transcripts in UBC with preliminary data suggesting an association with a better prognosis [60].

The interplay between inflammatory and tumour cells should also be assessed. PD-L1 is important as a marker and as a therapeutic target [61]. Interaction between PD-L1 and its receptor PD1 transmits an inhibitory signal to CD8⁺ lymphocytes, reducing their proliferation [62]. The three studies reviewed in this paper showed that PD-L1 tumour cell expression was associated with adverse outcome. Anti-PD-L1 antibodies have shown remarkable antitumour activity and safety in recent clinical trials in patients with advanced cancers [63]. In melanoma, anti-PD1 and anti-PD-L1 antibodies induced high rates of durable responses [64]. Clinical trials are now being conducted in metastatic UBC.

To translate knowledge into the management of patients with UBC, several specific challenges need to be addressed: Tumours are highly heterogeneous; the TURB generally disrupts the morphology of the tumour mass, which is fragmented; the natural history of the disease is long and subject to medical intervention, for example with nonsteroidal anti-inflammatory drugs; and chronic cystitis, associated with lymphocytic and lymphofollicular infiltrates, can occur in the setting of UBC. Immune editing secondary to BCG immunotherapy may be particularly relevant. Regarding MIBC, it will be important to assess incident tumours separately from those that progress from NMIBC [65]. These issues will require well-designed prospective multicentre studies of appropriate sample size.

This review has some limitations. Reporting and publication bias may result from the lack of information or unpublished negative studies. The reduced number of studies for each marker did not allow us to provide a quantitative assessment of the presence of bias. In addition, the power of meta-analyses is limited due to the small number of studies included, making assessment of heterogeneity and the interpretation of results difficult. The search, using very general keywords, demonstrated problems in the referencing of inflammatory-related articles in Medline. A hand search of reference lists of retrieved publications was necessary to identify most of the studies regarding CRP. This also outlines the difficulties of defining an inflammatory marker. Undoubtedly, some markers are directly implicated in immune response, such as CD3, CD8, HLA, or CRP. Others, such as HSP70, have more pleiotropic functions. Finally, markers included in Supplementary Table 1 are not necessarily devoid of interest: They just require more attention. A major emphasis should be placed on building partnerships to conduct larger and better studies. The study of the tumour inflammatory microenvironment promises novel insight into cancer biology and raises new opportunities for therapeutic intervention. The urologic community should contribute so that progress in the management of patients with UBC does not get "lost in translation."

4. Conclusions

InfBMs show promising usefulness in the management of patients with UBC, both for improved assessment of prognosis and to guide therapy. Serum CRP is one of the most promising InfBMs and independently associated with mortality in advanced UBC. However, these findings need to be interpreted with caution because of many methodological drawbacks. In the translational process of these markers into the clinical milieu, rigorous efforts should be placed in proper study design. Progress will be faster if researchers unite their efforts.

Author contributions: Núria Malats had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Masson-Lecomte, Allory, Malats.

Acquisition of data: Masson-Lecomte.

Analysis and interpretation of data: Masson-Lecomte, Rava, Real, Allory, Malats.

Drafting of the manuscript: Masson-Lecomte, Allory, Malats.

Critical revision of the manuscript for important intellectual content: Masson-Lecomte, Rava, Hartmann, Real, Allory, Malats.

Statistical analysis: Masson-Lecomte, Rava.

Obtaining funding: Masson-Lecomte, Allory, Malats.

- Administrative, technical, or material support: None.
- Supervision: Allory, Malats.

Other (specify): None.

Financial disclosures: Núria Malats certifies that all conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject matter or materials discussed in the manuscript (eg, employment/affiliation, grants or funding, consultancies, honoraria, stock ownership or options, expert testimony, royalties, or patents filed, received, or pending), are the following: None.

Funding/Support and role of the sponsor: This work was partially funded by a fellowship of the European Urological Scholarship Program for Research given to Alexandra Masson-Lecomte (EUSP Scholarship S-01-2013); Red Temática de Investigación Cooperativa en Cáncer (#RD12/ 0036/0050) for supporting the two groups at CNIO and Fondo de Investigaciones Sanitarias (FIS), Instituto de Salud Carlos III, Spain (Grant numbers #PI00-0745, #PI05-1436, and #PI06-1614); and EU-7FP-HEALTH-TransBioBC #601933 for all the background provided by these studies that is exploited here.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j. eururo.2014.07.033.

