UNIVERSIDAD AUTÓNOMA DE MADRID
FACULTY OF MEDICINE
Department of Preventive Medicine and Public Health

THE ROLE OF INFLAMMATION IN BLADDER CANCER DEVELOPMENT AND PROGRESSION: A MOLECULAR AND GENETIC EPIDEMIOLOGICAL ASSESSMENT

MACIEJ JAN CZACHOROWSKI

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ABSTRACT

Growing epidemiologic and experimental evidence implicates chronic inflammation in risk and progression of urothelial carcinoma of the bladder (UCB). This thesis investigates the association between inflammation related factors (environmental, molecular and genetic) and UCB development and progression in the Spanish population. The ultimate aim is to further elucidate the role of chronic inflammation in this disease. Epidemiologic, medical, clinical and genetic data was collected as part of the Spanish Bladder Cancer/EPICURO Study, a hospital-based, case-control study of nearly 1300 incident UCB cases, and age, gender and hospital matched controls from 18 hospitals in five regions of Spain.

In chapter 3, self-reported information on lifetime history of urinary tract infections (UTI) was used to investigate the association between bladder infection and risk of UCB. Nearly a 50% reduction in UCB risk was observed in patients who reported using UTI medication to treat episodes of infection as compared to uninfected individuals; while non-medicating patients exhibited a non-significant increase in UCB risk. Patients who used the urinary tract analgesic phenazopyridine also had a further reduction in UCB risk. In chapter 4, follow-up information spanning nearly ten years for almost 800 UCB patients with available tumor tissue was used to evaluate the prognostic utility of the inflammatory mediator COX2 in UCB. Tumor expression of COX2 did not confer independent prognostic utility over conventional clinicopathological factors for tumor recurrence and progression in patients with non-muscle invasive bladder cancer, or for progression or survival of patients with muscle-invasive bladder cancer. A meta-analysis that included 11 evaluable studies corroborated these findings.

In chapter 5, 911 genetic variants in 214 genes linked to the inflammatory response and relevant to cancer were assessed for heterogeneity in susceptibility to UCB subtypes characterized by their level of COX2 expression. Variants rs187084 and rs352139 in the gene encoding the innate immune receptor TLR9 conferred heterogeneity in susceptibility to UCB subtypes. These associations remained robust after multiple testing correction and were not modified by patient history of bladder infections. The variant allele of *TLR9*-rs187084 also independently conferred a reduced rate of tumor progression in patients with COX2-negative tumors but only in those treated with BCG immunotherapy; suggesting that it may be used as a predictive marker of BCG response in these patients.

The large patient size afforded by the Spanish Bladder Cancer / EPICURO Study enabled for a more precise evaluation of the prognostic potential of COX2 tumor expression than has been undertaken in the literature to date. Moreover, the association between UTI medication and UCB risk has never been evaluated in an observational study despite experimental evidence suggesting some common medications may confer urothelial tumor cytotoxicity. In this respect, the results presented herein are the first described using an observational approach to corroborate experimentally obtained data regarding the effect of UTI medication on UCB cytotoxicity. Despite the advantages afforded by a large sample size and extensive patient information, the observational nature of this study makes it susceptible to bias. Due to the self-reported nature of urinary tract infection history, there is potential for the misclassification of early UCB symptoms with those of infection. Moreover, although great effort was taken to minimize confounding by adjustment of all statistical models and by stratification, one cannot exclude that uncontrolled confounding influenced some of the results.

Collectively, these findings reiterate the complex interplay between inflammatory processes and UCB risk and progression and may have implications for antibiotic UCB prophylaxis and improvement of patient response to immunotherapy.

RESUMEN

Un creciente cuerpo de evidencias epidemiológicas y experimentales implica a la inflamación crónica en el desarrollo y progresión del carcinoma urotelial de vejiga (CUV). Esta tesis investiga la asociación entre los factores relacionados con la inflamación (ambientales, moleculares y genéticos) y el desarrollo y progresión del CUV en población española con el objetivo último de elucidar el papel de la inflamación crónica en esta enfermedad. Los datos epidemiológicos, médicos, clínicos y genéticos fueron recogidos como parte del Estudio Español de Cáncer de Vejiga/EPICURO, un estudio de casos y controles hospitalarios con casi 1300 casos incidentes de CUV y controles pareados por edad, género y hospital, procedentes de 18 hospitales de cinco regiones de España.

En el capítulo 3, se utilizó la información autoreportada sobre la historia de infecciones del tracto urinario (ITU) para investigar la asociación entre esta exposición y el riesgo de CUV. Se observó una reducción de casi un 50% en el riesgo de CUV en los pacientes que reportaron el uso de medicamentos para tratar la ITU, en comparación con las personas que no habían reportado ninguna infección, mientras que los pacientes que no usaron medicamentos para tratar la ITU mostraron un aumento, aunque no significativo, del riesgo de CUV. Los pacientes que usaron fenazopiridina, el analgésico del tracto urinario, también tuvieron una reducción en el riesgo de CUV.

En el capítulo 4, la información de seguimiento que abarca casi diez años para casi 800 pacientes con tejido tumoral disponible, se utilizó para evaluar la utilidad pronóstica del mediador inflamatorio COX2 en el CUV. La expresión tumoral de COX2 no mostró tener un valor pronóstica independiente de los factores clínico-patológicos convencionales para la recurrencia y progresión del tumor en pacientes con cáncer de vejiga no invasivo del músculo, ni para la progresión o la supervivencia de los pacientes con tumores de vejiga que invaden el músculo. Una meta-análisis que incluyó 11 estudios corroboró estos hallazgos.

En el capítulo 5, se estudiaron 911 variantes genéticas en 214 genes ligados a la respuesta inflamatoria y relevantes en cáncer para valorar su heterogeneidad genética en relación con la susceptibilidad a padecer CUV caracterizado por una alta o baja expresión de COX2. Variantes (rs187084 y rs352139) en el gen que codifica el receptor de la inmunidad innata, TLR9, confirieron heterogeneidad en la susceptibilidad a CUV. Las asociaciones se mantuvieron después de la corrección por comparaciones múltiples y no fueron modificadas por la historia de UTIs en los pacientes y otros transtornos uropatológicos. La variante TLR9-rs187084 también confirió, de forma independiente, un menor riesgo de progresión tumoral en pacientes con tumores negativos para COX2, pero sólo en los pacientes tratados con inmunoterapia de BCG; sugiriendo que esta variante podría ser utilizada como un marcador predictivo para la respuesta a BCG en estos pacientes.

El gran tamaño muestral del estudio Español de Cancer de Vejiga / Estudio EPICURO ha posibilitado una evaluación más precisa del potencial pronóstico de la expresión de COX2 de lo que se ha realizado en la literatura hasta ahora. Además, nunca se ha evaluado antes en un estudio observacional la asociación entre la medicación utilizada para la infección del tracto urinario y el riesgo de CUV, a pesar de la evidencia experimental que sugiere que algunos medicamentos comunes pueden conferir citotoxicidad tumoral. En este sentido, los resultados presentados en esta tesis son los primeros descritos utilizando un diseño observacional que corroboran los datos obtenidos experimentalmente con respecto al efecto de la medicación para la infección del tracto urinario sobre la citotoxicidad en el CUV. A pesar de las ventajas que ofrece un tamaño muestral grande y una amplia información sobre los pacientes, la naturaleza observacional de este estudio lo hace susceptible a sesgos. Debido a la naturaleza auto-reportada de la historia de infecciones del tracto urinario, es posible que se haya producido una clasificación errónea de los primeros síntomas de CUV con los de la infección. Por otra parte, a pesar de un gran esfuerzo en minimizar la confusión mediante el ajuste de todos los modelos estadísticos y la estratificación de los analisis, no se puede excluir que la existencia de confusión residual haya podido influir en algunos de los resultados.

Estos resultados muestran la relación compleja entre los procesos inflamatorios y el riesgo y progresión del CUV. Además, pueden tener implicaciones para la profilaxis antibiótica y la mejora de las tasas de respuesta a la inmunoterapia.

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CHAPTER 1: INTRODUCTION

1.1 BLADDER CANCER

1.1.1 Burden of disease

According to the International Agency for Research on Cancer (IARC) approximately 382,000 new cases of bladder cancer were diagnosed globally in 2008 and more than 150,000 individuals died from the disease; ranking bladder cancer the 9th most incident and 13th deadliest cancer worldwide (Ferlay et al., 2010). Crude estimates place the global prevalence of the disease at nearly 2.7 million individuals, the majority of which present with superficial tumors with a favorable prognosis following treatment (Ploeg et al., 2009). However, due to a high rate of recurrence and risk of tumor progression, patients need to be closely followed-up over the course of their lives; contributing to making bladder cancer one of the most expensive cancers to treat on a per patient basis (Botteman et al., 2003). Age-standardized bladder cancer incidence and mortality rates are highest in developed nations, while developing regions by comparison, observe reduced incidence and mortality rates by factors of three and two, respectively (Table 1.1).

Table 1.1 Age-standardized bladder cancer incidence and mortality rates by gender and region*

Region	Men		Women	
	Incidence	Mortality	Incidence	Mortality
World	9.1	3.3	2.2	0.9
More developed	16.6	4.6	3.6	1.0
Less developed	5.4	2.6	1.4	0.7
Spain	27.7	8.3	3.2	1.1

Adapted from Ferlay et al. 2010

A histological dichotomy is also apparent in these regions, with nearly 90% of all bladder cancer patients diagnosed in developed nations presenting with urothelial carcinoma of the bladder (UCB), while more than 70% of cases in developing nations manifest squamous cell carcinoma (SCC) (Schottenfeld and

Fraumeni, 2006).Globally, men are three to four times more likely than women to be diagnosed with bladder cancer, and three times as likely to die of their disease (Table 1.1). However, in developed nations like the United States, women and black patients consistently present with more aggressive tumors upon diagnosis and observe decreased UCB survival rates compared to white males which cannot be entirely explained by demographic differences alone (Fajkovic et al., 2011; Mallin et al., 2011).

In Spain, like in other developed nations, UCB is the predominant bladder cancer histology. Spanish men observe the highest UCB incidence rate in Europe (27.7 per 100,000 men) and the disease ranks as the fourth most incident tumor type following prostate, lung and colorectal cancers (Ferlay et al., 2010). In 2008, more than 13,000 individuals (men and women) were diagnosed with UCB in this country and nearly 5,000 died as a result of the disease (GLOBOCAN). Between 1970 and 2005, while UCB mortality rates in several Western European nations steadily declined, age-standardized mortality in both men and women has remained relatively stable in Spain (Bosetti et al., 2011; Yako-Suketomo and Katanoda, 2010). By some estimates, UCB-specific male incidence and mortality are expected to decrease in Spain in the period between 2007 to 2022, while increasing slightly in Spanish women (Bernal-Perez et al., 2012). The proportion of men:women who develop bladder cancer in Spain is, at nearly 7:1, disproportionately higher than the global average of about 3:1 (Table 1.1). Due to its high incidence and public health impact in Spain, this thesis will focus on UCB in the Spanish population.

1.1.2 Etiology of urothelial carcinoma of the bladder

UCB is a complex disease with both genetic and non-genetic risk factors contributing to its etiology. Cigarette smoking, occupational exposure to azo-based dyes and arsenic, are among the best established environmental risk factors (Burger et al., 2013). At present, several genetic variants, mainly single nucleotide polymorphisms (SNPs), have also been independently associated with the risk of UCB through various candidate gene and genome-wide approaches (Rothman et al., 2010). The complexity of the disease is further underscored by the ability of some of these factors to interact and thereby alter UCB susceptibility as demonstrated for cigarette smokers harboring the slow-acetylator phenotype of *N*-acetyl transferase 2 (NAT2); a phase-2 detoxification enzyme (Garcia-Closas et al., 2005).

Risk associations with environmental exposures and/or genetic aberrations in detoxifying enzymes have led to the proposal that UCB initiation may result from direct exposure of the urothelium to carcinogens excreted in the urine (Wu, 2005). UCBs are often multifocal with evidence to suggest that they may be monoclonal, arising from a single urothelial cell that spreads to other parts of the urothelium (Sidransky et al., 1992), or polyclonal, arising through a field effect through which carcinogens exposed to the urothelium initiate independent genetic alterations at several sites (Jones et al., 2005).

1.1.2.1 Environmental risk factors of UCB

Tobacco smoking is among the best established non-genetic risk factors of UCB having an estimated population attributable risk of about 50% men, and slightly less in Spanish women , with current smokers being 3 to 7 times more likely to develop UCB than never smokers (Freedman et al., 2011; Samanic et al., 2006). Risk of UCB has been reported to increase linearly with intensity and duration of smoking and drop accordingly in former smokers with time since cessation of smoking, but do not reach the level found in never-smokers (Brennan et al., 2000; Samanic et al., 2006). Samanic et al only noted the inverse trend in UCB risk with time since cessation in former smokers of blond tobacco, but observed no apparent trend in former smokers of black tobacco in Spain (Samanic et al., 2006). Recent evidence from patients in the United Kingdom also suggests that smokers are diagnosed with UCB at a younger age and present with more aggressive and larger malignant tumors than non-smokers (van Roekel et al., 2013). The particulate phase of cigarette smoke contains various compounds and carcinogens including polycyclic aromatic hydrocarbons (PAHs), *N*-nitrosamines, aromatic amines and metals (Pfeifer et al., 2002) which can contribute to bladder carcinogenesis by means that are not completely understood but may include aromatic amine induced urothelial cell DNA adduction, mutagenicity and oxidative damage (Besaratinia and Tommasi, 2013; Ohnishi et al., 2002).

Occupations with a high risk of exposure to various carcinogens, such as textile, metal working, machining, equipment transport, and mining have been attributed to account for between 5 to 10% of UCB cases in men in Western Europe (Kogevinas et al., 2003). Specifically, aromatic amines such as benzidine and beta-naphthylamine commonly used in the dye, chemical, and rubber industries, as well as PAHs, have been cited as the compounds responsible for increasing occupational UCB risk (Golka et al.,

2004). Bladder cancer specific mortality was reported to increase in dye stuff workers heavily exposed to aromatic amines with younger age at first exposure and increasing duration of exposure, with excess risk being apparent even 30 years after last exposure (Pira et al., 2010). Aromatic amines were also commonly used in hair dye products and two recent meta-analyses indicate that hairdressers observe at least a 30% increased risk of UCB (Harling et al., 2010; Takkouche et al., 2009); however no consistent associations have been reported between UCB risk and personal hair dye use (Koutros et al., 2011; Ros et al., 2012).

Various other suspected environmental and lifestyle risk factors have been explored for their association with UCB risk. Increased risk has been associated with high exposure (dermal or oral) to water disinfection by-products such as *trihalomethanes* (THMs) (Castano-Vinyals et al., 2011; Michaud et al., 2007; Villanueva et al., 2007) as well as trace metals such as *arsenic* (Chiou et al., 2001; Smith et al., 1998) and a constantly *low urinary pH* (Alguacil et al., 2011). Reduced UCB risk has been found associated with increased *nighttime urination frequency* (Silverman et al., 2008), high *fruit and vegetable intake* (Garcia-Closas et al., 2007) and long-term use of *non-aspirin non-steroidal anti-inflammatory drugs* (Fortuny et al., 2006); no apparent associations (or only modest associations in certain strata) with UCB were reported for coffee consumption (Villanueva et al., 2009) or air pollution (e.g. low-level industrial PAH and diesel emissions near residential areas) (Castano-Vinyals et al., 2008).

1.1.2.2 Genetic susceptibility factors

UCB is not considered a hereditary cancer; however several recent case-control studies indicate that familial history of UCB may increase the risk of the disease in first-degree relatives by two- to fivefold (Aben et al., 2002; Lin et al., 2006; Murta-Nascimento et al., 2007). Somatic alterations in chromosome 9 and mutations in the *RAS* family of oncogenes (*HRAS, KRAS* and *NRAS*) are prevalent in both superficial and muscle-invasive UCB (Jebar et al., 2005; Sauter et al., 1995). However, the majority (>50%) of NMIBC are characterized by oncogenic mutations in *FGFR3* (Cappellen et al., 1999; Jebar et al., 2005), while inactivating mutations in the tumor suppressor genes *TP53* and *RB* characterize muscle invasive UCB (Cote et al., 1998; Sidransky et al., 1991). Genetic variants in several carcinogen detoxification genes have been associated with UCB susceptibility. N-acetyltransferases (namely NAT1

and NAT2) detoxify aromatic amines via the covalent addition of an acetyl group. Individuals who harbor two variant alleles of *NAT2*, known as a slow acetylator phenotype, are at an increased risk of UCB) (Garcia-Closas et al., 2005; Okkels et al., 1997). Similarly, glutathione-S-transferase M1 (GSTM1) is an enzyme involved in the detoxification of electrophilic compounds like poly aromatic hydrocarbons (PAHs), and individuals that lack both copies of the gene encoding this enzyme (60% of the Spanish population) are nearly 1.7 times more likely to develop UCB compared to wildtype homozygotes/heterozygotes (Garcia-Closas et al., 2005). Lastly, numerous genome wide analysis studies (GWAS) have identified and corroborated at least a dozen UCB genetic susceptibility loci arising from SNPs that fall within or near the following genes: *MYC, TP63, PSCA,CLPTM1L, TERT, TACC3, FGFR3, CBX6, APOBEC3A,CCNE1 UGT1A* and *NAT2* (summarized in (Garcia-Closas et al., 2011; Gu and Wu, 2011)).

1.1.3 Chronic inflammation and UCB risk

Inflammation is an adaptive immune response responsible for protecting the body against extrinsic and endogenous stress and mediating the clearance and repair process that follows its successful resolution . Broadly, inflammation can be divided into a highly regulated acute phase and a sustained chronic inflammatory phase that persists when the acute response is insufficient to mitigate the inducing threat. The presence of chronic inflammation has been implicated in all facets of tumor development including tumor cell initiation, promotion and progression in a wide array of tissues. In the urinary bladder, factors that predispose the urothelium to a state of sustained chronic inflammation also appear to increase bladder cancer risk (Kundu and Surh, 2008; Mantovani et al., 2008; Michaud, 2007). For example, calculi in the bladder, kidney or ureters, can agitate the urothelium and lead to localized chronic inflammation as well as obstructive urinary stasis conducive to bacterial infection of the bladder (Burin et al., 1995). These conditions have been associated with increased UCB risk and their contribution to UCB risk in the Spanish population is explored in more detail in chapter 3 of this thesis

1.1.3.1 The inflammatory response

In normal tissue, the acute inflammatory response is activated upon recognition of exogenous factors which may be microbial or non-microbial in nature, or by endogenous factors resulting from cell

or tissue damage (e.g. necrotic tissue) (Kumar et al., 2010). Macrophages, mast cells and endothelial cells in the vicinity of the infection or injury release pro-inflammatory cytokines (TNFa, IL-1B, IL-6, IL-12) , chemokines (CXCL-1, -2, -8) (Cohen-Hillel et al., 2009) and vasodilators that signal for the recruitment of plasma proteins and phagocytic neutrophils to the afflicted region. Local vasodilation permits diffusion of plasma proteins and neutrophils from the capillary network into the tissue resulting in symptoms characteristic of the acute inflammatory response like redness, warmth, and swelling (Kumar et al., 2010). Direct contact with the irritant or with cytokines released by injured tissues, triggers neutrophilic release of enzymatic granules laden with reactive oxygen (ROS) and nitrogen species (RNS) and proteolytic enzymes capable of breaking down the pathogen or damaged tissue (Fialkow et al., 2007). Successful neutralization of the irritant initiates macrophage-mediated resolution of the acute inflammatory phase and enables tissue repair (Medzhitov, 2008). At this point, the balance of cytokine production shifts toward anti-inflammatory cytokines (IL-4, IL-10) that curtail the production of pro-inflammatory prostaglandins and limit neutrophil recruitment, thus allowing for the removal of dead cells and tissue remodelling by monocytes.

Recalcitrant infection or persistent tissue irritation that does not clear up, however, triggers a heightened inflammatory response characterized by neutrophil replacement by macrophages and generation of cytolytic T-cells (CTLs) (Medzhitov, 2008). Notwithstanding, unmitigated prolongation of this heightened inflammatory state leads to localized chronic inflammation at the afflicted area. In a localized chronic inflammatory state, prolonged exposure of the surrounding cells to ROS and RNS produced by immune cells at the site of inflammation can result in DNA strand breaks contributing to replication errors and genomic instability, the induction of oncogenic mutations and inactivation of tumor suppressor genes, which collectively contribute to tumor initiation (Michaud, 2007). Furthermore, these reactive species can impart damage to the cell membrane through lipid peroxidation reactions and interfere with protein processing and cellular homeostatic functions thereby contributing to tumor promotion (Kundu and Surh, 2008).

1.1.3.2 Pathogen Associated Molecular Patterns and Toll-like receptors

In the case of exogenous stimulation as observed in urinary tract infections (UTIs), pathogen associated microbial patterns (PAMPs) not found in the host organism are recognized by cognate pattern recognition receptors (PRRs) whose activation induces a proinflammatory signaling cascade (Tang et al., 2012). In humans, the Toll-like receptors (TLRs) are a family of ten integral membrane PRRs found expressed both at the cell membrane and, in the case of TLRs 3, 7 and 9, in endocytic vesicles (Takeda and Akira, 2004). TLRs are predominantly expressed on immune cells including dendritic cells, macrophages and lymphocytes, but are also prevalent in a broad range of tissues including healthy and tumor urothelium (Ayari et al., 2011). TLRs recognize a broad variety of PAMPs such as microbial lipopeptides (TLRs 1, 2 and 6), lipopolysaccharide (LPS) (TLR4), flagellin (TLR5), and different microbial nucleotides including double-stranded RNA (TLR3), single-stranded RNA (TLRs 7 and 8), and hypomethylated CpG DNA (TLR9) (Takeda and Akira, 2004). The binding of PAMPs to their cognate TLRs induces receptor dimerization and MyD88-mediated intracellular signaling terminating in the activation of pro-inflammatory transcription factors like NF-kB and AP-1 and subsequent expression of inflammatory mediators, cytokines and chemokines which act in concert to mitigate the inducing stress (Janssens and Beyaert, 2003).