References

- Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell 2012;21: 309–22.
- [2] Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer 2013;12:298–306.
- [3] Galon J, Pages F, Marincola FM, et al. Cancer classification using the Immunoscore: a worldwide task force. J Transl Med 2012;10:205.
- [4] Denkert C, Loibl S, Noske A, et al. Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. J Clin Oncol 2010;28:105–13.
- [5] Galon J, Costes A, Sanchez-Cabo F, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 2006;313:1960–4.
- [6] Wang E, Miller LD, Ohnmacht GA, et al. Prospective molecular profiling of melanoma metastases suggests classifiers of immune responsiveness. Cancer Res 2002;62:3581–6.
- [7] Kunath F, Krause SF, Wullich B, et al. Bladder cancer—the neglected tumor: a descriptive analysis of publications referenced in MEDLINE and data from the register clinicaltrials.gov. BMC Urol 2013;13:56.
- [8] Catalona WJ, Smolev JK, Harty JI. Prognostic value of host immunocompetence in urologic cancer patients. J Urol 1975;114:922–6.
- [9] Romics I, Feher J, Horvath J. Immunological studies of patients with tumours of the prostate and bladder (a retrospective analysis). Int Urol Nephrol 1983;15:339–45.
- [10] Sadoughi N, Mlsna J, Guinan P, Rubenstone A. Prognostic value of cell surface antigens using immunoperoxidase methods in bladder carcinoma. Urology 1982;20:143–6.
- [11] McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. Reporting recommendations for tumor marker prognostic studies. J Clin Oncol 2005;23:9067–72.
- [12] Higgins JP, Thompson SG. Quantifying heterogeneity in a metaanalysis. Stat Med 2002;21:1539–58.
- [13] Lau J, Ioannidis JP, Terrin N, Schmid CH, Olkin I. The case of the misleading funnel plot. BMJ 2006;333:597–600.
- [14] Czachorowski MJ, Amaral AF, Montes-Moreno S, et al. Cyclooxygenase-2 expression in bladder cancer and patient prognosis: results

from a large clinical cohort and meta-analysis. PLoS One 2012;7: e45025.

- [15] Ahirwar D, Kesarwani P, Manchanda PK, Mandhani A, Mittal RD. Anti- and proinflammatory cytokine gene polymorphism and genetic predisposition: association with smoking, tumor stage and grade, and bacillus Calmette-Guerin immunotherapy in bladder cancer. Cancer Genet Cytogenet 2008;184:1–8.
- [16] Leibovici D, Grossman HB, Dinney CP, et al. Polymorphisms in inflammation genes and bladder cancer: from initiation to recurrence, progression, and survival. J Clin Oncol 2005;23:5746–56.
- [17] Ahirwar DK, Agrahari A, Mandhani A, Mittal RD. Cytokine gene polymorphisms are associated with risk of urinary bladder cancer and recurrence after BCG immunotherapy. Biomarkers 2009;14: 213–8.
- [18] Gakis G, Todenhofer T, Renninger M, et al. Development of a new outcome prediction model in carcinoma invading the bladder based on preoperative serum C-reactive protein and standard pathological risk factors: the TNR-C score. BJU Int 2011;108:1800–5.
- [19] Gondo T, Nakashima J, Ohno Y, et al. Prognostic value of neutrophilto-lymphocyte ratio and establishment of novel preoperative risk stratification model in bladder cancer patients treated with radical cystectomy. Urology 2012;79:1085–91.
- [20] Hilmy M, Campbell R, Bartlett JM, McNicol AM, Underwood MA, McMillan DC. The relationship between the systemic inflammatory response, tumour proliferative activity, T-lymphocytic infiltration and COX-2 expression and survival in patients with transitional cell carcinoma of the urinary bladder. Br J Cancer 2006;95:1234–8.
- [21] Hwang EC, Hwang IS, Yu HS, et al. Utility of inflammation-based prognostic scoring in patients given systemic chemotherapy firstline for advanced inoperable bladder cancer. Jpn J Clin Oncol 2012;42:955–60.
- [22] Ishioka J, Saito K, Sakura M, et al. Development of a nomogram incorporating serum C-reactive protein level to predict overall survival of patients with advanced urothelial carcinoma and its evaluation by decision curve analysis. Br J Cancer 2012;107:1031–6.
- [23] Nakagawa T, Hara T, Kawahara T, et al. Prognostic risk stratification of patients with urothelial carcinoma of the bladder with recurrence after radical cystectomy. J Urol 2013;189:1275–81.
- [24] Saito K, Urakami S, Komai Y, et al. Impact of C-reactive protein kinetics on survival of patients with advanced urothelial carcinoma treated by second-line chemotherapy with gemcitabine, etoposide and cisplatin. BJU Int 2012;110:1478–84.
- [25] Yoshida S, Saito K, Koga F, et al. C-reactive protein level predicts prognosis in patients with muscle-invasive bladder cancer treated with chemoradiotherapy. BJU Int 2008;101:978–81.
- [26] Lin CT, Tung CL, Tsai YS, et al. Prognostic relevance of preoperative circulating CD8-positive lymphocytes in the urinary bladder recurrence of urothelial carcinoma. Urol Oncol 2012;30:680–7.
- [27] Krane LS, Richards KA, Kader AK, Davis R, Balaji KC, Hemal AK. Preoperative neutrophil/lymphocyte ratio predicts overall survival and extravesical disease in patients undergoing radical cystectomy. J Endourol 2013;27:1046–50.
- [28] Sharma P, Shen Y, Wen S, et al. CD8 tumor-infiltrating lymphocytes are predictive of survival in muscle-invasive urothelial carcinoma. Proc Natl Acad Sci U S A 2007;104:3967–72.
- [29] Winerdal ME, Marits P, Winerdal M, et al. FOXP3 and survival in urinary bladder cancer. BJU Int 2011;108:1672–8.
- [30] Cai T, Nesi G, Boddi V, Mazzoli S, Dal Canto M, Bartoletti R. Prognostic role of the tumor-associated tissue inflammatory reaction in transitional bladder cell carcinoma. Oncol Rep 2006;16:329–34.
- [31] Flamm J, Havelec L. Factors affecting survival in primary superficial bladder cancer. Eur Urol 1990;17:113–8.
- [32] Offersen BV, Knap MM, Marcussen N, Horsman MR, Hamilton-Dutoit S, Overgaard J. Intense inflammation in bladder carcinoma