1.1.4 Urinary tract infections and UCB risk

Urinary tract infections (UTI) of the bladder are mostly due to *Escherichia coli* and are frequently characterized by increased voiding frequency, urgency and/or dysuria (Dhakal et al., 2008). They are more frequent in women than in men, with nearly 50% of women experiencing at least one episode in their life and 20% of these observing a recurrent event (Stamm and Hooton, 1993). Treatment generally entails a short-term (3 day) regimen of oral antibiotics such as nitrofurantoin, trimethoprim and/or sulfonamides, or broader spectrum fluoroquinolones like ciprofloxacin or levofloxacin, with extended treatment periods in the case of recurrent episodes (Hummers-Pradier and Kochen, 2002; Stamm and Hooton, 1993). In Spain, urinary tract analgesics are often taken concurrently with antibiotics during episodes of bladder infection to alleviate symptoms prior to clearance of the uropathogen. Phenazopyridine is a commonly prescribed azo-based, orally administered analgesic that achieves high

concentrations in the urinary tract and stains the urine with a bright orange color, easily recognized by patients (Zelenitsky and Zhanel, 1996).

An increased risk of SCC exists in patients with *Schistosoma haematobium* infestation or protracted catheter use resulting from agitation of the urothelium, induction of chronic urothelial inflammation and/or concurrent infection(Michaud et al., 2007). However, the association between UTI and UCB is less well established. Acute bacterial infection of the urinary bladder induces an acute host inflammatory response which may become prolonged as a result of recurrent infections or uropathological conditions conducive to urinary stasis and extended bacterial dwell-time within the bladder. In experimental studies, rats with bladders physically or chemically agitated before exposure to attenuated or pathogenic strains of *E. coli* were more prone to develop neoplastic lesions and manifested a higher incidence of UCB than did uninfected rats (Davis et al., 1984; Higgy et al., 1987; Kawai et al., 1994; Yamamoto et al., 1992). However, epidemiologic studies examining the association between UTI and UCB in humans exhibit a high degree of inconsistency with several studies reporting a positive association (Dunham et al., 1968; Kantor et al., 1984; La Vecchia et al., 1991; Wynder et al., 1963), while others reported no association (Gonzalez et al., 1991; Jhamb et al., 2007; Kjaer et al., 1989; Piper et al., 1986) or an inverse association in women only (Jiang et al., 2009). Moreover, the temporal relationship between infection and UCB diagnosis was not considered in many early studies and the use of bladder infection medication, an important confounding factor, has not been evaluated in any studies to date. In chapter 3 of this thesis, the association between UTIs and UCB is evaluated in more detail with specific focus on the effect of UTI medications on UCB risk in the Spanish population.

1.1.5 Clinical manifestation, diagnosis and prognosis of UCB

Early clinical symptoms of bladder cancer most commonly manifest as painless haematuria (i.e. presence of erythrocytes in the urine), that may be accompanied by urinary frequency, urgency and/or dysuria (Kaufman et al., 2009). Early symptoms may often be misdiagnosed due to their similarity with other urogenital conditions such as urinary bladder infection or symptoms secondary to benign prostatic hyperplasia (BPH). Upon presentation of symptoms, a urinalysis is often done to evaluate urine erythrocyte concentration followed by visual inspection of the bladder urothelium with the aid of a

cystoscope (Cheung et al., 2013). A cytological evaluation of the urine may also be undertaken to evaluate the presence and type of exfoliated tumor tissue present. Diagnosis is made by histological examination of resected bladder tumor tissue with which tumor histology, stage and grade can be accurately determined by a pathologist. Bladder tumors are graded based on their level of cellular differentiation on a three-point (1-3) scale, with strongly differentiated tissues graded as 1. In the work presented herein the three grade redefinition provided by the WHO was used (Eble et al., 2004; Mostofi et al., 1999). The standard histological staging of bladder tumors is based on the TNM classification which considers the depth of tumor tissue invasion (T), degree of lymph node invasion (N), and whether metastasis to the surrounding tissues has occurred (M) (Epstein et al., 1998).

The majority of bladder tumors in developed nations present as UCB upon diagnosis (>90%), with less than 5% presenting as SCC and less than 2% presenting as adenocarcinomas (Kaufman et al., 2009). UCB predominantly manifests (70-80% of patients) as a non-muscle invasive tumor (NMIBC: pTa-pT1) characterized by good overall prognosis following transurethral resection (TUR) in patients with low-risk tumors (pTaG1/2), and intravesical chemotherapy and/or Bacillus Calmette Guerin (BCG) instillation in patients with high-risk tumors (pTaG3 or pT1G2/3) (Wu, 2005). The proportion of patients with NMIBC surviving after 15 years is very good: 100% in patients with low-grade Ta; 74% in patients with high-grade Ta, and; 62% in patients with T1 tumors (Kaufman et al., 2009). However, approximately 50-70% of NMIBC patients suffer a recurrence following treatment and a further 10-20% progress, developing new tumors exhibiting muscle invasion (MIBC: pT2-pT4); the risk of progression being higher among patients with high-grade tumors (Kaufman et al., 2009; Wu, 2005). A lower proportion (20-30%) of UCB patients are diagnosed with muscle invasive tumors (MIBC; pT2-pT4) characterized by poor prognosis: 50% of these patients die from their cancer and the five year relative survival rate for metastatic disease is only 5% (Michaud, 2007; Wu, 2005). Genomic profiling and gene expression analyses indicate a strong correlation between these pathologic classifications and the underlying molecular architecture of UCB (Lindgren et al., 2010).

1.1.6 UCB treatment

NMIBC treatment. While physical excision of papillary tumors by TUR is effective treatment for low-grade NMIBCs, TUR of high-grade NMIBCs and carcinoma *in situ* (CIS) is generally followed by intravesical instillation of attenuated *Mycobacterium bovis* BCG to reduce the chance of tumor recurrence; an approach that is effective in 50-60% of treated patients. Although the exact therapeutic mechanism of BCG instillation remains unclear, a functional host innate immune system is necessary to induce a localized Th1-polarized inflammatory response that promotes tumor ablation (reviewed in (Luo et al., 2011)). Chemotherapeutic agents such as mitomycin are also commonly administered to these patients, with instillation directly following TUR showing particular efficacy in reducing tumor recurrence (Kaufman et al., 2009). The high propensity of NMIBCs to recur also necessitates close and continual surveillance of treated patients and entails regularly scheduled cystoscopic examinations and urine cytology analyses.

MIBC treatment. Patients diagnosed with MIBCs are generally considered for radical cystectomy which entails surgical removal of the bladder, adjacent organs, and regional lymph nodes and the redirection of urine flow (Kaufman et al., 2009). UCB is highly sensitive to cisplatin chemotherapy and an assortment of chemotherapeutic regimens combining cisplatin and other chemotherapeutics have been developed in the treatment of metastatic disease with the most prevalent being a combination of methotrexate, vinblastine, doxorubicin and cisplatin (a.k.a. MVAC). Other cisplatin based chemotherapeutic regimens include gemcitabine and cisplatin (GC), and paclitaxel + GC (PGC) therapy which have shown therapeutic efficacy on the level of MVAC therapy (Kaufman et al., 2009).

1.1.7 Prognostic factors of UCB

Due to the aggressive nature of high grade UCB and the low survival rate associated with metastatic tumors, a means to accurately identify high-risk patients is imperative to tailor post-operative surveillance programs and diminish the burden of disease. The European Association of Urology (EAU) has established guidelines to predict recurrence and progression of NMIBC based on six of the most significant clinical and pathological prognostic factors determined from clinical studies evaluating patient response to intravesical therapy (Babjuk et al., 2008; Sylvester et al., 2006). These established

clinicopathological factors are directly associated with worse prognosis and include tumor multifocality, tumor size (</≥3cm), prior recurrence rate (≤/> 1 recurrence/year), T category, presence of concomitant *CIS* (worse prognosis in the presence of *CIS*) and tumor grade. Combinations of these factors are used to adjust the multivariate prognostic models of NMIBC recurrence or progression risk through nomograms/score approach.

The most effective adjuvant currently administered after resection of high-risk NMIBC is BCG instillation. However, one-third of the high-risk NMIBC patients that receive BCG treatment do not respond to BCG and 15% observe tumor progression to MIBC with very poor prognosis; classifying failure to respond to BCG instillation as a parameter of poor patient prognosis. Clinicopathologic parameters such as older age, concomitant CIS, tumor multiplicity, and high-grade tumors are all independent predictive factors of poor outcome following BCG. However, evidence related to the predictive utility of immunologic, intracellular or genetic biomarkers in response to BCG therapy is extensive and heterogeneous, with only the presence in the urine of the Th-1 response-mediating cytokine interleukin-2 (IL-2) predicting favorable response to therapy (reviewed in (Zuiverloon et al., 2012)).

The best established clinicopathological factors of progression and disease-specific survival (DSS) in patients with MIBC include the pathological stage parameters of T category, presence of nodes (N) and distant metastases (M), all of which associate with unfavorable prognosis. The presence of lymphovascular tumor invasion and resistance to neoadjuvant chemotherapy are also established indicators of poor prognosis but were not included as covariables in the SBC study.

1.1.8 Prognostic biomarkers of UCB

While clinicopathological parameters of the tumor are currently the most informative and widely used clinical prognostic factors of UCB; they do not account for all disease heterogeneity(Netto, 2012). In this regard, numerous studies have evaluated the prognostic utility of molecular biomarkers representing distinct carcinogenic pathways involved in UCB as a means of more accurately representing the biology and clinical course of the disease (Matsushita et al., 2011). Emerging molecular prognostic biomarkers of UCB tend to mirror the key molecular genes and proteins altered in the divergent

molecular pathways that characterize NMIBC and MIBC (Netto, 2012). For example, *FGFR3* activating mutations are characteristic of low-grade NMIBC, and evidence also suggests that they are associated with increased risk of recurrence in patients with TaG1 tumors (Hernandez et al., 2006). Conversely, *TP53* and *RB* inactivating mutations are characteristic of MIBC, and several studies have reported that the altered expression of their protein products, p53 and pRb1 respectively, is associated with tumor recurrence, progression and patient mortality (Cote et al., 1998; Shariat et al., 2004; Shariat et al., 2010). Other molecular biomarkers of NMIBC include: *HRAS* alterations, proliferation indices characterized by Ki67 expression, multimarker immunoexpression analyses of proteins such as p53, p27, KI67, pRb1, p21, overexpression of angiogenic markers like *VEGF*, and promoter hypermethylation. On the other hand, molecular biomarkers reported to associate with prognosis of MIBC include: alterations in the expression of p16, 21, *EGFR*, *ERBB2*, *VEGF* and mTOR (reviewed in (Netto, 2012))

There are no established guidelines for undertaking biomarker studies, and even the most widely studied prognostic biomarkers of UCB (e.g. p53) exhibit a large degree of heterogeneity in effect among studies that differ in methodology and sample size (Malats et al., 2005). Due to its mediatory role in inflammation and overexpression in UCB, cyclooxygenase-2 (see the following section) has been given substantial attention as a prognostic biomarker with an array of studies evaluating its role in UCB recurrence, progression and patient survival (Aziz et al., 2010; Bamias et al., 2008; Diamantopoulou et al., 2005; Eltze et al., 2005; Friedrich et al., 2003; Gudjonsson et al., 2011; Hilmy et al., 2006; Kim et al., 2002; Liedberg et al., 2008; Margulis et al., 2007; Mokos et al., 2006; Naruse et al., 2010; Shariat et al., 2003a; Shariat et al., 2003b; Shirahama et al., 2001; Tiguert et al., 2002; Wild et al., 2005; Wulfing et al., 2004; Yoshimura et al., 2001; Youssef et al., 2011). However, no clear consensus has been reached regarding the prognostic utility of COX2 in UCB as studies vary widely in antibodies and their concentrations, expression evaluation techniques, sample sizes, tumor subgroups analyzed, multivariable adjustment criteria and other factors that contribute to inter-study heterogeneity (see Table 4.4). In chapter 4 of this thesis, an attempt is made to homogenize and integrate the diversity of prognostic data available in the literature related to COX2 tumor expression in UCB through a systematic review of the literature and meta-analysis. Effectively, univariable and multivariable association analyses are modeled separately in NMIBC and MIBC to account for the higher COX2 expression levels in the latter group

(Czachorowski et al., 2012). Moreover, the largest sample of UCB patients to date is used to evaluate each of the pertinent prognostic endpoints and their association with tumor COX2 expression in the Spanish population.

1.2 CYCLOOXYGENASE-2

Cyclooxygenases (COX), also known as prostaglandin endoperoxide synthases (PTGS), are enzymes involved in the rate-limiting step of prostaglandin production from free arachidonic acid (Brock et al., 1999). Autocrine and/or paracrine secretion of prostaglandins and their activation of cognate membrane-bound or intracellular receptors mediates diverse homeostatic and immunoregulatory processes (Hata and Breyer, 2004). Deregulation of prostaglandin mediated signaling as a result of aberrant COX expression has been linked to various pathologies including autoimmune disease and cancer (reviewed in (Dubois et al., 1998)). Of the two primary COX isoforms (COX1/2) identified, COX1 exhibits constitutive expression in most human tissues and undertakes a variety of homeostatic functions labeling it a 'housekeeping' enzyme (Crofford, 1997). In contrast, COX2 becomes induced by a variety of endogenous and external stimuli that may include growth factors, tumor promoters, viral or bacterial infections as well as an assortment of proinflammatory cytokines (Bakhle and Botting, 1996; Marnett, 2009). COX2 induction also plays a role in the resolution of the inflammatory response through the production of anti-inflammatory prostaglandins suggesting that it has a mediatory role in inflammation (Gilroy et al., 1999). Due to its association with the inflammatory response under most physiological conditions as well as the overexpression of COX2 in many tumors, including UCB, this isoform will be given the principle focus throughout this thesis.

1.2.1 Cyclooxygenase gene structure

COX1 and 2 proteins are found highly conserved in vertebrates and have also been identified in some invertebrates such as coral, but are absent from insects, plants and unicellular organisms suggesting they resulted from a gene duplication event early during vertebrate speciation (Simmons et al., 2004). The genes representing each isoform are found at distinct genetic loci and differ substantially in size; while *COX1* maps to the long arm of chromosome 9 and measures 22kb, *COX2* maps to the long arm of

chromosome 1 and at 8.3kb is nearly three times smaller than *COX1*. However, both isoforms share a similar number of coding nucleotides (approx. 1800 bp each) spread over 11 exons in COX1 and 10 exons in COX2 that translate into similarly sized protein products consisting of 576 amino acids (68 kDa) and 604 amino acids (70 kDa), respectively (Tanabe and Tohnai, 2002). The *COX1* promoter contains multiple transcription start sites, is GC-rich, lacks a TATA-box promoter sequence, and contains a region delimited by two Sp1 binding sites whose integrity is important for maintaining constitutive expression of COX1; characteristics consistent with a constitutively active homeostatic gene (Mbonye and Song, 2009; Wang et al., 1993; Xu et al., 1997). Conversely, the *COX2* promoter contains various *cis* regulatory elements including a TATA-box and CAAT enhancer region as well as binding sites for important transcriptional activators including a cAMP response element (CRE), NFkB, NF-IL6 and AP-1, consistent with an immediate early gene (Appleby et al., 1994; Hla et al., 1999; Mbonye and Song, 2009). COX2 mRNA is relatively unstable (half-life of approx. 30 min.) in comparison to COX1 mRNA (half-life of approx. 4 hours) owing primarily to the presence of multiple copies of AU rich elements (ARE) found within the 3'-UTR of COX2 mRNA which promote mRNA degradation (Mbonye and Song, 2009; Roy et al., 1996). However, COX2 mRNA becomes stabilized by ARE binding proteins in response to proinflammatory factors that activate the p38 MAPK signaling cascade (Lasa et al., 2000). Moreover, COX2 can be expressed as three alternatively polyadenylated transcripts that vary in size and mRNA stability and may also confer subcellular and tissue-specific targeting properties (Hall-Pogar et al., 2005; Ristimaki et al., 1996; Simmons et al., 2004).

1.2.2 Cyclooxygenase protein structure

The protein structures of both COX isoforms are nearly superimposable and share approximately 60% amino acid similarity, with the most significant difference being a larger substrate binding site in COX2 resulting from the substitution of a Val residue in COX1 for an Ile at corresponding position 523 within the cyclooxygenase active site of COX2 (Kurumbail et al., 1996; Picot et al., 1994). As a result of a larger substrate binding site, COX2 displays less substrate specificity than COX1 and is more apt to synthesize prostaglandins using bigger substrates (Simmons et al., 2004). Both COX isoforms are heme-containing, integral membrane glycoproteins found inserted into the lumenal face of the endoplasmic

reticulum and within the perinuclear region. Morita et al observed that while both isozymes localize to the endoplasmic and nuclear membranes, COX1 displays activity predominantly at the ER membrane in contrast to COX2 which is active at both subcellular locations (Morita et al., 1995). Each isoform is a homodimer consisting of a short N-terminal domain, an amphipathic membrane binding domain, and a large catalytic domain that contains the active sites for cyclooxygenase and peroxidase activity on either side of a heme-moiety (Picot et al., 1994)

1.2.3 Cyclooxygenase activity and products.

Both COX isoforms catalyze the same reaction and their luminal positioning within the lipid membrane facilitates access to mobilized arachidonic acid freed from the phospholipid bilayer by phospholipase A_2. Arachidonic acid is oxygenated at the cyclooxygenase site to form the peroxide intermediate prostaglandin G_2 (PGG_2) which diffuses to the peroxidase active site where it is reduced to prostaglandin H_2 (PGH_2). PGH_2 acts as a substrate for several prostaglandin synthases as well as thromboxane synthase, by which it becomes converted into various primary prostanoids including PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2, and TXA_2 (Simmons et al., 2004). These prostaglandins bind to cognate membrane bound G-protein-coupled receptors in an autocrine or paracrine fashion. In contrast, some prostaglandins including PGJ_2, 15-deoxy-PGJ_2 and PGA_2 interact with intracellular peroxisomal proliferator activated receptors (PPAR) which induce transcriptional activity in the nucleus (Hla et al., 1999).

Prostaglandins regulate the biological processes associated with COX isoform expression in a broad range of tissues under both physiological (largely attributed to constitutive COX1 activity) and pathological conditions (largely attributed to the induction of COX2 activity). For example, PGE2 and PGI2 confer cytoprotective effects in the gastrointestinal tract by reducing gastric acid and increasing the release of mucous, while in the kidneys these prostaglandins function as vasodilators that maintain renal homeostasis (Simmons et al., 2004). Other peripheral tissues which require constitutive prostaglandin secretion for the maintenance of proper function include the cardiovascular system, the lungs, reproductive system and the central nervous system. Alternatively, PGE2 and PGI2 also have proinflammatory, hyperalgesic and pyretic roles under pathological conditions (Simmons et al., 2004).

1.2.4 Cyclooxygenase expression and signaling.

COX1 is found constitutively expressed in most normal tissues of the body with similar expression levels maintained in tissues representing pathophysiological conditions such as osteoarthritis, atherosclerosis, and cancer (Koki et al., 2002; O'Neill and Ford-Hutchinson, 1993). Conversely, with exception of the brain, kidney, placenta and female reproductive tract, COX2 is not expressed constitutively under basal conditions and becomes induced upon exposure to pathophysiological stress such as inflammation, infection or vascular shear stress (Harris et al., 1994; Hirst et al., 1995; Koki et al., 2002; O'Neill and Ford-Hutchinson, 1993). At the molecular level this stress may take the form of proinflammatory cytokines such as IL-1, TNF-α and IFN-γ, growth factors, tumor promoters, hormones or external factors such as endotoxins found on invading pathogens. Upon binding and activation of their respective cellular receptors these molecular factors induce receptor-mediated signaling cascades that terminate with transcriptional activation of COX2 via one of its promoter sites thereby facilitating prostaglandin mediated resolution of the stress-response. Receptor-mediated signaling cascades leading to COX2 transcriptional activation can be broadly classified as activating the NF-kB or mitogen activated protein kinase signaling (MAPK) pathways (Smith et al., 2000; Tanabe and Tohnai, 2002). Inflammatory factors such as TNF, IL-1 and other pro-inflammatory cytokines can activate both the NF-kB and MAPK signaling pathways, acting through any one of the three major MAPKs of the latter pathway (e.g. ERK1/2, JNK or p38). Pathogen-induced COX2 expression may similarly act through NF-kB and MAPK signaling pathways, but with signaling following the activation of pathogen specific toll-like receptors (TLR) by pathogen associated molecular patterns (PAMPs) and the recruitment of the adaptor protein MyD88. For example, endotoxins such as LPS found on some bacterial membranes specifically activate TLR4, while hypomethylated viral or bacterial DNA binds to TLR9 within the endosome, thereby facilitating MyD88 recruitment and initiation of COX2 expression via NF-kB, C/EBP or AP1. Conversely, growth factors such as PDGF and FGF typically signal via MAPK signal-transduction pathways (Smith et al., 2000).

1.2.5 COX inhibition.

COX isozymes have a limited catalytic turnover and undergo self-inactivation within 1-2 minutes at AA concentrations inducing maximum reactivity (Simmons et al., 2004). Self-inactivation has been proposed to result from oxidative damage to the enzymes incurred from the generation of radical and high-state oxidative heme intermediates during the catalytic process (Tsai and Kulmacz, 2010; Wu et al., 2007). Despite innate self-inactivation, a variety of medically available non-steroidal anti-inflammatory drugs (NSAIDs) targeting the cyclooxygenase active site of COX enzymes have been developed to provide immediate catalytic inhibition and relief of clinical symptoms that may include fever, pain and inflammation. The most widely used is acetylsalicylic acid or Aspirin, which is the only NSAID to covalently modify COX1/2 by acetylating a serine residue within the active site and thereby sterically block subsequent substrate binding and prostaglandin production (Loll et al., 1995). Aspirin completely inactivates COX1 activity, but due to conformational differences of several residues within the active sites of the two isozymes, COX2 is incompletely inactivated and capable of oxygenating arachidonic acid to produce lipoxins in lieu of prostaglandin H_2 (Johnson et al., 1985; Rowlinson et al., 2000). However, the majority of medically relevant NSAIDs compete with arachidonic acid for key amino acid residues within the cyclooxygenase active site in a manner that is reversible. The main difference among non-covalently binding NSAIDs is the rate at which they inhibit COX activity . Ibuprofen and piroxicam are two commonly administered NSAIDs that bind and dissociate rapidly from the COX active site and confer immediate enzyme inhibitory effects. Conversely, NSAIDs such as diclofenac, flurbiprofen and indomethacin exhibit a time-dependent inhibitory response proposed by Selinsky et al to correlate with the speed and efficiency by which these drugs can reach and interact with Arg120 and Tyr355 within the catalytic pocket (Selinsky et al., 2001).

1.2.6 Cyclooxygenase in the urinary bladder.

In the healthy urinary bladder, prostaglandin secretion maintains bladder tone and contributes to the normal functioning of the urinary process (reviewed in (Maggi, 1992)). The topical application of prostaglandins such as PGE2 and TXB2 to the urinary bladder of anesthetized rats was reported by Maggi et al to induce involuntary micturition (Maggi et al., 1988). Wheeler et al detected both COX1

and COX2 mRNA in the ureter, bladder muscle and urothelium of healthy individuals, but only the former isoform was detected in these tissues at the protein level (Wheeler et al., 2002). However, urinary PGE2 and COX2 protein expression in urine particulates was increased in patients with urogenital cancer, urinary tract infections and following processes that induced localized inflammation, such as BCG treatment, with no changes in COX1 expression reported. These results are consistent with those of Farkas et al who reported a significant increase in prostaglandin levels in patients with acute bacterial cystitis, compared to healthy controls, that correlated with the onset and duration of cystitis (Farkas et al., 1980). Park et al observed that increased mechanical stretching of cultured rat bladder smooth muscle cells induced COX2 mRNA and protein expression in a time-dependent manner but had no effect on COX1 expression levels (Park et al., 1999). Collectively, these studies appear to suggest that prostaglandins serve a cytoprotective role in the healthy urinary bladder in which their levels are maintained by the constitutive expression of COX1, with COX2 induction resulting from exposure of the bladder to mechanical, inflammatory or pathogenic stress

1.2.7 COX2 and (bladder) cancer.

In healthy tissues, transient COX2 expression generally occurs as part of an acute and highly regulated response to an assortment of predominantly pro-inflammatory stimuli (see above). However, aberrant overexpression of COX2, as in the case of recalcitrant infection or sustained exposure to pro-inflammatory stimuli, primarily promotes the production of PGE_2 which is sufficient to promote carcinogenesis by affecting the hallmarks of cancer in various tissues including the urothelium (reviewed in (Greenhough et al., 2009; Hanahan and Weinberg, 2011)). For example, using immortalized urothelial cells overexpressing COX2, Gee et al reported that PGE_2 levels correlated significantly with cellular invasion through an artificial extracellular matrix (Gee et al., 2008). Furthermore, Adhim et al demonstrated that COX2 inhibition in T24 urothelial cancer cell lines by the COXIB etodolac could suppress the epithelial to mesenchymal transition (EMT); a process implicated in carcinoma invasion and migration (Adhim et al., 2011). Other *in vitro* studies relying on COXIBs to inhibit COX2 activity in UCB cell lines have reported dose-dependent decreases in cellular proliferation (Mohseni et al., 2004; Smakman et al., 2005) and promotion of apoptosis through the down regulation of the anti-apoptotic

protein Bcl-2 (Gee et al., 2006). Using NMIBC samples taken from 110 patients, Friedrich et al also observed a significant correlation between COX2 expression and blood vessel proliferation in the tumor zone suggesting a link between COX2 and angiogenesis in UCB (Friedrich et al., 2003). Moreover, PGE2 secreted by COX2 overexpressing tumors has immunoregulatory properties which promote tumor evasion by polarizing cytokine production towards a Th2 immune response and impair dendritic cell maturation and tumor infiltration (Ahmadi et al., 2008; Sharma et al., 2003). While PGE2 levels are kept in check by another COX2 prostaglandin product, 15-PGDH, which contributes to PGE2 degradation, 15-PGDH activity has been found inhibited in bladder tumors (Gee et al., 2003).

While the majority of the preceding studies relied on *in vitro* techniques to draw conclusions, they highlight the diverse roles of COX2/PGE_2 expression in bladder carcinogenesis, and collectively suggest that increased COX2 expression could be conducive to bladder tumor development and progression *in vivo.* Strong evidence in support of this view came from the group of Klein et al, who developed a transgenic mouse that overexpressed COX2 under the control of the keratin-5 promoter. COX2 overexpression in the basal epithelial cells was sufficient to cause transitional cell hyperplasia in 75% of homozygous transgenic mice and UCB in about 10% of mice; with significant increases in vascular endothelial growth factor (VEGF) expression above wild-type urinary bladders (Klein et al., 2005). In another approach, tumor prone transgenic mice treated with a tumor promoter were significantly more likely to develop bladder tumors than mice concomitantly administered the COX2-specific inhibitor rofecoxib (D'Arca et al., 2010). COX2 expression has been reported in various premalignant lesions and tumors including urinary bladder dysplasia and cancer (reviewed in (Subbaramaiah and Dannenberg, 2003)). Urothelial carcinomas *in situ* and squamous cell carcinomas express COX2 more highly relative to UCB, with expression generally accepted to increase with urinary tumor stage and grade (Shirahama and Sakakura, 2001).

1.3 SPANISH BLADDER CANCER/EPICURO STUDY

1.3.1 Study design and population.

The analyses carried out in the body of this thesis relied almost entirely on patient information collected as part of the Spanish Bladder Cancer (SBC)/EPICURO study. The SBC/EPICURO study is a hospital-based case-control study with 1219 incident UCB cases and 1271 hospital controls recruited from 18 Spanish hospitals in five regions of Spain (Asturias, Barcelona, Elche, Tenerife and Valles) between 1998-2001. Controls were inpatients with conditions unrelated to bladder cancer risk and were matched to cases on age (+/- 5 years), gender, hospital region and ethnicity (99% of all patients were of Caucasian ancestry). The majority of study participants were men (87%) and ranged in age from 21-80 years old, with the median age of cases being slightly older than that of controls (66 years versus 65 years, respectively). These general patient characteristics may vary slightly from the patient sub-groups used for the analyses in the body of this thesis depending on the completeness of the data for the variables considered but the show no significant demographic differences (see Chapters 3, 4 and 5).

1.3.2 Information.

Patient information concerning various bladder cancer risk factors was collected by trained monitors using a questionnaire administered via computer assisted personal interview (CAPI) at hospital admission to which 84% of cases and 88% controls approached responded. Information collected from patients regarded a broad range of environmental, occupational, lifestyle and medical risk factors. The CAPI data used in the analyses undertaken in this thesis (with pertinent references to the literature) includes factors relevant to: smoking (Samanic et al., 2006); drug use: non-aspirin NSAIDs (Fortuny et al., 2006); medical history regarding urinary tract infections; water intake; frequency of nocturia (Silverman et al., 2008); and constant urinary pH readings (Alguacil et al., 2011).

Clinicopathological data regarding tumor characteristics was collected from cases after resection, with all tumors uniformly classified by a panel of pathologists according to the criteria of the TNM classification and the WHO-ISUP using the thre-grade redefinition provided by the ISUP-WHO as described above. All bladder tumor samples used in the study were collected prior to the administration

of any intravesical or systemic therapy. Other clinicopathological data collected that is used in this thesis includes tumor size and multiplicity and the type of treatment that patients were administered to treat their cancer.

Patients were also followed-up yearly by telephone interview and review of clinical charts following diagnosis to ascertain the state of their cancer, the number of tumors that may have had developed since diagnosis or the last follow-up, and the type of treatment they were receiving. Follow-up information ranged from 1 to 117 months, with a median follow-up time of 70.7 months.

Several different types of biological samples were collected from patients, of which lymphocyte based blood samples, buccal swabs, and tumor tissue samples embedded in paraffin blocks were directly used for the analyses in this thesis. Specifically, lymphocytes and buccal swabs were collected for DNA extraction used for genotyping of the genes evaluated in chapter 5; while paraffin tissue blocks were used to construct tissue microarrays which were subsequently evaluated for tumor COX2 expression as detailed in chapters 4 and 5.

Genotypes for genes of interest, as described in Chapter 3, were obtained using four different genotyping platforms at the Core Genotyping Facility of the U.S National Cancer Institute (NCI), or the Core Genotyping Centre of the Spanish National Cancer Research Centre (CNIO). These included the TaqMan, Illumina GoldenGate and Illumina Infinium HumanMap 1M platforms.

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CHAPTER 2: HYPOTHESIS AND OBJECTIVES

2.1 HYPOTHESIS

Environmental or endogenous factors that induce or contribute to sustained urothelial inflammation can incite tumor cell initiation and promote tumor progression. Consequently, exposed individuals, or those harboring molecular or genetic aberrations that promote chronic inflammation in the bladder will have a higher risk of UCB and suffer from a worse prognosis.

2.2 OBJECTIVES

2.2.1 General objective

To assess the involvement of local chronic inflammation in the development and clinical evolution of urothelial carcinoma of the bladder (UCB) by analyzing the association between inflammation-related factors (environmental, molecular and genetic) and UCB risk and prognosis in the Spanish population.

2.2.1 Specific objectives

1.) Evaluate the association between urinary tract conditions, specifically bladder infection, and UCB risk

2.) Determine whether the use of urinary tract infection medications influences the association between bladder infection and UCB risk

3.) Assess COX2 protein expression in UCB and its association with tumor clinicopathological factors

4.) Evaluate the prognostic value of COX2 tumor expression in the recurrence and progression of NMIBC, and in the progression and survival of patients with MIBC

5.) Determine whether SNPs in inflammatory genes confer heterogeneity in susceptibility to UCB characterized by low or high levels of COX2 tumor expression

6.) Determine whether COX2 tumor expression levels considered together with SNPs in inflammatory genes have prognostic value in UCB and/or predict responsiveness to BCG immunotherapy

CHAPTER 3: REVISITING THE ASSOCIATION BETWEEN URINARY TRACT INFECTIONS AND BLADDER CANCER RISK

Maciej J Czachorowski[1], Debra T Silverman[2], Jesus Herranz[1], Sirish Kishore[1], Josep Lloreta[3,4,5], Alfredo Carrato[6,7], Adonina Tardón[8], Juan Alguacil[9], Nathaniel Rothman[2], Montserrat Garcia-Closas[2], Manolis Kogevinas[10], Francisco X Real[5,11], Núria Malats[1] for the SBC/EPICURO investigators[12]

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

[2] Dept. of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, United States.

[3] Institut Municipal d'Investigació Mèdica – Hospital del Mar, Barcelona, Spain.

[4] Departament de Patologia, Hospital del Mar – Parc de Salut Mar, Barcelona, Spain.

[5] Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Barcelona, Spain.

[6] Hospital General Universitario de Elche, Elche, Spain.

[7] Hospital Ramon y Cajal, Madrid, Spain.

[8] Universidad de Oviedo, Oviedo, Spain.

[9] Centro de Investigación en Salud y Medio Ambiente (CYSMA), University of Huelva, Huelva, Spain.

[10] Centre de Recerca en Epidemiologia Ambiental (CREAL), Barcelona, Spain.

[11] Epithelial Carcinogenesis Group, Spanish National Cancer Research Centre (CNIO), Madrid, Spain.

[12] Spanish Bladder Cancer (SBC)/EPICURO Study investigators.

(*submitted*)

3.1 SUMMARY

Experimental models suggest that bacterial urinary tract infections (UTI) promote urothelial carcinoma of the bladder (UCB). However, epidemiologic studies provide conflicting results, suggesting a more complex relationship. In the following study the association between UTI and the UCB risk was examined with emphasis on the effect of UTI medication. Newly diagnosed UCB patients (n=1096) recruited between 1998-2001 from 18 Spanish hospitals and 1259 age, gender and hospital-matched controls were included in the study. Information regarding UTIs was collected in person by trained interviewers through personal interviews. Clinical data was retrieved from hospital charts. Unconditional logistic regression analysis was used to estimate odds ratios (OR) and 95% confidence intervals (95%CIs). A history of at least one UTI was reported in 182 (17.1%) cases and 255 (20.5%) controls yielding a 24% reduction in UCB risk (OR=0.76, 95%CI 0.60-0.95, P=0.014). The decreased risk was mainly observed in patients who reported $\geq$2 infections (OR=0.51, 95%CI 0.28-0.93, P=0.029) and among those who reported using medication to treat their UTIs (OR=0.51, 95%CI 0.37-0.70, P=1.0e-04). Use of the azo-based urinary tract analgesic phenazopyridine, in particular, was associated with a reduction in UBC risk (OR=0.35, 95%CI 0.20-0.62, P=2.40e-4); however risk did not decrease with increased use of phenazopyridine. The main limitations were 1) the retrospective and self-reported nature of UTI history and use of UTI medication 2) limited statistical power in some stratified analyses. The cytotoxic properties of some commonly administered UTI medications may contribute to modifying the association between UTI and UCB risk. A protective effect was most pronounced among those individuals who received analgesic treatment with phenazopyridine. Given the uro-carcinogenic properties of azo-based compounds, uncontrolled confounding cannot be excluded and this association should be interpreted cautiously and investigated further experimentally and in future observational studies

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3.2 INTRODUCTION

Urinary tract infections (UTI) of the bladder are mostly due to *Escherichia coli* and are frequently characterized by increased voiding frequency, urgency and/or dysuria (Dhakal et al., 2008). They are more frequent in women than in men, with nearly 50% of women experiencing at least one episode in their life and 20% of these observing a recurrent event (Stamm and Hooton, 1993). Treatment generally entails a short-term (3 day) regimen of oral antibiotics such as nitrofurantoin, trimethoprim and/or sulfonamides, or broader spectrum fluoroquinolones like ciprofloxacin or levofloxacin, with extended treatment periods in the case of recurrent episodes (Hummers-Pradier and Kochen, 2002; Stamm and Hooton, 1993). In Spain, urinary tract analgesics are often taken concurrently with antibiotics during episodes of bladder infection to alleviate symptoms prior to clearance of the uropathogen. Phenazopyridine is a commonly prescribed azo-based, orally administered analgesic that achieves high concentrations in the urinary tract and stains the urine with a bright orange color, easily recognized by patients (Zelenitsky and Zhanel, 1996).

Strong associations between chronic UTI and squamous cell carcinoma have been established in cases of *Schistosoma haematobium* infestation and in spinal cord injury patients with protracted catheter use (Michaud et al., 2007). However, the association between UTI and urothelial carcinoma of the bladder (UCB), the predominant bladder cancer histology in developed nations, is less well established. In experimental studies, rats with bladders physically or chemically agitated before exposure to attenuated or pathogenic strains of *E. coli* were more prone to develop neoplastic lesions and manifested a higher incidence of UCB than did uninfected rats (Davis et al., 1984; Higgy et al., 1987; Kawai et al., 1994; Yamamoto et al., 1992). However, epidemiologic studies examining the association between UTI and UCB in humans exhibit a high degree of inconsistency with several studies reporting a positive association (Dunham et al., 1968; Kantor et al., 1984; La Vecchia et al., 1991; Wynder et al., 1963), while others reported no association (Gonzalez et al., 1991; Jhamb et al., 2007; Kjaer et al., 1989; Piper et al., 1986) or an inverse association in women only (Jiang et al., 2009). Importantly, the temporal relationship between UTI and UCB diagnosis was not considered in many early studies and the use of UTI medication, has not been evaluated in any study to date.

The aim of this study was to investigate whether reported UTI and medication taken to treat and alleviate them are associated with UCB risk.

3.3. MATERIALS AND METHODS

3.3.1 Population

Patients were recruited from 18 hospitals in five regions of Spain as part of the Spanish Bladder Cancer (SBC)/EPICURO Study, a hospital-based case-control study described elsewhere (Garcia-Closas et al., 2005). Eligible cases (N=1,096) were men and women newly diagnosed with UCB between 1998-2001 and aged 22-81 years at the time of diagnosis. A pathologist review panel uniformly classified the level of invasiveness (T) and grade (G) of each tumor biopsy based on the TNM classification and the WHO-ISUP 1999 (Eble et al., 2004; Mostofi et al., 1999). Individuals previously diagnosed with cancer of the urinary tract were not eligible for the study. Eligible controls (N=1,259) were selected from in-patients at similar times as cases and from those individuals admitted to the hospital for conditions/diseases thought to be unrelated to the exposures under study. They were matched on age at diagnosis/interview, gender, hospital and ethnicity.

The study was approved by the IRBs of participating centers and the U.S. National Cancer Institute. Prior to being interviewed, informed written consent was obtained from all study participants in accordance with the Ethics Committees of each participating hospital.

3.3.2. Information

Subjects were interviewed in each participating hospital using a computerized questionnaire (CAPI) with response rates for cases and controls of 84% and 88%, respectively. They were asked for past history of UTIs and their treatment, to characterize this exposure. Patients were specifically asked whether any of the medications they had taken turned their urine orange , indicating treatment with the urinary tract analgesic phenazopyridine. Additionally, data on kidney infection, bladder or kidney stones, and in men, prostatic symptoms secondary to BPH was also obtained. Information was collected regarding patient demographics and other factors previously found to be associated with UCB risk including smoking status, occupational exposures, fruit and vegetable consumption, daily water consumption, night-time urination frequency,urinary pH and non-steroidal anti-inflammatory drug (NSAID) use.

3.3.3 Statistical methods

Odds ratios (ORs) and 95% confidence intervals (CIs) were used to estimate the relative risk of UCB and were computed for the exposure of interest using unconditional logistic regression models adjusting for age, gender, hospital region, and smoking status. UCB risk due to infection was also assessed distinctly in low-risk (pTaG1/G2) and high-risk (pTa/pT1G3) non-muscle invasive UCB (NMIBC) and muscle invasive UCB (MIBC). A history of prostatic symptoms secondary to BPH was included in final models for men.

Interaction between two risk factors was tested by adding a cross-product term to the logistic model and conducting a likelihood ratio test. All statistical tests were done using R statistical software.

3.4 RESULTS

Characteristics of the studied population (cases and controls) is shown in Table 3.1.

3.4.1. Risk of UCB conferred by a history of UTI

A history of UTI was reported by 17% of cases and 21% of controls, yielding a significant reduction in bladder cancer risk by 24% (OR=0.76, 95%CI 0.60-0.95) (Table 3.2). This effect was strengthened in women (OR=0.53, 95%CI 0.32-0.89) but not observed in men (OR=0.92, 95%CI 0.68-1.23). Histories of other urinary tract disorders were not significantly associated with UCB risk in men or women (Table 3.2) and they were not confounding factors for the UTI association either (Table 3.3). However, men reporting prostatic symptoms secondary to BPH had a significant reduction in UCB risk (OR=0.68, 95%CI 0.49-0.94; Table 3.2) and their exclusion from the analysis attenuated the inverse association for UTI among men (Table 3.3), suggesting it was a confounder.

To reduce the potential that UTI were misdiagnosed as, or resulted from, the early clinical manifestation of the tumor, UCB risk was evaluated considering the time between the last reported bladder infection and cancer diagnosis/interview (Table 3.4). While, compared to individuals with no reported UTI history, patients with an infection reported <1 year prior to diagnosis/interview did not have a reduction in UCB risk (OR=1.36, 95%CI 0.87-2.13), whereas those who reported bladder infections ≥1 year prior to cancer diagnosis/interview had a reduced risk (OR=0.58, 95%CI 0.43-0.79); which was apparent in both women (OR=0.44, 95CI 0.23-0.86) and men (OR=0.66; 95%CI 0.47-0.94).

We next investigated whether the reduced UCB risk was associated with the number of episodes of reported UTI (Table 3.5). Patients reporting >2 episodes of UTI exhibited a more pronounced reduction in bladder cancer risk (OR=0.51, 95%CI 0.28-0.93) than those reporting ≤2 infections (OR=0.62, 95%CI 0.44-0.87). This pattern was observed for men but the reduced risks were no longer statistically significant.