is associated with angiogenesis and indicates good prognosis. Br J Cancer 2002;87:1422–30.

- [33] Samaratunga H, Fairweather P, Purdie D. Significance of stromal reaction patterns in invasive urothelial carcinoma. Am J Clin Pathol 2005;123:851–7.
- [34] Sanchez de la Muela P, Rosell D, Aguera L, et al. Superficial bladder cancer: survival and prognostic factors. Eur Urol 1991;20:184–91.
- [35] Flamm J. The value of tumor-associated tissue inflammatory reaction in primary superficial bladder cancer. Urol Res 1990;18:113–7.
- [36] Ayari C, LaRue H, Hovington H, et al. Bladder tumor infiltrating mature dendritic cells and macrophages as predictors of response to bacillus Calmette-Guerin immunotherapy. Eur Urol 2009;55: 1386–96.
- [37] Ayari C, LaRue H, Hovington H, et al. High level of mature tumorinfiltrating dendritic cells predicts progression to muscle invasion in bladder cancer. Hum Pathol 2013;44:1630–7.
- [38] Takayama H, Nishimura K, Tsujimura A, et al. Increased infiltration of tumor associated macrophages is associated with poor prognosis of bladder carcinoma in situ after intravesical bacillus Calmette-Guerin instillation. J Urol 2009;181:1894–900.
- [39] Hanada T, Nakagawa M, Emoto A, Nomura T, Nasu N, Nomura Y. Prognostic value of tumor-associated macrophage count in human bladder cancer. Int J Urol 2000;7:263–9.
- [40] Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003;299: 1057–61.
- [41] Kitamura H, Torigoe T, Honma I, et al. Effect of human leukocyte antigen class I expression of tumor cells on outcome of intravesical instillation of bacillus Calmette-Guerin immunotherapy for bladder cancer. Clin Cancer Res 2006;12:4641–4.
- [42] Boorjian SA, Sheinin Y, Crispen PL, et al. T-cell coregulatory molecule expression in urothelial cell carcinoma: clinicopathologic correlations and association with survival. Clin Cancer Res 2008;14:4800–8.
- [43] Nakanishi J, Wada Y, Matsumoto K, Azuma M, Kikuchi K, Ueda S. Overexpression of B7-H1 (PD-L1) significantly associates with tumor grade and postoperative prognosis in human urothelial cancers. Cancer Immunol Immunother 2007;56:1173–82.
- [44] Wang Y, Zhuang Q, Zhou S, Hu Z, Lan R. Costimulatory molecule B7-H1 on the immune escape of bladder cancer and its clinical significance. J Huazhong Univ Sci Technolog Med Sci 2009;29:77–9.
- [45] Levin I, Klein T, Goldstein J, Kuperman O, Kanetti J, Klein B. Expression of class I histocompatibility antigens in transitional cell carcinoma of the urinary bladder in relation to survival. Cancer 1991;68:2591–4.
- [46] Homma I, Kitamura H, Torigoe T, et al. Human leukocyte antigen class I down-regulation in muscle-invasive bladder cancer: its association with clinical characteristics and survival after cystectomy. Cancer Sci 2009;100:2331–4.
- [47] Calderwood SK, Murshid A, Gong J. Heat shock proteins: conditional mediators of inflammation in tumor immunity. Front Immunol 2012;3:75.
- [48] Syrigos KN, Harrington KJ, Karayiannakis AJ, et al. Clinical significance of heat shock protein-70 expression in bladder cancer. Urology 2003;61:677–80.
- [49] Yu HJ, Chang YH, Pan CC. Prognostic significance of heat shock proteins in urothelial carcinoma of the urinary bladder. Histopathology 2013;62:788–98.
- [50] Malats N, Bustos A, Nascimento CM, et al. P53 as a prognostic marker for bladder cancer: a meta-analysis and review. Lancet Oncol 2005;6:678–86.
- [51] Sjodahl G, Lauss M, Lovgren K, et al. A molecular taxonomy for urothelial carcinoma. Clin Cancer Res 2012;18:3377–86.
- [52] Volkmer JP, Sahoo D, Chin RK, et al. Three differentiation states riskstratify bladder cancer into distinct subtypes. Proc Natl Acad Sci U S A 2012;109:2078–83.