Table 3.1. Distribution of selected characteristics of urothelial bladder cancer cases and controls

	Cases		Controls		*P*-value[a]
	(*n*=1096)	%	(*n*=1259)	%	
Gender					0.495
Men	963	87.86	1094	86.89	
Women	133	12.14	165	13.11	
Region					0.835
Barcelona	207	18.89	247	19.62	
Valles	185	16.88	190	15.09	
Elche	73	6.66	84	6.67	
Tenerife	192	17.52	223	17.71	
Asturias	439	40.05	515	40.91	
Age (yrs.), mean (±SD)	66.11	9.86	64.78	9.93	0.0012
Smoking status					<0.0001
Non-smokers	142	12.96	367	29.15	
Occasional	50	4.56	97	7.70	
Former	428	39.05	474	37.65	
Current	476	43.43	321	25.50	
Ever smoker					<0.0001
Never	142	12.96	367	29.15	
Ever	954	87.04	892	70.85	
History of bladder infection					0.038
No	885	82.85	990	79.52	
Yes	182	17.05	255	20.48	
missing	29		14		
Bladder infection medication					0.002
No infection	885	86,34	990	82,98	
Yes	109	10,63	181	15.17	
No	31	30,23	22	1.84	
missing	71		66		
Urinary pH					0.010
>6.0	379	54.14	410	61.10	
≤6.0	321	45.86	261	38.90	
missing	396		588		
Tumor subtype					
No tumor	-		1259	100.00	
NMIBC low risk	603	55.02	-		
NMIBC high risk	235	21.44	-		
MIBC	258	23.54	-		

[a] From Fisher's exact test or t-test as appropriate; missing values excluded

Table 3.2: Urinary tract disorders and risk of urothelial bladder cancer by gender

	All					Men					Women				
Variables[a]	**Cases**	**Controls**	**OR**[b]	**95%CI**	**P**	**Cases**	**Controls**	**OR**	**95%CI**	**P**	**Cases**	**Controls**	**OR**	**95%CI**	**P**
Bladder Infection[c]															
No	885(82.9%)	990(79.5%)	1,00	REF		610(85%)	728(84.1%)	1,00	REF		86(66.7%)	88(54%)	1,00		
Yes	182(17.1%)	255(20.5%)	0,76	(0,6-0,95)	0,014	108(15%)	138(15.9%)	0,92	(0,68-1,23)	0,554	43(33.3%)	75(46%)	0,53	(0,32-0,89)	0,015
Kidney Infection															
No	796(95.6%)	963(94.3%)	1,00	REF		694(96%)	822(94.3%)	1,00	REF		102(92.7%)	141(94.6%)	1,00		
Yes	37(4.4%)	58(5.7%)	0,8	(0,51-1,24)	0,314	29(4%)	50(5.7%)	0,73	(0,45-1,2)	0,220	8(7.3%)	8(5.4%)	1,2	(0,41-3,5)	0,737
Bladder Stones															
No	814(97.6%)	996(97.7%)	1,00	REF		704(97.4%)	851(97.7%)	1,00	REF		110(99.1%)	145(98%)	1,00		
Yes	20(2.4%)	23(2.3%)	1,04	(0,55-1,96)	0,908	19(2.6%)	20(2.3%)	1,1	(0,56-2,16)	0,777	1(0.9%)	3(2%)	0,56	(0,06-5,67)	0,626
Kidney Stones															
No	710(85%)	852(83.5%)	1,00	REF		615(84.9%)	725(83%)	1,00	REF		95(85.6%)	127(86.4%)	1,00		
Yes	125(15%)	168(16.5%)	0,97	(0,74-1,27)	0,824	109(15.1%)	148(17%)	0,95	(0,71-1,26)	0,713	16(14.4%)	20(13.6%)	1,13	(0,54-2,34)	0,748
Prostatic Symptoms															
No						643(89.4%)	742(85.6%)	1,00	REF						
Yes						76(10.6%)	125(14.4%)	0,68	(0,49-0,94)	0,021					

[a] Data was missing for 2 cases on kidney infection; for 1 case and 2 controls on bladder stones; for 1 control on kidney stones; and 2 cases missing data on prostatic symptoms were also excluded for analyses in men [b] Unconditional logistic regression adjusted for age, gender, region, smoking status [c] Analysis in men additionally adjusted for history of prostatic symptoms; 1 case and 1 control missing bladder infection data but not prostatic symptoms data.

Table 3.3. Association between bladder infections and UCB considering effect of other Urinary tract disorders and diabetes medication

		Cases	Controls			
		N(%)	N(%)	OR[a]	95%CI	P
Bladder infection - *All Cases*						
	No	890(83%)	994(79.5%)	1		
	Yes	182(17%)	257(20.5%)	0,76	(0,6-0,95)	0,014
Bladder infection - *excluding bladder stones*						
	No	676(83.3%)	798(80.3%)	1		
	Yes	136(16.7%)	196(19.7%)	0,77	(0,6-1)	0,049
Bladder infection - *excluding kidney stones*						
	No	591(83.6%)	691(81.2%)	1		
	Yes	116(16.4%)	160(18.8%)	0,82	(0,62-1,08)	0,164
Bladder infection - *excluding kidney infections*						
	No	660(83.2%)	779(81%)	1		
	Yes	133(16.8%)	183(19%)	0,84	(0,65-1,09)	0,199
Bladder infection - *excluding prostatic symptoms*						
	No	557(86.6%)	648(87.3%)	1		
	Yes	86(13.4%)	94(12.7%)	1,03	(0,74-1,43)	0,852
Bladder infection - *excluding diabetes medications*						
	No	573(82%)	694(80.1%)	1		
	Yes	126(18%)	172(19.9%)	0,8	(0,61-1,05)	0,110
Bladder infection - *excluding all urinary tract disorders and diabetes meds*						
	No	364(87.1%)	433(87.3%)	1		
	Yes	54(12.9%)	63(12.7%)	0,98	(0,65-1,48)	0,931
Bladder infection - *excluding all urinary tract disorders and diabetes meds but including prostatic symptoms*						
	No	469(83.2%)	569(82%)	1		
	Yes	95(16.8%)	125(18%)	0,86	(0,63-1,17)	0,343

[a] Unconditional logistic regression adjusted for age, gender, region, smoking status with sequential exclusion of patients with indicated conditions

Table 3.4 Bladder infection and risk of bladder cancer based on time since last infection

	All					Men					Women				
Time[a]	**Cases**	**Controls**	**OR[b]**	**95%CI**	**P**	**Cases**	**Controls**	**OR[c]**	**95%CI**	**P**	**Cases**	**Controls**	**OR[b]**	**95%CI**	**P**
No infection	885	990	1,00			610	728	1,00			86	88	1,00		
< 1 year	49	39	1,36	(0,87-2,13)	0,174	38	25	1,70	(0,99-2,93)	0,054	11	14	0,89	(0,37-2,14)	0,788
≥ 1 year	81	154	0,58	(0,43-0,79)	4,10E-04	61	109	0,66	(0,47-0,94)	0,021	20	44	0,44	(0,23-0,86)	0,017
≥ 5 years	48	83	0,62	(0,42-0,92)	0,016	34	69	0,57	(0,36-0,89)	0,013	14	14	1,05	(0,43-2,55)	0,920
≥ 10 years	39	61	0,70	(0,46-1,09)	0,115	27	50	0,63	(0,38-1,05)	0,076	12	11	1,07	(0,41-2,8)	0,895

[a] Individuals diagnosed with bladder infection within 1, more than or equal to 1, 5 or 10 years of interview were compared in turn to individuals without a history of bladder infections; 52 cases and 62 controls did not provide data on time since last infection b Unconditional logistic regression adjusted for age, gender, region, smoking status c Additional adjustment for history of prostatic symptoms.

Table 3.5 Bladder infection and risk of urothelial bladder cancer (excluding patients with last bladder infection within 1 year of diagnosis/treatment)

	All					Men					Women				
Variables[a]	**Cases**	**Controls**	**OR[b]**	**95%CI**	**P**	**Cases**	**Controls**	**OR[c]**	**95%CI**	**P**	**Cases**	**Controls**	**OR[b]**	**95%CI**	**P**
Bladder infection frequency															
no infection	885	990	1	REF		610	728	1	REF		86	88	1	REF	
1 to 2	64	114	0,62	(0,44-0,87)	0,006	51	88	0,7	(0,47-1,02)	0,064	13	26	0,45	(0,2-1,01)	0,054
> 2	17	37	0,51	(0,28-0,93)	0,029	10	20	0,56	(0,25-1,24)	0,151	7	17	0,46	(0,17-1,22)	0,120
Medication for bladder infection															
yes	64	136	0,51	(0,37-0,7)	5.25E-5	48	94	0,59	(0,4-0,87)	0,007	16	42	0,36	(0,18-0,74)	0,005
no	15	16	1,3	(0,61-2,79)	0,497	12	14	1,2	(0,52-2,8)	0,669	3	2	2,21	(0,35-14,03)	0,400
Specific infection medication taken															
phenazopyridine	19	57	0,35	(0,2-0,62)	0,000	11	35	0,34	(0,17-0,7)	0,003	8	22	0,38	(0,15-0,97)	0,042
other medication	37	54	0,76	(0,49-1,19)	0,232	30	40	0,94	(0,56-1,58)	0,825	7	14	0,47	(0,17-1,29)	0,143
no medication	15	16	1,29	(0,6-2,77)	0,509	12	14	1,2	(0,51-2,79)	0,675	3	2	2,23	(0,35-14,14)	0,395

[a] Data missing for 3 controls on bladder infection frequency; for 2 cases and 2 controls on medication for bladder infection; for 10 cases and 27 controls on specific medication taken. b Unconditional logistic regression adjusted for age, gender, region, smoking status. c Additional adjustment for history of prostatic symptoms.

3.4.2 The effect of UTI treatment

A significant reduction in UCB risk was observed only in patients who reported taking medication to treat their infection (OR=0.51, 95%CI 0.37-0.70) (Table 3.5). In contrast, those patients that did not use any medication showed a non-significant increased UCB risk (OR=1.30, 95%CI 0.61-2.79). More specifically, the protective effect conferred by medication use was confined to patients who used the urinary tract analgesic phenazopyridine (OR=0.35, 95%CI 0.20-0.62); while patients who took other medications did not observe a significant reduction in UCB risk (OR=0.76, 95%CI 0.49-1.19). The observed reduced risk associated with phenazopyridine use was apparent in both sexes.

Individuals who medicated with phenazopyridine for each one of multiple reported episodes of UTI did not appear to benefit from a greater reduction in UCB risk compared to those who used the drug only once (P_{het}=0.873) (Table 3.6). The reduction in UCB risk was restricted to individuals who had medicated with phenazopyridine between one to five years prior to UCB diagnosis/interview (OR=0.11, 95%CI 0.02-0.45). Comparable effects were observed in analyses stratified by gender, although small sample sizes affected the significance values in several strata.

3.4.3 The effect of cigarette smoking and other UCB risk factors

Cigarette smoking, the best established risk factor for UCB, did not significantly modify the reduction in UCB risk attributed to UTI medication ($P_{interaction}$= 0.218) (Table 3.7). Phenazopyridine reduced UCB risk in all individuals irrespective of smoking status but the reduction was slightly larger in ever smokers (OR=0.35; 95% CI, 0.17-0.69) than in never smokers (OR=0.42, 95%CI 0.17-1.03). In contrast, only never smokers who reported using other medications had a reduction in UCB risk (OR=0.30, 95%CI 0.10-0.92), while no association between other medication use and UCB risk was observed in ever smokers (OR=0.95, 95%CI 0.58-1.57). Never smokers who did not report any treatment for their UTI showed a non-significant increased risk (OR=1,93, 95%CI 0,55-6,75, P=0,300). No significant interaction was observed between smoking*UTI treatment on UCB risk. Similar effects were observed in males and females.

Table 3.6 Phenazopyridine (DPP) medication in urinary bladder infection and risk of bladder cancer (excluding patients with last bladder infection within 1 year of diagnosis/treatment)

	All					Men					Women				
Variables[a]	**Cases**	**Controls**	**OR**[b]	**95%CI**	**P**	**Cases**	**Controls**	**OR**[c]	**95%CI**	**P**	**Cases**	**Controls**	**OR**[b]	**95%CI**	**P**
Frequency of DPP treatment for bladder infection															
no infection	885	990	1	REF		610	728	1,00	REF		86	88	1,00	REF	
1	10	30	0,33	(0,15-0,7)	0,004	7	20	0,35	(0,14-0,84)	0,019	3	10	0,31	(0,07-1,32)	0,113
> 1	6	15	0,53	(0,2-1,43)	0,213	3	7	0,60	(0,14-2,54)	0,490	3	8	0,44	(0,11-1,77)	0,247
no treated/with DPP	52	70	0,87	(0,59-1,28)	0,481	42	54	1	(0,64-1,57)	0,990	10	16	0,65	(0,27-1,58)	0,344
$P_{heterogeneity}$[d]					0,873										
Age at first DPP treatment for bladder infection															
< 43 yrs.	9	24	0,47	(0,21-1,07)	0,073	5	14	0,42	(0,15-1,22)	0,112	4	9	0,54	(0,14-2,06)	0,366
≥ 43 yrs.	9	25	0,34	(0,15-0,75)	0,008	6	16	0,40	(0,15-1,07)	0,068	3	10	0,26	(0,06-1,11)	0,070
no treated/with DPP	52	70	0,87	(0,59-1,28)	0,481	42	54	1,01	(0,64-1,57)	0,982	10	16	0,66	(0,27-1,6)	0,357
$P_{heterogeneity}$					0,996										
Age at last DPP treatment for bladder infection															
< 50 yrs.	8	23	0,49	(0,21-1,15)	0,103	4	14	0,37	(0,12-1,18)	0,093	4	9	0,68	(0,18-2,51)	0,563
≥ 50 yrs.	8	24	0,32	(0,14-0,74)	0,008	6	15	0,45	(0,17-1,22)	0,118	2	9	0,17	(0,03-0,93)	0,041
no treated/with DPP	52	70	0,87	(0,59-1,28)	0,483	42	54	1,01	(0,64-1,57)	0,977	10	16	0,67	(0,27-1,62)	0,371
$P_{heterogeneity}$					0,827										
Time since last DPP treatment															
≥ 1 to < 5 yrs.	2	19	0,1	(0,02-0,45)	0,003	2	9	0,30	(0,06-1,46)	0,135	0	10	0,00	NA	0,987
≥ 5 to < 20 yrs.	6	8	0,82	(0,28-2,45)	0,725	3	7	0,39	(0,1-1,54)	0,180	3	1	6,30	(0,57-69,8)	0,134
≥ 20 yrs.	8	20	0,52	(0,22-1,22)	0,130	5	13	0,52	(0,17-1,53)	0,231	3	7	0,63	(0,15-2,67)	0,533
no treated/with DPP	52	70	0,87	(0,59-1,28)	0,478	42	54	1,00	(0,64-1,57)	0,983	10	16	0,69	(0,28-1,71)	0,425

[a] Data missing for 3 cases and 12 controls on frequency of DPP treatment; for 1 case and 8 controls on age at first DPP treatment; for 3 cases and 10 controls on age at last DPP treatment; for 3 cases and 10 controls for time since last DPP treatment b Unconditional logistic regression adjusted for age, gender, region, smoking status. c Additional adjustment for history of prostatic symptoms. d P-values for tests of heterogeneity were calculated by excluding individuals without a history of bladder infection and those not treated/with DPP

Table 3.7 Urinary tract infection and risk of bladder cancer (excluding patients with last bladder infection within 1 year of diagnosis/treatment)

	All					
	Cases	**Controls**	**OR[a]**	**95%CI**	**P**	**P-int.**
Smoking status						
never smokers						0.218
no infection	109(87.2%)	274(83%)	1,00	REF		
phenazopyridine	7(5.6%)	27(8.2%)	0,42	(0,17-1,03)	0,057	
other medication	4(3.2%)	22(6.7%)	0,30	(0,10-0,92)	0,035	
no medication	5(4%)	7(2.1%)	1,93	(0,55-6,75)	0,300	
ever smokers						
no infection	776(93.4%)	716(91%)	1,00	REF		
phenazopyridine	12(1.4%)	30(3.8%)	0,35	(0,17-0,69)	0,002	
other medication	33(4%)	32(4.1%)	0,95	(0,58-1,57)	0,847	
no medication	10(1.2%)	9(1.1%)	1,05	(0,42-2,61)	0,922	
Urinary pH[c]						0.217
>= 6.0						
no infection	308(90.9%)	325(87.4%)	1	REF		
phenazopyridine	13(3.8%)	18(4.8%)	0,62	(0,28-1,34)	0,224	
other medication	12(3.5%)	23(6.2%)	0,5	(0,23-1,06)	0,069	
no medication	6(1.8%)	6(1.6%)	1,45	(0,41-5,16)	0,564	
< 6.0						
no infection	260(91.9%)	202(85.6%)	1	REF		
phenazopyridine	3(1.1%)	14(5.9%)	0,14	(0,04-0,51)	0,003	
other medication	15(5.3%)	16(6.8%)	0,59	(0,27-1,30)	0,188	
no medication	5(1.8%)	4(1.7%)	1,12	(0,26-4,90)	0,877	
Tumor stage/grade						NA
NMIBC Low Risk						
no infection	491(91.8%)	990(88.6%)	1,00	REF		
phenazopyridine	14(2.6%)	57(5.1%)	0,47	(0,25-0,87)	0,016	
other medication	21(3.9%)	54(4.8%)	0,78	(0,46-1,33)	0,362	
no medication	9(1.7%)	16(1.4%)	1,27	(0,53-3,04)	0,585	
NMIBC High Risk						
no infection	190(94.1%)	990(88.6%)	1,00	REF		
phenazopyridine	3(1.5%)	57(5.1%)	0,25	(0,07-0,82)	0,023	
other medication	5(2.5%)	54(4.8%)	0,45	(0,17-1,16)	0,098	
no medication	4(2%)	16(1.4%)	1,63	(0,50-5,25)	0,417	
MIBC						
no infection	204(93.2%)	990(88.6%)	1,00	REF		
phenazopyridine	2(0.9%)	57(5.1%)	0,16	(0,04-0,69)	0,014	
other medication	11(5%)	54(4.8%)	1,08	(0,54-2,17)	0,822	
no medication	2(0.9%)	16(1.4%)	0,68	(0,15-3,16)	0,620	
MIBC and NMIBC High Risk						
no infection	394(93.6%)	990(88.6%)	1,00	REF		
phenazopyridine	5(1.2%)	57(5.1%)	0,20	(0,08-0,53)	0,001	
other medication	16(3.8%)	54(4.8%)	0,75	(0,41-1,35)	0,335	
no medication	6(1.4%)	16(1.4%)	1,20	(0,43-3,31)	0,726	
$P_{heterogeneity}$ [b]					0,271	

[a] Unconditional logistic regression adjusted for age, gender, region, smoking status

[b] Multinomial logistic regression constraining the type of medication used in each tumor subtype examined [c] Constant urinary pH data was available for 700 cases (63.9%) and 671 (53.3%) of original study population

The inverse association between UTI medication/phenazopyridine and UCB risk was maintained in nearly all strata examined of fruit and vegetable consumption, daily water consumption, night-time voiding frequency, history of NSAID, use and constant urinary pH reading (Table 3.8). Reported users of phenazopyridine exhibited a significant reduction in UCB risk when their urinary pH was <6.0 (OR=0.14, 95%CI 0.04-0.51); those who had a constant urinary pH≥6.0 observed a less pronounced non significant reduction in UCB risk (OR=0.62, 95%CI 0.28-1.34) (Table 3.7). However, urinary pH did not significantly modify the association between UTI medication use and UCB risk (P=0.217).

3.4.4 The risk according to UCB subphenotypes

We also examined whether the observed reduction in cancer risk was maintained in all pathological subtypes of UCB (Table 3.7). The magnitude of the association between UTI medication and UCB risk was similar in all tumor subtypes evaluated (P_{het}=0.271). Compared to individuals with no reported history of UTI, patients who used phenazopyridine to treat their infection observed a significant reduction in UCB risk that strengthened with increasing tumor severity. Conversely, no significant associations with UCB risk were observed in any of the tumor subtypes examined in individuals who used medications other than phenazopyridine or no medication at all (Table 3.7). Similar associations were observed in males and females.

Table 3.8: UCB risk depending on urinary tract infection medication taken by strata of other UCB risk factors

Factor		Cases N(%)	Controls N(%)	OR[a]	95%CI	P	P-interaction
All Cases							
	no infection	885(92.6%)	990(88.6%)	1			
	phenazopyridine	19(2%)	57(5.1%)	0,4	(0,2-0,62)	0,000	
	other medication	37(3.9%)	54(4.8%)	0,8	(0,49-1,19)	0,232	
	no medication	15(1.6%)	16(1.4%)	1,3	(0,6-2,77)	0,509	
urinary pH							0,217
pH >= 6.0							
	no infection	308(90.9%)	325(87.4%)	1			
	phenazopyridine	13(3.8%)	18(4.8%)	0,6	(0,28-1,34)	0,224	
	other medication	12(3.5%)	23(6.2%)	0,5	(0,23-1,06)	0,069	
	no medication	6(1.8%)	6(1.6%)	1,5	(0,41-5,16)	0,564	
pH < 6.0							
	no infection	260(91.9%)	202(85.6%)	1			
	phenazopyridine	3(1.1%)	14(5.9%)	0,1	(0,04-0,51)	0,003	
	other medication	15(5.3%)	16(6.8%)	0,6	(0,27-1,3)	0,188	
	no medication	5(1.8%)	4(1.7%)	1,1	(0,26-4,9)	0,877	
NSAIDs							NA
never users							
	no infection	621(90.7%)	709(87.1%)	1			
	phenazopyridine	18(2.6%)	44(5.4%)	0,4	(0,23-0,74)	0,003	
	other medication	31(4.5%)	46(5.7%)	0,7	(0,44-1,15)	0,164	
	no medication	15(2.2%)	15(1.8%)	1,3	(0,6-2,82)	0,498	
ever users							
	no infection	29(90.6%)	49(76.6%)	1			
	phenazopyridine	1(3.1%)	8(12.5%)	0,4	(0,04-4,16)	0,450	
	other medication	2(6.2%)	6(9.4%)	1,2	(0,19-7,98)	0,839	
	no medication	0(0%)	1(1.6%)	0	(0-Inf)	0,995	
vegetable and fruit consumption							0,262
1st quartile							
	no infection	200(94.3%)	169(86.2%)	1			
	phenazopyridine	1(0.5%)	9(4.6%)	0,1	(0,01-0,78)	0,029	
	other medication	8(3.8%)	16(8.2%)	0,3	(0,13-0,84)	0,021	
	no medication	3(1.4%)	2(1%)	1,1	(0,15-7,71)	0,931	
2nd quartile							
	no infection	160(87.9%)	174(88.8%)	1			
	phenazopyridine	7(3.8%)	9(4.6%)	0,7	(0,25-2,21)	0,585	
	other medication	13(7.1%)	11(5.6%)	1,4	(0,58-3,43)	0,443	
	no medication	2(1.1%)	2(1%)	1,6	(0,19-13,95)	0,652	
3rd quartile							
	no infection	167(91.8%)	176(90.7%)	1			
	phenazopyridine	8(4.4%)	10(5.2%)	0,7	(0,25-1,93)	0,486	
	other medication	2(1.1%)	3(1.5%)	0,7	(0,1-4,28)	0,663	
	no medication	5(2.7%)	5(2.6%)	1,4	(0,35-5,69)	0,626	

*(**Table 3.8 cont'd**)*

Factor		Cases N(%)	Controls N(%)	OR[a]	95%CI	P	P-interaction
4th quartile							
	no infection	143(92.9%)	161(84.3%)	1			
	phenazopyridine	2(1.3%)	12(6.3%)	0,2	(0,05-1,04)	0,056	
	other medication	6(3.9%)	14(7.3%)	0,5	(0,17-1,34)	0,160	
	no medication	3(1.9%)	4(2.1%)	0,8	(0,13-4,15)	0,739	
water consumption							0,370
<400mL/day							
	no infection	58(89.2%)	79(87.8%)	1			
	phenazopyridine	4(6.2%)	4(4.4%)	1,4	(0,3-6,52)	0,679	
	other medication	2(3.1%)	6(6.7%)	0,4	(0,06-2,28)	0,292	
	no medication	1(1.5%)	1(1.1%)	1,6	(0,08-33,83)	0,761	
400-1399mL/day							
	no infection	170(88.5%)	273(87.5%)	1			
	phenazopyridine	5(2.6%)	9(2.9%)	0,7	(0,22-2,42)	0,601	
	other medication	14(7.3%)	23(7.4%)	0,9	(0,45-1,93)	0,839	
	no medication	3(1.6%)	7(2.2%)	0,7	(0,17-3,04)	0,662	
>=1400mL/day							
	no infection	83(93.3%)	197(88.3%)	1			
	phenazopyridine	1(1.1%)	15(6.7%)	0,1	(0,02-1,09)	0,060	
	other medication	3(3.4%)	9(4%)	0,5	(0,11-2,11)	0,339	
	no medication	2(2.2%)	2(0.9%)	3,3	(0,41-26,15)	0,263	
Nocturnal urination frequency							0,942
0							
	no infection	443(90.4%)	451(85.6%)	1			
	phenazopyridine	11(2.2%)	29(5.5%)	0,4	(0,17-0,76)	0,008	
	other medication	25(5.1%)	37(7%)	0,7	(0,38-1,15)	0,141	
	no medication	11(2.2%)	10(1.9%)	1,1	(0,43-2,73)	0,875	
1							
	no infection	146(90.7%)	183(85.9%)	1			
	phenazopyridine	6(3.7%)	15(7%)	0,4	(0,13-1,12)	0,081	
	other medication	7(4.3%)	12(5.6%)	0,6	(0,23-1,64)	0,326	
	no medication	2(1.2%)	3(1.4%)	1,5	(0,21-10,22)	0,699	
2+							
	no infection	88(92.6%)	170(89.9%)	1			
	phenazopyridine	1(1.1%)	11(5.8%)	0,2	(0,02-1,46)	0,109	
	other medication	4(4.2%)	5(2.6%)	1,7	(0,4-7,49)	0,468	
	no medication	2(2.1%)	3(1.6%)	2,2	(0,3-16,21)	0,436	

[a] Unconditional logistic regression adjusted for age, gender, region, smoking status and stratifying for each factor indicated in turn

3.5 DISCUSSION

In this study, a reduced UCB risk was observed in patients reporting a UTI one or more years before cancer diagnosis. This inverse association was restricted to individuals who used UTI medication, particularly those with a history of phenazopyridine use. Moreover, the reduction in UCB risk was not materially affected by a history of other urinary tract disorders in the studied population. However, given the low incidence of UTI in men compared to women, and the increased risk of UTI reported for men with BPH, the reduced UCB risk observed in men with prostatic symptoms secondary to prostate enlargement in this study was mediated by UTI.

Early studies reported an increased risk of UCB in patients with a history of UTI (Dunham et al., 1968; Kantor et al., 1984; Wynder et al., 1963), while more recent investigations excluding patients with recent infections reported no association (Gonzalez et al., 1991; Jhamb et al., 2007; Kjaer et al., 1989; Piper et al., 1986). These discrepancies may be due to a misclassification of early clinical symptoms of cancer with those of UTI. Similarly, we observed an increased albeit non-significant risk of UCB in patients reporting infections within a year of cancer diagnosis while an inverse association was observed when this group was excluded from the analysis. Jiang et al. reported an inverse association between UCB risk and UTI confined to women who had their last infection $\geq$5 years prior to cancer diagnosis (Jiang et al., 2009). These authors hypothesized that the UCB protection could be attributed to the anti-tumorigenic effects of antibiotics taken to treat UTI. Consistent with this hypothesis we observed an inverse association between UCB risk and UTI in individuals who reported using medication (i.e. antibiotics and/or analgesics) for UTI. Interestingly, a non-significant increased risk was seen among those individuals that reported not having received treatment for UTI.

Antibiotics used to treat UTI may have at least a two-fold effect that could account for the protective properties conferred against UCB in this study. Firstly, by clearing uropathogens in the bladder, antibiotics could indirectly decrease UCB risk though reducing pathogen induced promotion of initiated tumors as observed in animal models of urinary bladder infection and UCB (Davis et al., 1984; Higgy et al., 1987; Kawai et al., 1994; Yamamoto et al., 1992). On the other hand, antibiotics may confer

cytotoxic effects directly against initiated urothelial tumor cells. Combination treatments of sulfamethoxazole and trimethoprim for UTI have significantly inhibited cell proliferation and induced cytotoxicity of human UCB cell lines in a dose-dependent manner (Casini et al., 2002; Kamat and Lamm, 2004; Seay et al., 1996). As for phenazopyridine, this drug has been available in Spain for more than 50 years as a dual combination for UTI therapy together with the short duration sulfonamide sulfamethizole. A high localized concentration of active sulfamethizole in the bladders of patients who used the phenazopyridine combination therapy may efficiently induce cytotoxicity of initiated bladder tumors and contribute to the observed reduction in UCB risk in this group. The increased absorption of sulfamethizole through urothelial tumor tissue in patients having constantly low urinary pH would presumably further contribute to tumor cell cytotoxicity (Mishina et al., 1986).

It may also be phenazopyridine that is the active component of the combination therapy conferring tumor reduction through a mechanism that is distinct from, or complemented by, the sulfonamide. Experimental evidence indicates that the physiological reduction of phenazopyridine generates reactive oxygen species (ROS) like superoxide as well as hydrogen peroxide; the latter of which can accumulate to high levels in the peroxidase-free urinary environment (Munday and Fowke, 1994). Localized accumulation of ROS in the bladder could tip initiated bladder tumors beyond a sustainable oxidative threshold, while not adversely affecting benign urothelial cells subsisting at an inherently lower oxidative level (Trachootham et al., 2009).

An oxidative-stress induced tumor cytotoxic response may also offer a clue as to why a more pronounced reduction in tumor risk was observed by phenazopyridine users who were ever smokers, as compared to never smokers. Cigarette smoke increases free radical levels in urothelial cell lines (Ohnishi et al., 2002; Onol et al., 2007) while reducing the total radical trapping antioxidant potential (TRAP) of smokers (Chelchowska et al., 2011; Sharpe et al., 1996); presumably, conditions favorable for the further accumulation of free radicals and oxidative stress induced tumor cytotoxicity. The reduction in UCB risk was further strengthened in patients with constantly low urinary pH. Oxidative-stress induced tumor cytotoxicity may be most efficient at a low urinary pH in which nitrogen containing nucleophilic

functional groups (e.g. TAP) would not be influenced by the underlying chemical properties of glucuronide.

Whether the inverse association observed can be attributed to sulfamethizole, phenazopyridine or the concerted and complementary action of both drugs remains to be determined. Our observations suggest that the combination therapy may target pre-existing bladder tumors rather than preventing tumor formation. Sujects that benefitted of the greatest reduction in UCB risk were those who used the medication later in life. Consistent with this hypothesis, is the observation that treatment conferred the greatest protection against high-risk NMIBC and MIBC.

The current study is the largest to evaluate the relationship between UTI on UCB risk and the first to investigate the effect of UTI medication on this association. Information regarding the temporal relationship between the last infection and recruitment in the study was collected so as to minimize misclassification between UTI and UCB symptoms. Other urinary tract disorders, as well as use of diabetes medication were also investigated to rule out potential confounding. Moreover, the sample population accurately represents UCB in the general population as they were included from a good mix of referral centers and county hospitals. Despite these considerations, information collected about history of UTIs and medications was entirely self-reported. Recall bias is unlikely to influence the results substantially as UTI and bladder cancer were inversely associated. However, individuals with frequent UTIs may be more likely to receive cytoscopic examination for early signs of UCB resulting in selection bias that could contribute to the observed reduction in UCB risk. Lastly, although our study population was large, upon sub-stratification in patients who reported taking phenazopyridine several of the subgroups were small and the risk estimates should be interpreted with caution.

This is the first epidemiologic study to identify a protective effect against UCB resulting from medication taken to treat UTI. The greatest reduction in cancer risk was observed in patients who reported taking the urinary tract analgesic phenazopyridine through a mechanism that we propose may be dependent on the cytotoxic properties of its free radical generating metabolites. These results should be

examined in more detail in experimental studies and may have implications for the potential use of UTI medication as a prophylactic therapy against UCB.

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CHAPTER 4: CYCLOOXYGENASE-2 EXPRESSION IN BLADDER CANCER AND PATIENT PROGNOSIS: RESULTS FROM A LARGE CLINICAL COHORT AND META-ANALYSIS

Maciej J Czachorowski[1], André FS Amaral[1], Santiago Montes-Moreno[2], Josep Lloreta[3,4,5], Alfredo Carrato[6,7], Adonina Tardón[8], Manuel M Morente[9], Manolis Kogevinas[10], Francisco X Real[5,11], Núria Malats[1] for the SBC/EPICURO investigators[12]

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

2 Servicio de Anatomía Patológica, Hospital Universitario Marqués de Valdecilla, Santander, Spain.

3 Institut Municipal d'Investigació Mèdica – Hospital del Mar, Barcelona, Spain.

4 Departament de Patologia, Hospital del Mar – Parc de Salut Mar, Barcelona, Spain.

5 Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Barcelona, Spain.

6 Hospital General Universitario de Elche, Elche, Spain.

7 Hospital Ramon y Cajal, Madrid, Spain.

8 Universidad de Oviedo, Oviedo, Spain.

9 Tumor Bank Unit, Spanish National Cancer Research Centre (CNIO), Madrid, Spain.

10 Centre de Recerca en Epidemiologia Ambiental (CREAL), Barcelona, Spain.

11 Epithelial Carcinogenesis Group, Spanish National Cancer Research Centre (CNIO), Madrid, Spain.

12 Spanish Bladder Cancer (SBC)/EPICURO Study investigators.

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4.1 SUMMARY

Aberrant overexpression of cyclooxygenase-2 (COX2) is observed in urothelial carcinoma of the bladder (UCB). Studies evaluating COX2 as a prognostic marker in UCB report contradictory results. We determined the prognostic potential of COX2 expression in UCB and quantitatively summarize the results with those of the literature through a meta-analysis. Newly diagnosed UCB patients recruited between 1998-2001 in 18 Spanish hospitals were prospectively included in the study and followed-up (median, 70.7 months). Diagnostic slides were reviewed and uniformly classified by expert pathologists. Clinical data was retrieved from hospital charts. Tissue microarrays containing non-muscle invasive (n=557) and muscle invasive (n=216) tumours were analyzed by immunohistochemistry using quantitative image analysis. Expression was evaluated in Cox regression models to assess the risk of recurrence, progression and disease-specific mortality. Meta-hazard ratios were estimated using our results and those from 11 additional evaluable studies. COX2 expression was observed in 38% (211/557) of non-muscle invasive and 63% (137/216) of muscle invasive tumors. Expression was associated with advanced pathological stage and grade ($p<0.0001$). In the univariable analyses, COX2 expression - as a categorical variable - was not associated with any of the outcomes analyzed. As a continuous variable, a weak association with recurrence in non-muscle invasive tumors was observed (p-value=0.048). In the multivariable analyses, COX2 expression did not independently predict any of the considered outcomes. The meta-analysis confirmed these results. We did not find evidence that COX2 expression is an independent prognostic marker of recurrence, progression or survival in patients with UCB.

4.2 INTRODUCTION

Urothelial carcinoma of the bladder (UCB) is the most common bladder cancer type in developed nations (Jemal et al., 2011). UCB predominantly manifests (70-80% of patients) as a non-muscle invasive tumor (NMIBC: pTa-pT1) characterized by an overall good prognosis following transurethral resection in patients with low-grade tumors (pTaG1/2), and intravesical chemotherapy and/or Bacillus Calmette Guerin (BCG) instillation in patients with high-grade tumors (pTaG3 or pT1G2/3) (Wu, 2005). Approximately 70% of NMIBC patients suffer a recurrence following treatment and a further 15% progress, developing new tumors exhibiting muscle invasion (MIBC: pT2-pT4); the risk of progression being higher among patients with high-grade tumors (Wu, 2005). Due to a high rate of recurrence and the need for close follow-up over a patient's lifetime, UCB remains one of the most expensive tumors to treat on a per patient basis (Botteman et al., 2003). A lower proportion (20-30%) of UCB patients are diagnosed with muscle invasive tumors (MIBC; pT2-pT4) characterized by poor prognosis: 50% of these patients die from their cancer (Wu, 2005). Genomic profiling and gene expression analyses indicate a strong correlation between these pathologic classifications and the underlying molecular architecture of UCB (Lindgren et al., 2010).

Growing evidence indicates that chronic inflammation may increase the risk of UCB (Michaud, 2007). Studies investigating the prolonged use of cyclooxygenase-2 (COX2) inhibiting non-steroidal anti-inflammatory drugs (NSAIDs) have reported a decrease in UCB risk (Daugherty et al., 2011; Fortuny et al., 2006). COX2 is a prostaglandin endoperoxide synthetase that catalyzes the production of prostanoids upon induction by proinflammatory cytokines, growth factors, tumor promoters and other external stimuli (Harris, 2007). COX2 activation mediates cellular processes also implicated in carcinogenesis such as angiogenesis, cell survival/proliferation and apoptosis (Greenhough et al., 2009). Moreover, studies have shown that bladder tissue from patients with cystitis or UCB exhibits elevated COX2 levels in contrast to benign bladder tissue (Shirahama, 2000; Wheeler et al., 2002).

While numerous groups have investigated the prognostic potential of COX2 expression in UCB (Aziz et al., 2010; Bamias et al., 2008; Diamantopoulou et al., 2005; Eltze et al., 2005; Friedrich et al., 2003; Gudjonsson et al., 2011; Hilmy et al., 2006; Kim et al., 2002; Liedberg et al., 2008; Margulis et al., 2007; Mokos et al., 2006; Naruse et al., 2010; Shariat et al., 2003a; Shariat et al., 2003b; Shirahama et al., 2001; Tiguert et al., 2002; Wild et al., 2005; Wulfing et al., 2004; Yoshimura et al., 2001; Youssef et al., 2011), there is no clear consensus on its utility. The objective of this study was to assess whether COX2 protein expression in UCB cells is associated with prognosis using a large and standardized cohort of newly diagnosed bladder cancer patients. A meta-analysis was also done to summarize these results together with those from other studies published on the topic.

4.3 MATERIALS AND METHODS

4.3.1 Study population

A total of 773 newly diagnosed UCB cases aged 22-80 years (mean ± SD = 66 ± 10 yrs) with a median follow-up of 70.7 months (range 0.7 - 117.7 months) and available tumor tissue were used in the current analysis. All cases were recruited between 1998 and 2001 from 18 hospitals in five regions of Spain as part of the Spanish Bladder Cancer (SBC) / EPIdemiology of Cancer of the UROthelium (EPICURO) study, a hospital-based case-control study described previously (Garcia-Closas et al., 2005). A pathologist review panel uniformly classified the T stage and grade (G) of each tumor biopsy according to the criteria of the TNM classification and the WHO-ISUP (Epstein et al., 1998), using the three grade redefinition provided by the WHO (Eble et al., 2004; Mostofi et al., 1999). All bladder tumor samples used in the study were collected prior to the administration of any intravesical or systemic therapy. Clinical information related to diagnostic procedures, tumor characteristics and treatment was collected from medical records, and a computerized questionnaire was used for the collection of sociodemographic data. NMIBCs were removed by transurethral resection and patients received intravesical chemo- or immunotherapy (i.e. BCG) as appropriate. The majority of patients presenting with MIBCs were treated by radical cystectomy; in cases where surgery was not possible, radiotherapy or systemic chemotherapy were administered. Follow-up information was collected annually from hospital records and through direct telephone interviews by trained monitors using structured questionnaires. Among NMIBCs, recurrence was defined as the appearance of a new NMIBC following a previous negative follow-up cystoscopy, and progression, as the development of a MIBC. In patients initially presenting with MIBCs, any tumor reappearance after treatment was considered progression, regardless of whether the tumor relapse was local or distal. Tumour-specific survival was assessed only for patients with MIBCs. Informed written consent was obtained from study participants in accordance with the Ethics Committees of each participating hospital.

4.3.2 Immunohistochemistry

Tissue blocks of formalin-fixed, paraffin-embedded primary bladder tumors were used to construct tissue microarrays (TMA) containing tumor cores of 0.6-mm in diameter represented in duplicate and selected from the most representative regions of the tumor on which T and G were based. After deparaffinisation and heat-induced antigen retrieval, all slides were stained simultaneously at the Histology and Immunohistochemistry Core Unit of the CNIO using the PT LINK system as per manufacturer's instructions (Dako Inc., Glostrup, Denmark). Briefly, tissue sections were incubated with anti-COX2 rabbit monoclonal antibody (ThermoFisher Scientific, Fremont, CA, USA; #RM-9121-R7; pre-diluted, ready-to-use) at room temperature, followed by visualization using the EnVision Flex Visualization system (Dako Inc., Glostrup, Denmark) and exposure to diaminobenzedine. Tissues were then counterstained with haematoxylin, dehydrated and mounted. A section of colon tissue was used as a positive control.

4.3.3 Evaluation of COX2 immunostaining

COX2 expression was quantified using the Ariol SL-50 (version 3.1.2, Applied Imaging Corp., San Jose, CA, USA) high-throughput slide imaging scanner. All cores were imaged and processed using a light microscope and the accompanying TMA Multistain Imaging software. The program was trained by a pathologist (SM) to maximize the inclusion of positively stained tumor epithelium while minimizing stromal material, as described previously (Wahlin et al., 2010). COX2 expression score was calculated as the product of the mean intensity of staining (by defining the background and saturation limits of the antibody and imaging sensor, respectively) and the proportion of cellular antibody-positive area divided by total cellular area. Values from replicate cores were averaged to provide a final expression score for each patient. Furthermore, one randomly selected TMA (representing 10% of all cores) was analyzed by direct visual microscopic inspection by an independent pathologist (MMM) to enable comparison with the automated scoring approach. The pathologist-derived score was calculated as the product of COX2 staining intensity (1=weak, 2=intermediate, 3=strong) and a quartile of the percentage of epithelial tumor cells stained (0-4; with 0 representing 0% staining), providing

a final categorical score in the range of 0-12. There was a high and significant correlation between the machine and pathologist derived scores (Spearman rho=0.85; 95%CI=0.79-0.90; p-value<0.00001). COX2 expression was analyzed as both a continuous variable and categorical variables partitioned at the median and extreme tertiles. Additionally, expression was examined as a categorical variable dichotomized at a threshold (0.340 arbitrary units [au]) above which COX2 expression was considered to be *positive*. This expression threshold was derived by comparing the pathologist's (MMM) binary assignment of positive expression (i.e. score of 0 vs. score ≥1, as described above) to the machine-derived continuous score using receiver operating characteristic (ROC) curve analysis (area under the curve = 0.95; 86% sensitivity and 92% specificity) (Metz, 1978).