- [53] Shariat SF, Lotan Y, Vickers A, et al. Statistical consideration for clinical biomarker research in bladder cancer. Urol Oncol 2010;28: 389–400.
- [54] Tripepi G, Jager KJ, Dekker FW, Zoccali C. Statistical methods for the assessment of prognostic biomarkers (part I): discrimination. Nephrol Dial Transplant 2010;25:1399–401.
- [55] de Maturana EL, Ye Y, Calle ML, et al. Application of multi-SNP approaches bayesian LASSO and AUC-RF to detect main effects of inflammatory-gene variants associated with bladder cancer risk. PLoS One 2013;8:e83745.
- [56] Dalpiaz O, Pichler M, Mannweiler S, et al. Validation of the pretreatment derived neutrophil-lymphocyte ratio as a prognostic factor in a European cohort of patients with upper tract urothelial carcinoma. Br | Cancer 2014;110:2531–6.
- [57] Viers BR, Boorjian SA, Frank I, et al. Pretreatment neutrophil-tolymphocyte ratio is associated with advanced pathologic tumor stage and increased cancer-specific mortality among patients with urothelial carcinoma of the bladder undergoing radical cystectomy. Eur Urol 2014;66:1157–64.
- [58] Wei Y, Jiang YZ, Qian WH. Prognostic role of NLR in urinary cancers: a meta-analysis PLoS One 2014; 2014;9:e92079.
- [59] Pages F, Kirilovsky A, Mlecnik B, et al. In situ cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer. J Clin Oncol 2009;27:5944–51.

- [60] Sjodahl G, Lauss M, Lovgren K, et al. A molecular taxonomy for urothelial carcinoma Clin Cancer Res 2012; 2012;18:3377–86.
- [61] Butte MJ, Pena-Cruz V, Kim MJ, Freeman GJ, Sharpe AH. Interaction of human PD-L1 and B7-1. Mol Immunol 2008;45:3567–72.
- [62] Butte MJ, Keir ME, Phamduy TB, Sharpe AH, Freeman GJ. Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. Immunity 2007;27:111–22.
- [63] Brahmer JR, Tykodi SS, Chow LQ, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med 2012; 366:2455–65.
- [64] Eggermont AM, Spatz A, Robert C. Cutaneous melanoma. Lancet 2014;383:816–27.
- [65] Schrier BP, Hollander MP, van Rhijn BW, Kiemeney LA, Witjes JA. Prognosis of muscle-invasive bladder cancer: difference between primary and progressive tumours and implications for therapy. Eur Urol 2004;45:292–6.
- [66] Andrews B, Shariat SF, Kim JH, Wheeler TM, Slawin KM, Lerner SP. Preoperative plasma levels of interleukin-6 and its soluble receptor predict disease recurrence and survival of patients with bladder cancer. J Urol 2002;167:1475–81.
- [67] Otto W, Denzinger S, Wieland WF, Hartmann A. First analysis of immune cell infiltration in stage pT1 urothelial bladder carcinoma: CD3 positivity as a prognostic marker for cancer-specific survival. World J Urol 2012;30:875–7.

http://esudavos.uroweb.org European Urology Forum 2015

Challenge the experts

7-10 February 2015, Davos, Switzerland



European Association of Urology

EAU meetings and courses are accredited by the EBU in compliance with the UEMS/EACCME regulations

<u>Résumé</u>

L'objectif du travail a été d'explorer la valeur pronostique pour les tumeurs de la vessie des polymorphismes de gènes associés à l'inflammation et du microenvironnement tumoral lymphocytaire CD8+. Pour les marqueurs constitutionnels, deux approches ont été conduites concomitamment, l'une explorant de façon globale les gènes associés à l'inflammation, l'autre ciblant un gène inflammatoire d'intérêt, *PDL1*, impliqué dans des points de contrôles immunologiques. A l'échelle du génome, en utilisant des méthodes statistiques soit classiques soit innovantes (dites multi marqueurs), nous avons démontré que les variants (SNP) dans les gènes *TNIP1*, *CD5* et *JAK3* étaient associés au risque de récidive des tumeurs de vessie non invasives du muscle alors que les variants dans les gènes *MASP1*, *AIRE* et *CD3* étaient associés au risque de progression. Dans un deuxième temps, l'association entre variants dans le gène de *PDL1* et pronostic des tumeurs de vessie a été explorée en appliquant une méthode classique « SNP par SNP » et une approche à l'échelle du gène. Nous avons identifié une forte association entre des variants de *PDL1* et le pronostic de tumeurs de vessie envahissant le muscle dans une large cohorte prospective, mais sans pouvoir répliquer ce résultat dans une série issue du consortium TCGA.

Dans les tumeurs n'envahissant pas le muscle, nous avons développé et évalué une méthode d'évaluation standardisée de l'infiltrat lymphocytaire CD8+, cellules T-cytotoxiques impliquées dans la mort des cellules tumorales. L'analyse morphométrique après double immuno-marquage des cellules tumorales et des lymphocytes CD8+ et numérisation a permis d'estimer de façon séparée le compte des cellules inflammatoires dans la tumeur et le stroma, et d'estimer l'hétérogénéité spatiale intra-tumorale. Nous avons montré que cette hétérogénéité limite les estimations de l'infiltrat CD8+ sur les puces tissulaires (Tissue Micro Array) qui échantillonnent les tumeurs de façon restrictive. Sous cette réserve, nous avons identifié dans les tumeurs n'envahissant pas le muscle une association entre l'infiltrat lymphocytaire CD8+ le stade tumoral Ta/T1, ainsi qu'avec le risque de récidive des tumeurs T1. A l'avenir, variations génétiques constitutionnelles dans les gènes de l'inflammation et évaluation de l'infiltrat tumoral inflammatoire pourraient être intégrées en vue d'améliorer la prédiction du pronostic des tumeurs vésicales.

Summary

The aim of this study was to explore prognostic value for bladder cancer of germline polymorphisms in inflammatory genes and tumor CD8+ lymphocytic microenvironment. For constitutional markers, two approaches were conducted jointly: one genome-based using specific GWAS statistical methods, the other gene-based focusing on *PDL1*, an inflammatory gene implicated in immune checkpoints. At the genome level, using both standard and innovative statistical methods (multi marker methods Bayesian Lasso and Bayes A) we demonstrated that variants (SNPs) in TNIP1, CD5 and JAK3 were associated with the risk of recurrence of non-muscle invasive bladder cancer (NMIBC) while SNPs in MASP1, AIRE and CD3 were associated with risk of progression. Meanwhile, association between *PDL1* and prognosis of NMIBC and muscle invasive BC (MIBC) was explored using classical SNPS by SNP investigations and a gene based approach. We identified a very strong association between PDL1 variants and MIBC prognosis in a large prospective cohort but failed replicating those results in the TCGA consortium series.

In non-muscle invasive bladder cancer, we developed and evaluated a standardized counting approach of CD8+ cells, T-cytotoxic lymphocytes implicated in tumor cells death. Morphometric analysis after double immuno-staining of tumor cells and digitalization allowed separate estimation of CD8+ cells in the tumor and stroma compartment and estimation of spatial intra tumoral heterogeneity. We demonstrated that this heterogeneity compromised CD8+ estimation on Tissue Micro Arrays, which sample the tumors in a restrictive manner. Keeping those limitations in mind, we identified an association between CD8+ inflammatory infiltrate and both NMIBC stage and T1 tumours risk of recurrence. In the future, germline variation in inflammatory genes and evaluation of tumor inflammatory infiltrate could be integrated for better prediction of bladder cancer prognosis.