4.3.4 Meta-analysis

The meta-analysis included COX2 expression results from our own series (using the ROC-derived categorical expression variable) and relevant studies published before 1 January 2012 identified by searching PubMed and ISI Web of Knowledge. The search string used was: (cox2 OR cox-2 OR cyclooxygenase-2 OR "cyclooxygenase 2" OR ptgs2) AND (prognos* OR survival OR mortality OR recurrence OR relapse OR progression) AND ("bladder cancer"). Studies were considered eligible if: (i) they reported the effect measure (as HRs, survival curves or log-rank p-values) of COX2 protein expression on recurrence, progression or disease-specific survival; (ii) COX2 was assessed in primary tumors exhibiting homogeneity in tumor histology (≥75% UCB), and subphenotype (≥75% NMIBC *or* MIBC); (iii) they were written in English or Spanish (Table 4.4). Reviews, abstracts, non-clinical studies, and duplicate publications were excluded. HRs and 95%CIs were directly extracted from the publications whenever available. For those reporting only the log-rank p-value or the Kaplan–Meier survival curves, the HRs and 95%CIs were independently calculated by two of the co-authors (MJC, AFSA) using the spreadsheet prepared by Sydes and Tierney with any discrepancies resolved by discussion (Tierney et al., 2007). In a few indicated cases, authors were directly contacted for clarification or provision of data not shown in the published manuscripts (Table 4.4). The level of heterogeneity among studies was calculated

by means of the I^2 statistic (Higgins and Thompson, 2002), and publication bias was assessed by analyzing funnel plots and Egger's asymmetry test (Egger et al., 1997).

4.3.5 Statistical analysis

Associations between demographic and clinico-pathological parameters and COX2 expression were assessed using Fisher's exact test. In NMIBCs, expression was also assessed distinctly in low-grade/risk (pTaG1/G2) and high-grade/risk (pTa/pT2G3) tumors, based on our previous evidence suggesting differential prognostic, genetic and molecular profiles between these subgroups (Hernandez et al., 2006; Lopez-Knowles et al., 2006). Recurrence-free, progression-free, and overall disease-specific survival curves were generated using the Kaplan-Meier method, with statistical significance assessed using the log-rank test. Time to each endpoint was calculated from date of primary treatment to the date of event, date of last follow-up, or date of patient's death. Individuals who did not present any event until the end of the study, those lost to follow-up, or those who died from other causes were censored either at the time of last medical visit or at death. Time to recurrence and progression were defined by applying the "mid-time" between the date of the previous disease-free visit and that when a new event was diagnosed. Survival time was measured as the time from initial treatment to death resulting from cancer. Univariable and multivariable Cox-proportional hazards analysis was used to calculate hazard ratios (HR) and 95% confidence intervals (CI). Schoenfeld residual analysis did not suggest any departure from the proportional hazards assumption in multivariable models.

All statistical analyses were done using STATA (version 10.1 SE, StataCorp, College Station, TX, USA). Statistical tests were two-sided and p-values less than 0.05 were considered significant. The REMARK (McShane et al., 2005) guidelines for prognostic studies as well as the PRISMA (Moher et al., 2009) guidelines for systematic reviews and meta-analyses were adhered to in the preparation of the manuscript.

4.4 RESULTS

4.4.1 Patients and COX2 expression in bladder cancer TMAs

COX2 expression was assessed in 557 patients with NMIBCs and 216 individuals with MIBCs. Median COX2 expression was 0.121 au (range 0-42.590; interquartile range 1.382) in NMIBCs, and 0.760 au (0-30.806; 3.600) in MIBCs (p-value=$4x10^{-12}$). Representative COX2 immunostaining patterns in UCBs are shown in Figure 4.1. Of patients with NMIBCs, 41% (230/557) were treated only by transurethral resection, with the remainder (56%) receiving endovesical BCG immunotherapy and/or chemotherapy following transurethral resection, or other treatment (3%; Table 4.1). Nearly half (46%) of patients with MIBCs were treated by cystectomy, with the remainder receiving systemic chemotherapy, radiotherapy, superficial or other treatment, or some combination thereof (Table 4.2).

4.4.2 COX2 expression and clinicopathological features

Two-hundred eleven (38%) NMIBCs and 137 (58%) MIBCs expressed COX2 (Tables 4.1 and 4.2, respectively), with positive expression defined as a score equal to or greater than the ROC-derived threshold of 0.340 au. Patient and tumor characteristics in the analyzed sample did not differ significantly from the initial SBC/EPICURO study population with the exception of geographic region and tumor size in NMIBC patients (data not shown). The distribution of COX2 positivity was assessed according to established bladder cancer prognosticators including tumor invasion and grade, tumor multiplicity, tumor size and treatment, among others. Demographic factors like age, gender and region were not associated with COX2 expression, nor was the type of primary treatment received by patients (Tables 4.1 and 4.2). In NMIBCs, COX2 expression was significantly associated only with T and G; being more prominent in low-grade/risk pTaG1/2 tumors than in high-grade/risk pTa/pT1G3 tumors (p-value<0.0001; Table 4.1). Further assessment of COX2 distribution in relevant molecular subtypes of UCB (Lindgren et al., 2010), revealed a greater proportion of pTaG2 than pTaG1 tumors positively expressing COX2 in low-grade NMIBCs (p<0.0001, subtype 1; Figure 4.2). COX2 expression did not differ among high-grade/risk NMIBCs (p=0.075), but a

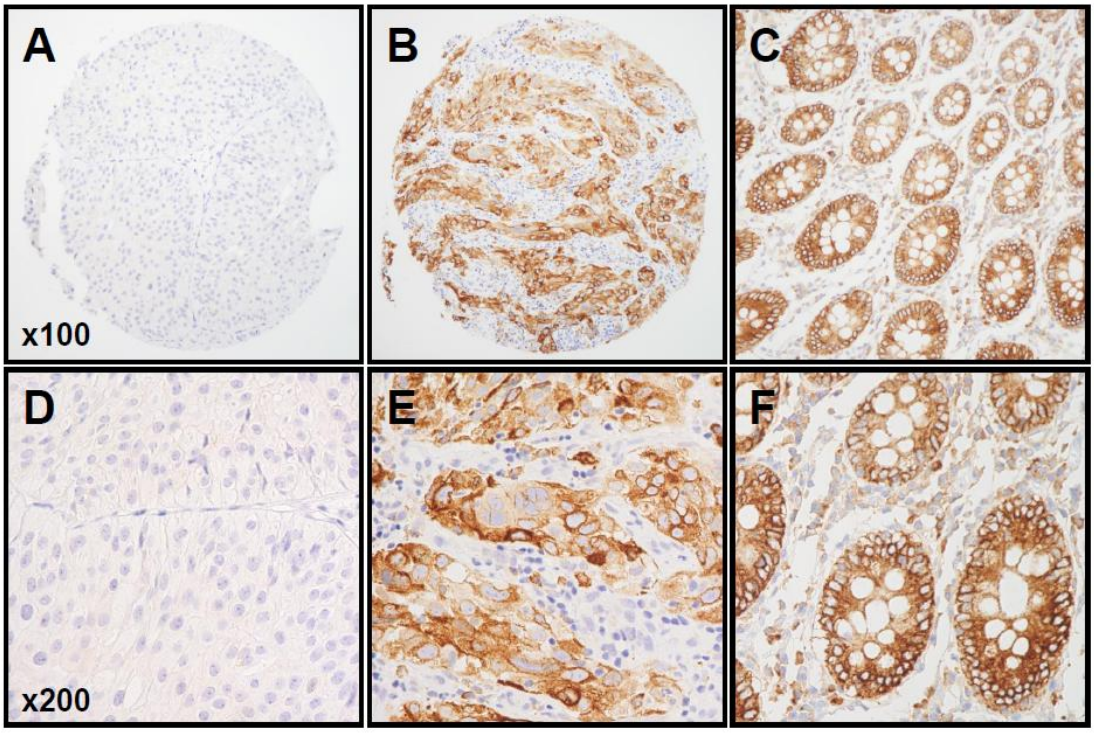


Figure 4.1. Immunohistochemical staining of COX2 in primary UCBs on TMAs. Expression was scored as a product of the percentage of epithelial area stained and the staining intensity using automated imaging analysis. A score of < 0.340 au was considered negative for COX2 expression, while a score of ≥ 0.340 was considered positive. Representative sections of a pTaG1 UCB lacking COX2 expression (**A and D**) and a pT2G3 UCB expressing COX2 (**B and E**) are shown. Normal colon tissue was used as a positive control (**C and F**). Upper panels show sections under 100x magnification (**A-C**); lower panels show sections under 200x magnification (**D-F**).

Table 4.1. Distribution of characteristics of patients with NMIBCs by COX2 expression.

		COX2 expression*		
Patient characteristics	**Total, N**	**negative, n**	**positive, n**	**P value†**
	557	346	211	
Area				0,506
Barcelona	98	68	30	
Valles	105	66	39	
Elche	51	32	19	
Tenerife	122	71	51	
Asturias	181	109	72	
Age (yrs.)				0,385
≤60	140	81	59	
>60 and ≤70	210	130	80	
>70	207	135	72	
Gender				0,891
Men	494	306	188	
Women	63	40	23	
Tumor Invasion				<0,0001
Ta	477	277	200	
T1	80	69	11	
Grade				<0,0001
GI	200	131	69	
GII	219	95	124	
GIII	138	120	18	
Low/High Grade				<0,0001
Low (TaG1/TaG2)	408	221	187	
High (TaG3/T1G2/T1G3)	149	125	24	
Number of tumors				0,106
1	348	209	139	
>1	178	120	58	
missing	31	17	14	
Tumour Size				0,564
≤3 cm	294	188	106	
>3 cm	111	67	44	
missing	152	91	61	
Number of Recurrences				0,409
none	366	232	134	
at least 1	191	114	77	
Treatment‡				0,393
TUR	230	133	97	
TUR+BCG	158	105	53	
TUR+Chem.	132	83	49	
TUR+BCG+Chem.	19	14	5	
Other	18	11	7	

* COX2 expression score dichotomised at the threshold of positivitiy (0,340 au)

† Fisher's exact test comparing distribution of COX-2 negative versus positive patients; missing values excluded from analysis where applicable

‡ TUR: transurethral resection; BCG: Bacillus Calmette-Guerin instillation; Chem.: chemotherapy via endovesical instillation

Table 4.2. Distribution of characteristics of patients with MIBCs by COX2 expression.

		COX2 expression*		
Patient Characteristics	**Total, N**	**negative, N**	**positive, N**	**P value†**
	216	79	137	
Area				0,207
Barcelona	39	16	23	
Valles	36	9	27	
Elche	15	9	6	
Tenerife	39	14	25	
Asturias	87	31	56	
Gender				0,816
Men	194	72	122	
Women	22	7	15	
Age (yrs.)				0,426
≤60	45	14	31	
>60 and ≤70	84	35	49	
>70	87	30	57	
Tumor invasion				0,896
T2	114	42	72	
T3	55	21	34	
T4	47	16	31	
Grade				0,326
GII	19	9	10	
GIII	197	70	127	
Metastases				0,296
M0	168	57	111	
M1	29	13	16	
Mx	19	9	10	
Lymphatic invasion				0,862
N0	141	50	91	
N1, N3	49	16	33	
Nx	26	13	13	
Number of tumors				0,008
1	146	63	83	
>1	54	12	42	
missing	16	4	12	
Tumour size				0,572
≤3 cm	53	19	34	
>3 cm	66	28	38	
missing	97	32	65	
Treatment‡				0,417
Cystectomy	67	19	48	
Cystectomy+Chem.	32	15	17	
Chem. only	23	9	14	
RT +/- Chem.	19	7	12	
Superficial Treatment	13	3	10	
Others	61	25	36	
missing	1	1	0	

* COX2 expression score dichotomised at the threshold of positivity (0,340 au)

† Fisher's exact test comparing distribution of patients with negative or positive COX-2 expression; missing, Nx and Mx values excluded from analysis where applicable

‡ Chem.: Systemic chemotherapy; RT: Radiation therapy

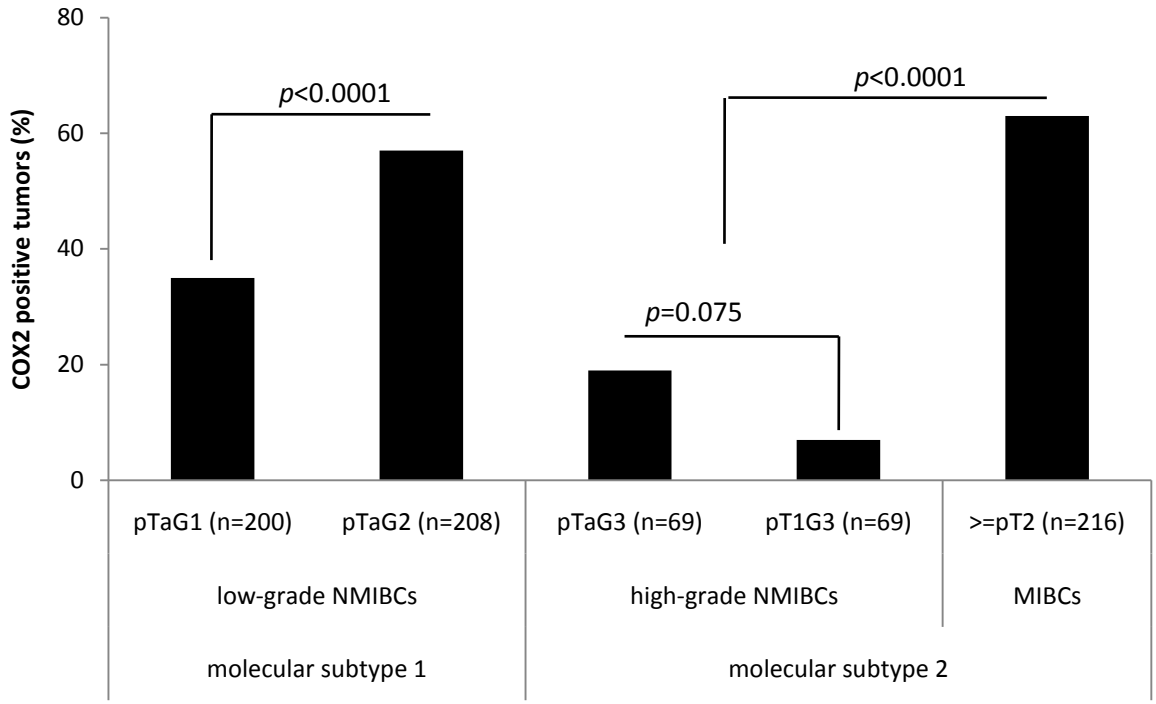


Figure 4.2. Distribution of positive COX2 expression in urothelial carcionomas of the bladder classified by their molecular and pathological stage-grade subtypes. Positive COX2 expression assessed as described in Figure 4.1. Statistical significance assessed using Fisher's exact test with a 0.05 significance level. pT1G2 tumors excluded due to low sample size in the current study (n=11), and a reported tendency to overlap both molecular subtypes.

greater proportion of MIBCs positively expressed COX2 than did all high-grade/risk NMIBCs combined ($p<0.0001$, subtype 2; Figure 4.2). Only tumor multiplicity was associated with positive COX2 expression in MIBC patients (p-value=0.008; Table 4.2).

4.4.3 COX2 expression and prognosis in bladder cancer patients

We analyzed the association of COX2 expression with tumor recurrence and progression in patients with NMIBCs and with progression and disease-specific survival in patients with MIBCs (Table 4.3; Figure 4.3). When considered as a continuous variable in the univariable analysis, COX2 expression was marginally associated with an increased risk of recurrence in NMIBCs (HR=1.02, 95%CI=1.00-1.04, p-value=0.048; Table 4.3). However, this association disappeared upon multivariable analysis when adjusting for region, gender, tumor stage and grade, multiplicity, tumor size, and treatment. Moreover, COX2 expression was not significantly associated with recurrence in NMIBCs when considered as a categorical variable, neither in the univariable nor multivariable analyses (Figure 4.3A; Figure 4.4AC; Table 4.3). Lastly, no significant association between COX2 expression and progression or survival was observed in patients with NMIBCs or MIBCs, regardless of whether expression was considered as a continuous or categorical variable in non-adjusted or adjusted analyses (Figure 4.3B-D; Figure 4.4B, 4.4D-H; Table 4.3).

4.4.4 Meta-analysis of COX2 expression and bladder cancer prognosis

Twenty publications on COX2 expression and bladder cancer prognosis were identified through the literature review (Table 4.4) (Aziz et al., 2010; Bamias et al., 2008; Diamantopoulou et al., 2005; Eltze et al., 2005; Friedrich et al., 2003; Gudjonsson et al., 2011; Hilmy et al., 2006; Kim et al., 2002; Liedberg et al., 2008; Margulis et al., 2007; Mokos et al., 2006; Naruse et al., 2010; Shariat et al., 2003a; Shariat et al., 2003b; Shirahama et al., 2001; Tiguert et al., 2002; Wild et al., 2005; Wulfing et al., 2004; Yoshimura et al., 2001; Youssef et al., 2011). Three of them lacked prognostic data, two overlapped with other larger studies and four included patient cohorts that did not meet the eligibility criteria outlined earlier, leaving 11

Table 4.3. Analysis of COX2 expression in NMIBCs and MIBCs; univariable and multivariable analyses

	Univariate COX-regression					Multivariate COX-regression[†]				
Score Categorization*	**Patients, n**	**Events, n**	**HR**	**(95% CI)**	**P value[‡]**	**Patients, n**	**Events, n**	**HR**	**(95% CI)**	**P value[‡]**
Non-muscle invasive tumors										
Recurrence [§]										
Continuous	556	191	1,02	1,00 - 1,04	0,048	401	141	1,02	1,00 - 1,04	0,140
Negative vs. Positive	556	191	1,08	0,81 - 1,44	0,612	401	141	1,11	0,78 - 1,59	0,555
Median	556	191	1,08	0,82 - 1,44	0,583	401	141	1,17	0,82 - 1,67	0,390
Extreme tertiles	370	127	1,06	0,89 - 1,27	0,483	268	94	1,08	0,86 - 1,37	0,510
Progression										
Continuous	557	48	0,92	0,84 - 1,01	0,094	526	43	0,96	0,87 - 1,05	0,350
Negative vs. Positive	557	48	0,72	0,39 - 1,33	0,302	526	43	1,38	0,61 - 3,11	0,434
Median	557	48	0,67	0,38 - 1,20	0,181	526	43	1,11	0,53 - 2,33	0,780
Extreme tertiles	371	33	0,71	0,49 - 1,01	0,059	351	29	0,92	0,54 - 1,56	0,750
Muscle invasive tumors										
Progression										
Continuous	216	131	0,99	0,96 - 1,03	0,617	189	110	0,99	0,96 - 1,03	0,750
Negative vs. Positive	216	131	0,94	0,66 - 1,34	0,734	189	110	0,85	0,56 - 1,29	0,448
Median	216	131	0,97	0,69 - 1,37	0,869	189	110	0,89	0,60 - 1,32	0,560
Extreme tertiles	144	85	0,92	0,75 - 1,14	0,464	128	75	0,90	0,70 - 1,15	0,410
Disease specific survival										
Continuous	216	110	1,00	0,97 - 1,04	0,908	187	89	1,01	0,97 - 1,05	0,730
Negative vs. Positive	216	110	0,91	0,61 - 1,34	0,627	187	89	0,77	0,48 - 1,23	0,267
Median	216	110	0,94	0,64 - 1,36	0,726	187	89	0,78	0,50 - 1,23	0,290
Extreme tertiles	144	68	0,90	0,71 - 1,15	0,407	126	57	0,78	0,58 - 1,04	0,090

[*] Expression cut-points used for categorical variables: "Neg. vs. Pos." - NMIBC/MIBC: 0.340; "Median" - NMIBC: 0.121, MIBC: 0.760; "Extreme tertiles" - NMIBC: (<0.0239, >0.586), MIBC: (<0.270, >2.149)

[†] Multivariate models adjusted for established bladder cancer prognostic factors as follows: NMIBC Recurrence adjusted by region, gender, tumour stage and grade, # tumours, size of tumours, and treatment; NMIBC Progression adjusted by region, # recurrences, age, tumour stage and grade, # tumours, and treatment; MIBC Progression adjusted by region, tumour stage, treatment, and presence of nodes; MIBC Survival adjusted by region, tumour stage, treatment, presence of nodes, and metastases

[‡] Cox proportional hazards analysis

[§] One patient excluded due to incomplete follow-up record

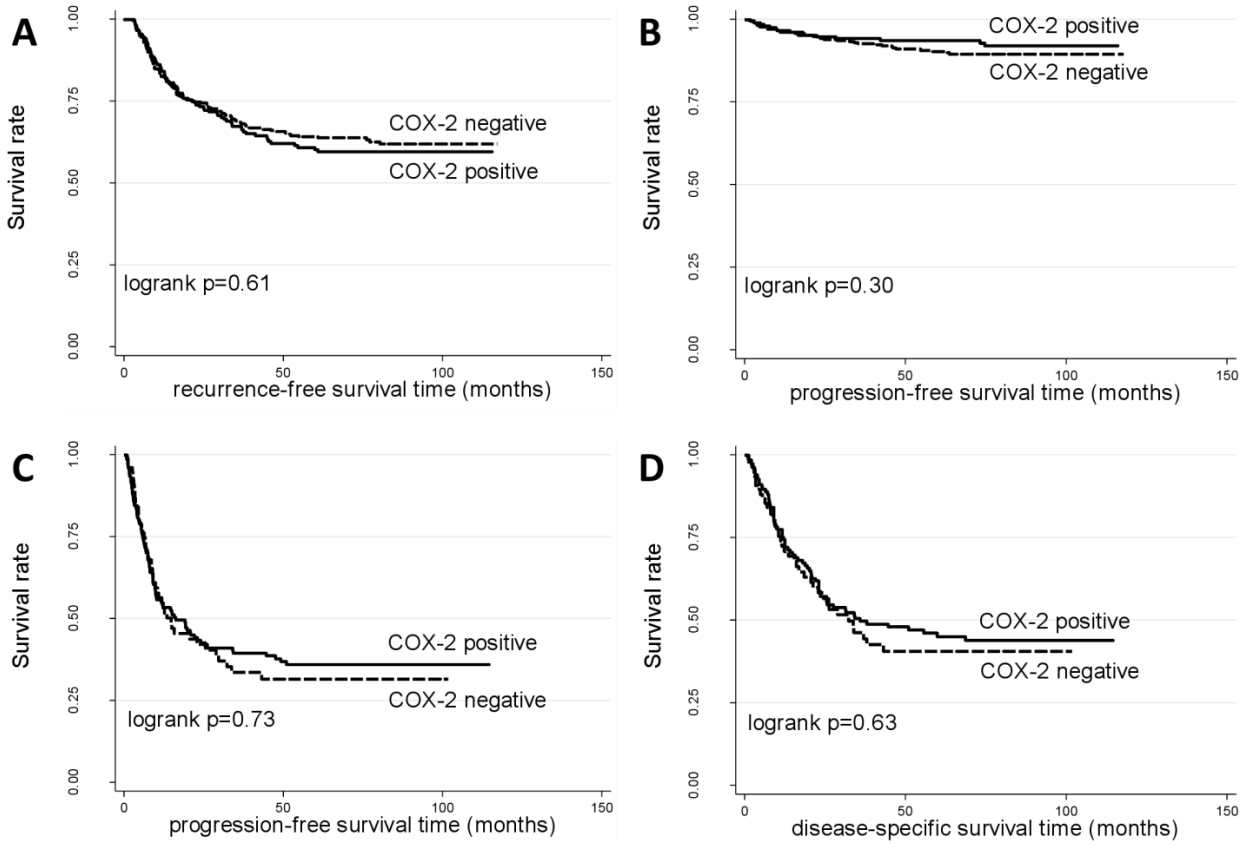


Figure 4.3. Kaplan-Meier survival curves corresponding to failures in superficial (**A, B**) and invasive (**C, D**) tumors for specified prognostic endpoints. Dashed curves: patients with tumors positive for COX2 protein staining; solid curves: patients with tumors negative for COX2 protein staining. Significance values from two-sided logrank test.

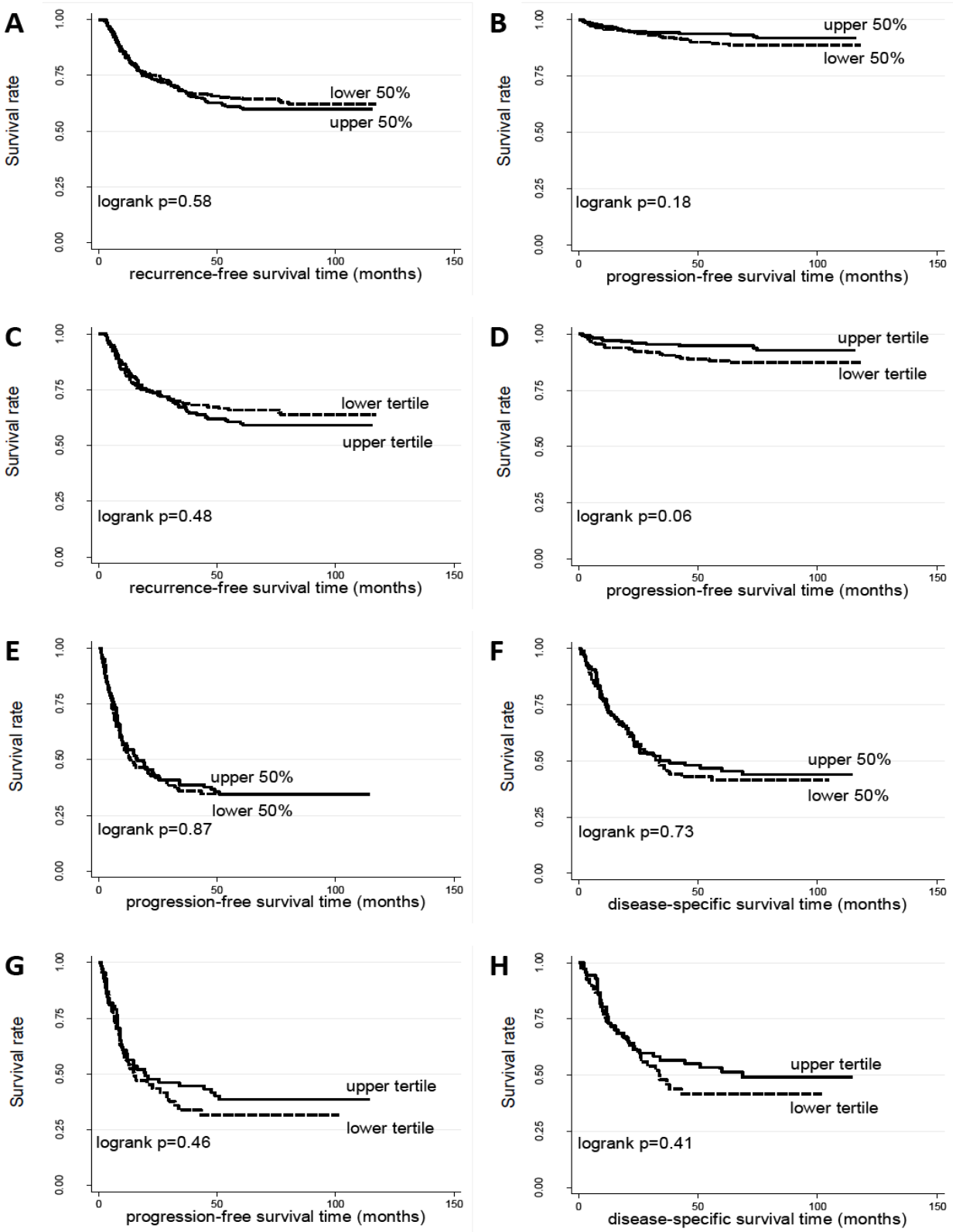


Figure 4.4. Kaplan-Meier survival curves corresponding to failures in superficial (**A, B, C, D**) and invasive (**E, F, G, H**) tumors for specified prognostic endpoints and quantiles of COX2 expression. Dashed curves: patients with tumors expressing COX2 at lower specified quantiles; solid curves: patients with tumors expressing COX2 at upper specified quantiles. Significance values from two-sided logrank test.

Table 4.4. Main characteristics of eligible studies used in meta-analysis

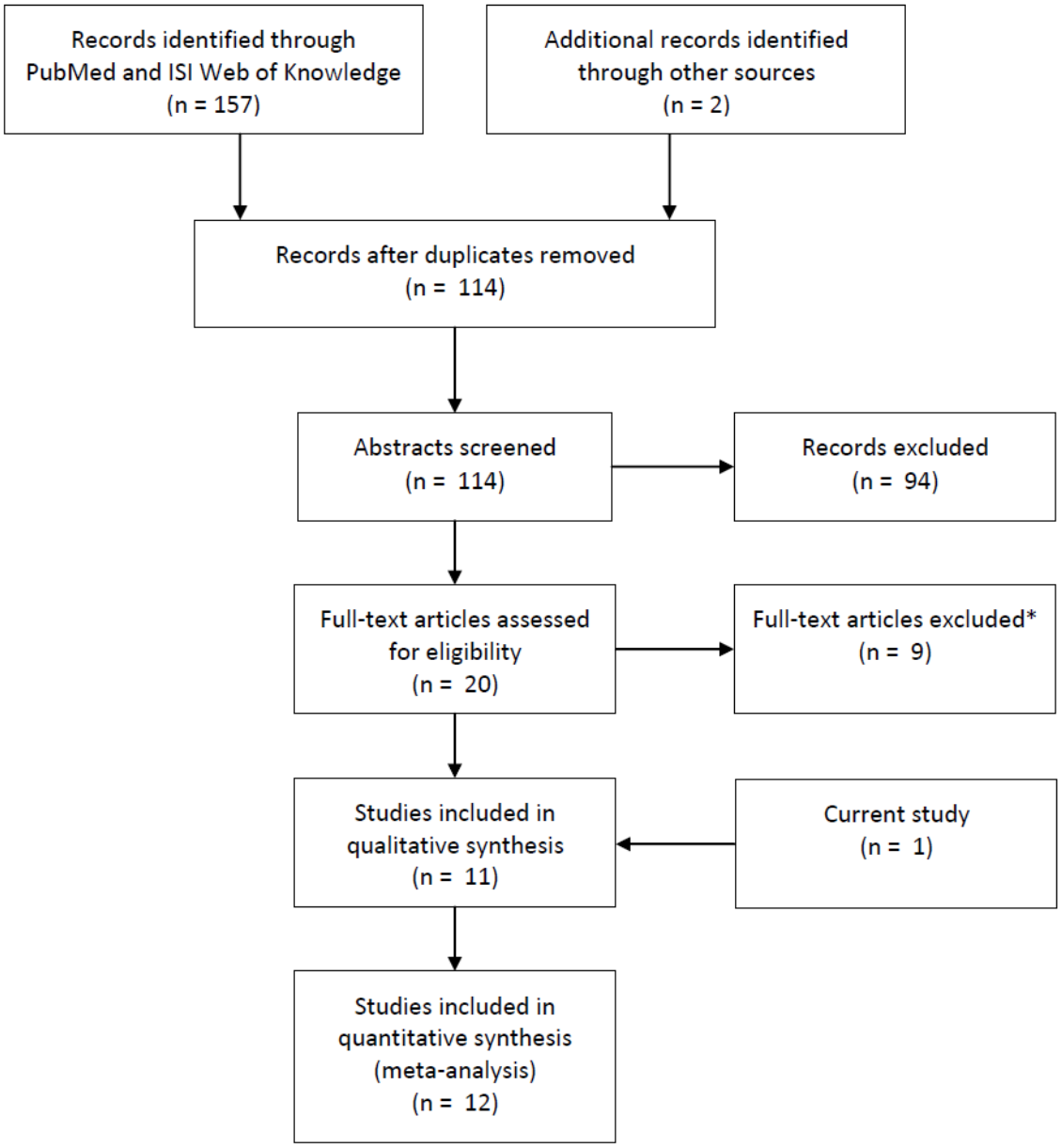
First Authour	Year	Country	Patients w/ follow-up	Median follow-up (months)	Histology	Stage	Tissue	Antibody for IHC, dilution	Definition of COX2 positivity	HR Estimation	Evaluable	Notes/reason excluded	Ref.
Aziz	2010	Canada	266	NS	TCC (>75%), SCC	T2-T4	whole section	Cayman, 200	> 5%	HRs/95% CIs	Yes		12
Bamias	2008	Greece	42	72	TCC	pT3, pT4	whole section	SantaCruz, 50	> 10%	HRs/95% CIs; from author	Yes	only bladder tumours analyzed	13
Diamantopoulou	2005	Greece	55	48	TCC	pTa, pT1; pT2-T4	whole section	SantaCruz, 130	>= 10%	HRs/95% CIs; from author	Yes		14
Eltze	2005	Germany	132	88	TCC	pT1; pT2-pT4	whole section	Cayman, 200	> 0, categorical	Surv. curves	No	overlap with Wulfing et al 2004	15
Friedrich	2003	Germany	91	26	TCC	pTa, pT1	NS	Oxford, 200	> 10%	No data	No	insufficient prognostic data	16
Gudjonsson	2011	Sweden	52	37,2	TCC	Ta	TMA	Dako, 5	1, categorical	HRs/95% CIs	No	38% primary tumours in cohort	17
Hilmy	2006	UK	103	60	TCC	Tis, pTa, pT1; pT2-pT4	whole section	Cayman, NS	tertiles examined	HRs/95% CIs	No	pooled cohort of N/MITs	18
Kim	2002	South Korea	37	27	TCC	T1G3	whole section	SantaCruz, 200	> 5%	Surv. curves	Yes		19
Liedberg	2008	Sweden	128	16	TCC	Tis, pT0, pT1; pT2-pT4	TMA	Dako, 5	> 0, categorical	No data	No	no prognostic data	20
Margulis	2007	USA	157	66	TCC	Ta, Tis; T1-T4 (>75%)	TMA	SantaCruz, NS	> 10%	Surv. curves; HRs/95% CIs	Yes		21
Mokos	2006	Croatia	70	24	TCC	Tis, Ta, T1	whole section	Oxford, 50	> 0%, categorical	Surv. curves	Yes	negative vs. positive staining	22
Naruse	2010	Japan	46	NS	TCC	pT2-pT4	whole section	SantaCruz, 20	> 10%	Surv. curves	Yes		23
Shariat	2003a	USA	37	120	TCC	Tis, pT1	whole section	NS, 100	> 10% rec.; > 20% prog.	Surv. curves	Yes		24
Shariat	2003b	USA	80	101	TCC	Tis, pTa, pT1; pT2-pT4 (>75%)	whole section	SantaCruz, 100	>10% of cells	Surv. curves	Yes		25
Shirahama	2001	Japan	108	75	TCC	pT1; pT2-T4	whole section	NS, 200	>= 5%	HRs/95% CIs	No	pooled cohort of N/MITs	26
Tiguert	2002	Canada	172	NS	TCC	MIT	NS	NS, 100	>= 5% of cells	Surv. curves	No	overlap with Aziz et al 2010	27
Wild	2005	Germany	617	39	TCC	pTa, pT1; pT2-4	TMA	Cayman, 5ug/ml	> 0, categorical	HRs/95% CIs; from author	Yes		28
Wulfing	2004	Germany	157	88	TCC (>75%), SCC	Tis, pT1; pT2-pT4 (>75%)	whole section	Cayman, 200	> 0, categorical	Surv. curves; HRs/95% CIs	Yes	negative vs. positive staining	29
Yoshimura	2001	Japan	118	NS	TCC, SCC	pTis, pT0, pT1; pT2-pT4	whole section	"in house", 100	> 0, categorical	No data	No	no prognostic data	30
Youssef	2010	Egypt	315	63,2	TCC (<75%), SCC, AD	pT1; pT2-pT4	TMA	ThermoFisher, 200	> 20%	Surv. curves; HRs/95% CIs	No	TCC (<75%)	31

Abbreviations: AD, adenocarcinoma; HRs/95% CIs, hazard ratios/95% confidence intervals; MIT, muscle invasive tumour; NMIT, non-muscle invasive tumour; NS, not specified; Ref., publication reference; Surv. Curves, survival curves; SCC, squamous cell carcinoma; TCC, transitional cell carcinoma; TMA, tissue microarray.

evaluable publications (Aziz et al., 2010; Bamias et al., 2008; Diamantopoulou et al., 2005; Kim et al., 2002; Margulis et al., 2007; Mokos et al., 2006; Naruse et al., 2010; Shariat et al., 2003a; Shariat et al., 2003b; Wild et al., 2005; Wulfing et al., 2004) plus the current study for the meta-analysis (Figure 4.5). Studies were classified by the tumor subtype(s) they reported on (i.e. NMIBC or MIBC), and whether adjustment for covariates was considered for each prognostic endpoint examined (i.e. univariable or multivariable; Figures 4.6 and 4.7). Of the four meta-analyses conducted with univariable data, only the metaHR of the association between COX2 expression and recurrence in NMIBCs showed marginal significance (metaHR=1.35, 95%CI=1.00-1.83; Figure 4.6). This result was not affected by study heterogeneity (I^2 p-value=0.13) but exhibited significant publication bias, as evidenced by Egger's test (p-value=0.019). The remaining meta-analyses considering univariable data suggested increased, albeit non-significant, risks of tumor progression in patients with NMIBCs (metaHR=2.07, 95%CI=0.76-5.64) and MIBCs (metaHR=1.45, 95%CI=0.77-2.74), and death in patients with MIBCs (metaHR=1.13, 95%CI=0.8-1.59; Figure 4.6). Notably, the summary effect for progression in NMIBCs and that observed for survival in MIBCs were both significantly affected by study heterogeneity (I^2 p-values: 0.006 and 0.004, respectively), with the former also significantly influenced by publication bias (Egger's test p-value=0.001).

Due to a paucity of published prognostic studies performing multivariable analysis on patients with NMIBCs, we could only address the multivariable meta-association with progression and survival in patients with MIBCs (Figure 4.7). A small, non-significant increased summary risk of progression (metaHR=1.12, 95%CI=0.53-2.35; Figure 4.7) was observed in COX2 expressing MIBCs that was unaffected by study heterogeneity (I^2 p-value=0.139). Similarly, a null summary effect was observed for survival (metaHR=0.97, 95%CI=0.69-1.36; Figure 4.7). This effect was influenced neither by study heterogeneity (I^2 p-value=0.114) nor by publication bias (Egger's test p-value=0.108).

Figure 4.5. Flow diagram of study selection and inclusion in meta-analysis



*reasons for exclusion of full-text articles included: no reported prognostic data (n = 3), patient overlap with a larger study (n = 2), pooled analysis on superficial and invasive bladder tumors (n = 2), inclusion of tumors other than UCB in analysis (n = 1), inclusion of non-primary tumors in analysis (n = 1)

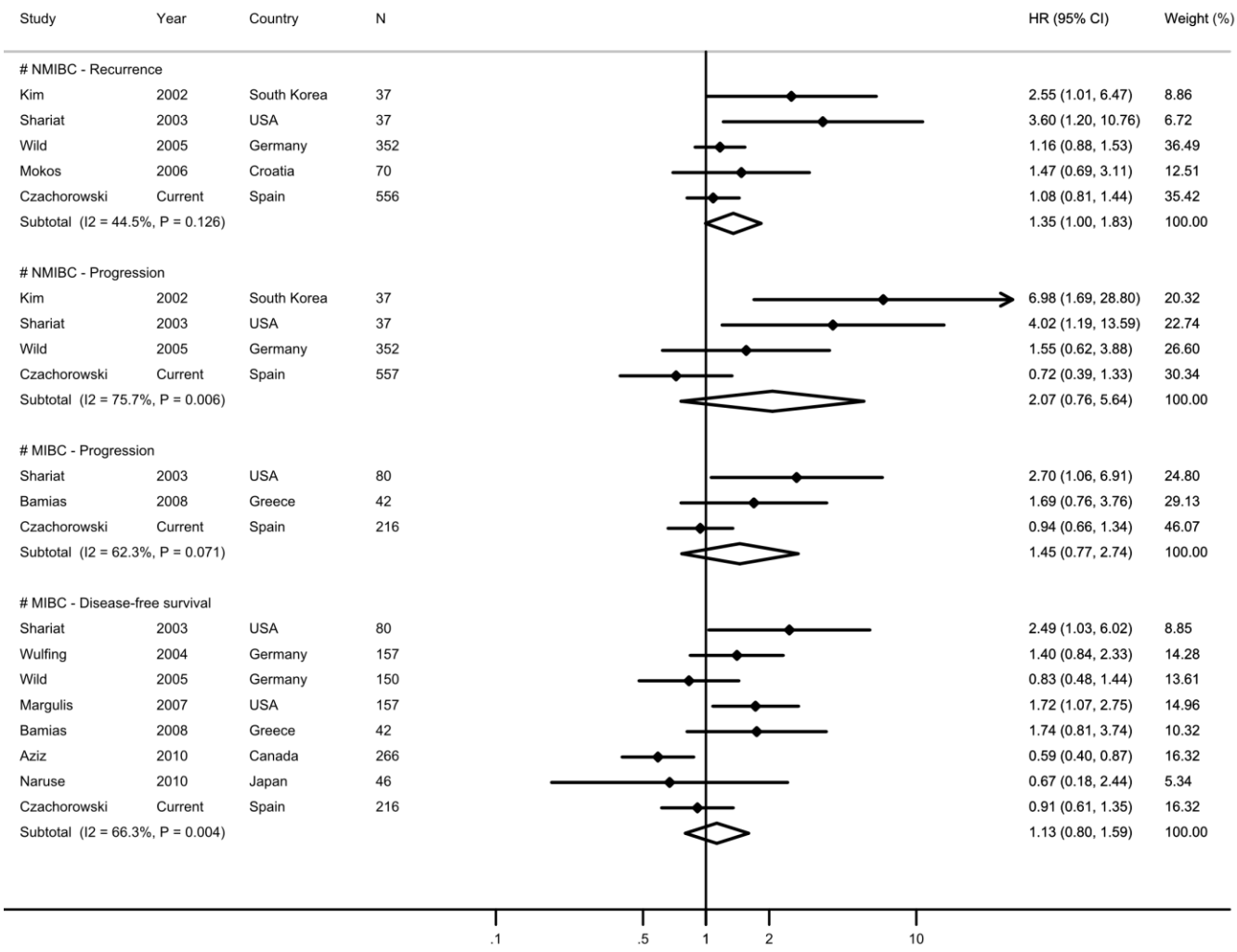


Figure 4.6. Forest plots from selected univariable studies indicating the risk of reaching the indicated prognostic endpoints in non-muscle invasive (NMIBC; two upper panels), and muscle invasive (MIBC; two lower panels) UCBs in the presence of urothelial COX2 expression.

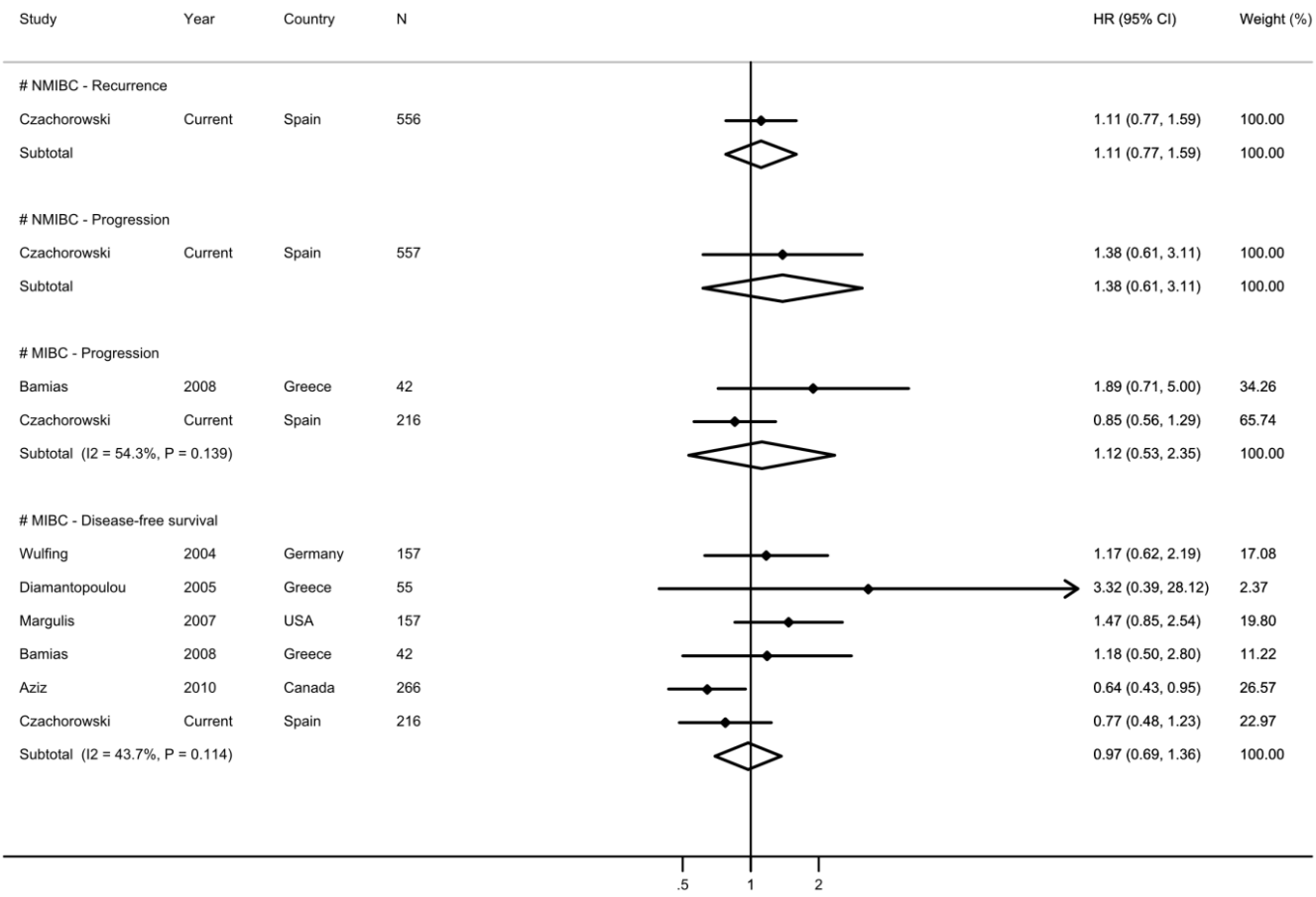


Figure 4.7. Forest plots from selected multivariable studies indicating the risk of reaching the indicated prognostic endpoints in non-muscle invasive (NMIBC; two upper panels), and muscle invasive (MIBC; two lower panels) UCBs in the presence of urothelial COX2 expression.

4.5 DISCUSSION

Despite many published studies, contradictory findings prevail on COX2 expression as an independent prognostic marker in patients with UCB. The current study suggests that COX2 expression is not an independent marker associated with recurrence, progression or survival in patients with UCB.

Using the largest cohort of patients with NMIBCs evaluated for COX2 expression to date, we observed that 38% of these tumors expressed the protein. Other groups have reported frequencies ranging from 53-88%; however, these studies used different COX2 antibodies and expression evaluation techniques and had smaller sample sizes (Friedrich et al., 2003; Mohammed et al., 1999; Ristimaki et al., 2001; Wild et al., 2005; Wulfing et al., 2004). In accordance with reported results (Hilmy et al., 2006; Ristimaki et al., 2001; Shirahama, 2000) we observed significantly higher COX2 expression in MIBCs (58%) than in NMIBCs. This frequency is similar to that observed in other large, histologically homogeneous studies (Margulis et al., 2007; Wild et al., 2005), while groups using heterogeneous cohorts of squamous and transitional cell carcinomas report frequencies different from our own (Aziz et al., 2010; Wulfing et al., 2004). Collectively, these findings reiterate the importance of homogeneity, or stratification, in tumor marker studies.

The association between COX2 and clinico-pathological characteristics remains a contentious issue in the literature. The majority of studies report an association between COX2 overexpression and advanced tumor invasion and grade, but use heterogeneous populations of NMIBCs *and* MIBCs in their assessments (Komhoff et al., 2000; Margulis et al., 2007; Shariat et al., 2003b; Shirahama et al., 2001; Wild et al., 2005). Given the known disparity in COX2 expression between NMIBCs and MIBCs, an association of this type would be expected in a mixed tumor population. After pooling NMIBCs and MIBCs in our study we also observe a strong significant association between COX2 overexpression and advanced tumor invasion ($p>0.0001$) and grade ($p>0.0001$). Notably, several groups report no association between COX2 expression and T and G (Diamantopoulou et al., 2005; Ristimaki et al., 2001; Wulfing et al.,

2004); especially those working strictly with homogeneous cohorts of MIBCs (Aziz et al., 2010; Naruse et al., 2010). Similarly, in our study, COX2 expression did not differ significantly among pT2, pT3 and pT4 tumors (p=0.896). Interestingly, we observed lower COX2 positivity in pT1 and high-grade/risk NMIBCs, than in pTa and low-grade/risk NMIBCs tumors. This result may seem counterintuitive if grade progression is considered a linear trait and COX2 expression is deemed to increase linearly with T and G. However, there is strong evidence indicating that UCB exists as two molecularly distinct subtypes, with high-grade/risk NMIBCs having a molecular signature more similar to MIBCs than to low-grade/risk NMIBCs (Lindgren et al., 2010; Lopez-Knowles et al., 2006). In this respect, we observed that COX2 positivity increased significantly with increasing T and G within each molecular tumor subtype (Figure 4.2). Shirahama et al. (Shirahama et al., 2001) reported a COX2 distribution similar to ours, observing 8% positivity in pT1 tumors and 50% in MIBCs when using whole section staining and a 5% expression threshold. Collectively, these results reiterate the disparity in COX2 expression between NMIBCs and MIBCs first reported by Komhoff et al. (Komhoff et al., 2000), and highlight the importance of considering expression within the proper molecular context.

To minimize the effects resulting from selecting an arbitrary expression threshold, we investigated COX2 protein expression as a continuous variable and three categorical variables. Only when considered as a continuous variable in the univariable analysis was COX2 expression found to be associated with a slight increase in the risk of recurrence. The meta-analysis, consisting of five other univariable studies, reiterated this association and showed a 35% increased risk of recurrence in patients with COX2 expressing NMIBCs. However, both effect estimates exhibit only marginal significance, suggesting that the observed associations may be due to chance. Moreover, the association observed in the univariable analysis did not hold after adjustment for conventional prognostic factors of recurrence in the multivariable analysis. Lastly, the summary effect observed in the meta-analysis may have been skewed by two small studies which selected only high risk NMIBCs (T1G3 (Kim et al., 2002) and Cis (Shariat et al., 2003a)). When a sensitivity analysis was performed removing these two studies from the meta-

analysis, the association between recurrence and COX2 expression was no longer maintained (metaHR=1.14, 95%CI=0.94-1.38). The observed disparity between effect estimates of progression in the present study and the meta-analysis could also be attributed to the inclusion of these two studies. Upon their exclusion, the summary HR showed no association with progression (metaHR=0.98, 95%CI=0.47-2.03). These results do not support a role for COX2 expression in NMIBCs as an independent prognostic marker of recurrence or progression.

Several groups have investigated the ability of COX2 expression to predict outcome in patients with MIBCs. Despite wide inter-study variation in methodology, antibodies used, sample size, and adjustment parameters in the case of multivariable analyses, the majority of these studies did not identify any significant association between COX2 expression and progression or survival, consistent with our findings (Bamias et al., 2008; Diamantopoulou et al., 2005; Naruse et al., 2010; Wild et al., 2005; Wulfing et al., 2004). Shariat and Margulis and their colleagues observed a negative association between high COX2 expression and tumor progression and mortality (Margulis et al., 2007; Shariat et al., 2003b). However, both studies relied on heterogeneous sample populations which included a small proportion of patients with NMIBCs; potentially accounting for the observed associations given the disparity in COX2 expression between superficial and advanced bladder tumors (Ristimaki et al., 2001). In another study, Wulfing et al. reported that high COX2 expression was an independent predictor of poor overall survival in a subgroup of 62 patients with MIBC treated with cisplatin-based chemotherapy (Wulfing et al., 2004). We did not identify any meaningful interaction between COX2 expression and treatment (data not shown), and were unable to replicate their findings in a smaller subset of 39 patients treated with cisplatin (HR=1.47, 95%CI=0.48-4.51, p-value=0.497). Aziz et al. reported a 36% survival advantage associated with increased COX2 levels in a cohort of 266 patients with MIBCs (221 with UCB) that was independent of lymph node status and neo/adjuvant chemotherapy (Aziz et al., 2010). While we also observed improved survival among patients with COX2 overexpressing MIBCs, this association did not

reach significance, consistent with other univariable (Naruse et al., 2010; Wild et al., 2005) and multivariable (Shirahama et al., 2001) analyses.

Our study had a large sample size, included only incident cases and relied on extensive and accurately acquired follow-up information spanning ten years. Additionally, we used automated scoring of immunostained TMAs, a strategy providing a reproducible assessment of expression that correlated highly with the independent evaluation of a subset of samples by an independent pathologist. COX2 staining was done in one laboratory to avoid heterogeneity in immunohistochemical staining and scoring, and evaluated as a continuous variable in the prognostic analyses to avoid potential bias related to selection of an expression threshold. Moreover, the sample population provides an accurate representation of bladder cancer in the general population as no inclusion criteria were applied in the recruitment process which included a good mix of referral centers and county hospitals. Lastly, the recommendations of the REMARK and PRISMA studies were followed in all of the reported analyses.

Despite these considerations and attempts to accurately quantify COX2 expression only in epithelial cells, the pathologist-trained automated imaging system may have incorporated some immunostained stromal material found on the tissue core, thereby increasing type I error. To reduce potential error we averaged the expression scores from duplicate cores and also explored a method investigated by Henriksen et al. (Henriksen et al., 2007) in which the higher score was used (data not shown). Both methods produced similar material associations between COX2 expression and clinico-pathological parameters or HRs. Moreover, adjusted analyses for progression in NMIBCs should be interpreted cautiously given the low number of events in relation to covariates. Also, different patient management practices across recruitment hospitals could increase sample heterogeneity, necessitating the inclusion of both recruitment area and treatment regimen in our multivariable analyses.

The results presented herein focus on COX2 expression levels measured in tumor epithelial cells – only one aspect of the complex interplay between the tumor and the host

immune/inflammatory response (Mantovani et al., 2008). The prognostic potential of COX2 (if any) may only be revealed when considered together with other tumoral markers. When investigating several potential prognostic parameters in UCB, Hilmy et al. concluded that systemic factors of the inflammatory response such as levels of C-reactive protein were superior to tumor-based factors such as grade, COX2 expression or T-lymphocytic infiltration (Hilmy et al., 2006). Moreover, in models of cervical cancer, Ferrandina et al. observed that while COX2 expression was mutually exclusive in the tumor and stromal inflammatory cells, high expression in both cell types could be used as an independent marker of poor survival (Ferrandina et al., 2002). Future studies investigating the prognostic value of COX2 expression in UCB should take into consideration the multi-factorial and multi-dimensional context of the inflammatory response during carcinogenesis.

The current study is the largest to investigate COX2 expression as an independent marker of outcome in a prospective cohort of UCB patients. These findings, supported by a meta-analysis that included our own data and that from other relevant studies, do not support COX2 tumor cell expression being an independent prognosticator of UCB.

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CHAPTER 5: GERMLINE GENETIC VARIATION IN *TLR9* AND DIFFERENTIAL COX2 EXPRESSION IN BLADDER CANCER: IMPLICATIONS FOR CANCER SUSCEPTIBILITY AND BCG THERAPY

Maciej J Czachorowski[1], Evangelina Lopez de Maturana[1], Deborah Silverman[2], Josep Lloreta[3,4,5], Alfredo Carrato[6,7], Adonina Tardón[8], Manolis Kogevinas[9], Francisco X Real[5,10], Núria Malats[1]* for the SBC/EPICURO investigators[11]

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

[2] Dept. of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, United States.

[3] Institut Municipal d'Investigació Mèdica – Hospital del Mar, Barcelona, Spain.

[4] Departament de Patologia, Hospital del Mar – Parc de Salut Mar, Barcelona, Spain.

[5] Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Barcelona, Spain.

[6] Hospital General Universitario de Elche, Elche, Spain.

[7] Hospital Ramon y Cajal, Madrid, Spain.

[8] Universidad de Oviedo, Oviedo, Spain.

[9] Centre de Recerca en Epidemiologia Ambiental (CREAL), Barcelona, Spain.

[10] Epithelial Carcinogenesis Group, Spanish National Cancer Research Centre (CNIO), Madrid, Spain.

[11] Spanish Bladder Cancer (SBC)/EPICURO Study investigators.

(*submitted*)

5.1 SUMMARY

Cyclooxygenase-2 (COX2) mediates the acute inflammatory response and exhibits elevated expression in urothelial carcinoma of the bladder (UCB) in contrast to benign bladder tissue. We aimed to determine whether genetic variants (SNPs) in inflammatory pathway genes are differentially associated with UCB risk according to COX2 protein expression levels in tumor tissue. SNPs identified as conferring heterogeneity in genetic susceptibility to UCB subtypes defined by COX2 expression were also evaluated for their prognostic and/or predictive utility in these tumors, with emphasis on patient response to BCG immunotherapy. COX2 expression was determined in 604 bladder cancer cases from the Spanish Bladder Cancer/EPICURO study using immunohistochemical analysis of tissue microarrays (TMA). In total, 331 COX2-negative and 273 COX2-positive cases and 988 matched controls were included in the analysis. Nine-hundred eleven SNPs in 214 inflammatory genes were genotyped in the same population. Polytomous logistic and Cox regression models were applied to estimate the odds ratios (OR) and hazard ratios (HR) for cancer risk and progression, respectively. Two common SNPs in *TLR9* (rs187084; rs352139) showed evidence of risk heterogeneity for UCB subtypes defined by COX2 positivity. The variant allele of rs187084 reduced the risk of COX2-negative UCB (OR=0.63; $P_{heterogeneity}$= 1.47E-06), while the variant of rs352139 increased risk of COX2-negative tumors (OR=1.44) but decreased risk of COX2-positive tumors (OR=0.81) ($P_{het.}$= 1.67E-06). The observed heterogeneity in risk remained robust after correction for multiple testing. Interestingly, patients harboring the variant allele in *TLR9*-rs187084 and manifesting COX2-negative tumors were less likely to exhibit cancer progression (HR=0.39, 95%CI 0.15-0.94, p=0.036) and were more responsive to BCG immunotherapy (HR=0.16, 95%CI 0.03-0.81, p=0.027) than wild-type patients or patients with COX2-positive tumors. We report for the first time that UCB subphenotypes defined by COX2 expression present differential risk according to genetic variation in the innate immune response gene *TLR9*. Moreover, the variant allele of *TLR9*-rs187084 exhibits potential as a predictive marker of good prognosis in patients manifesting COX-negative tumors treated with BCG treatment response in patients manifesting COX2-negative tumors.

5.2 INTRODUCTION

The majority of patients with urothelial carcinoma of the bladder (UCB) (70-80%) present with non-muscle invasive disease (NMIBC) confined to the bladder mucosa (pTa, pT1), with a smaller proportion (20-30%) observing tumors that invade into or beyond the surrounding muscle layer (MIBC; pT2-pT4). Tumors are further subcategorized according to their degree of differentiation: G1, G2, G3 or low/high grade (Eble et al., 2004; Mostofi et al., 1999). Following resection, about 70% of patients with NMIBC observe tumor recurrence, while a further 15% with high-grade NMIBC will experience tumor progression into the muscle layer (Wu, 2005).Tumor progression is particularly frequent (50%) among patients with high-grade NMIBC (TaG3 and T1G2/3) These high-risk patients are commonly treated with *Mycobacterium bovis* Bacillus Calmette Guerin (BCG) instillation therapy and 50-60% respond positively. Although the exact therapeutic mechanism of BCG instillation remains unclear, a functional host innate immune system is necessary to induce a localized Th1-polarized inflammatory response leading to tumor ablation (Luo et al., 2011).

Well established UCB risk factors include a dozen genetic susceptibility loci (Rothman et al., 2010) and environmental factors like smoking (Samanic et al., 2006), which can interact to further modulate disease risk as observed in smokers harbouring the NAT2 slow acetylator phenotype (Garcia-Closas et al., 2005; Garcia-Closas et al., 2013). Conditions that induce chronic inflammation of the bladder mucosa, such as *Schistosoma haematobium* infestation or extended catheter use in spinal cord injury patients also contribute to increased bladder cancer risk, albeit of the squamous cell type (SCC) (Michaud, 2007). Furthermore, recurrent cystitis has been associated with UCB, though results from the published studies are inconclusive (Chang and Parsonnet, 2010). Conversely, prolonged use of cyclooxygenase targeting non-steroidal anti-inflammatory drugs (NSAIDs) has been associated with a reduction in UCB risk (Daugherty et al., 2011). This evidence, together with findings that common sequence variants in inflammation regulating genes (e.g. *PTGS2/COX2*) modulate UCB risk (Yang et al., 2008), further implicate inflammation in the etiology of this complex disease.

Cyclooxygenase-2 (COX2) is a prostaglandin endoperoxide synthase induced by proinflammatory cytokines, growth factors and tumor promoters, among other factors (Harris, 2007). Together with the prostanoid products of arachidonic acid metabolism, COX2 signaling mediates the inflammatory response under physiological conditions, but its aberrant overexpression has been implicated in tumor promotion through the induction of angiogenesis, stimulation of cellular proliferation and inhibition of apoptosis (Greenhough et al., 2009). Animal models overexpressing COX2 in basal epithelial cells manifest increased transitional cell hyperplasia and carcinoma (Klein et al., 2005) while in humans, COX2 levels are elevated in bladder tissue from patients with cystitis and in UCB (Shirahama, 2000; Wheeler et al., 2002).

Given the association between COX2, inflammation and bladder cancer, we wanted to determine whether genetic variation in genes involved in inflammatory processes could differentially predispose individuals to discrete subgroups of UCB characterized by the presence of COX2 expression. This information was subsequently evaluated for its prognostic utility in UCB patients from 18 hospitals in Spain, with emphasis placed on response to BCG immunotherapy.

5.3. MATERIALS AND METHODS

5.3.1 Population

All patients were of white ethnicity and recruited between 1998 and 2001 from 18 hospitals in five regions of Spain as part of the Spanish Bladder Cancer (SBC) / EPIdemiology of Cancer of the UROthelium (EPICURO) study, a hospital-based case-control study described previously (Garcia-Closas et al., 2005). A set of 608 newly diagnosed UCB cases with available tumor tissue and genotype information were included in the current analysis. All tumor samples were reviewed by a panel of expert pathologists to confirm diagnosis and were collected prior to the administration of any intravesical or systemic therapy. The T stage and grade (G) of each tumor was assessed according to the criteria of the TNM classification and the ISUP-WHO (Epstein et al., 1998), using the three grade redefinition provided by the WHO (Eble et al., 2004; Mostofi et al., 1999). Subsequently, cases were immunohistochemically categorized based on COX2 positivity (negative, *n*=334; positive, *n*=274). Nine-hundred eighty-eight inpatients with complete genotype information and diagnoses unrelated to the exposures of interest were included as controls and matched to cases on age, gender and hospital region. Informed written consent was obtained from study participants in accordance with the Ethics Committees of each participating hospital.

5.3.2 Immunohistochemistry and evaluation of COX2 expression

COX2 immunostaining of patient tumors and subsequent evaluation of epithelial protein expression has been described previously (Czachorowski et al., 2012). Briefly, paraffin-embedded primary bladder tumors were used to construct tissue microarrays (TMA) containing tumor cores of 0.6-mm in diameter represented in duplicate. Tissue sections were incubated with anti-COX2 rabbit monoclonal antibody (ThermoFisher Scientific, Fremont, CA, USA; #RM-9121-R7; pre-diluted, ready-to-use) at room temperature. Epithelial COX2 expression was quantified using the Ariol SL-50 (version 3.1.2) high-throughput slide imaging scanner. The threshold of positive COX2 expression (>0.340 au) was determined by applying receiver operating characteristics (ROC) analysis (Metz, 1978) by comparing the machine-derived

expression score to a pathologist's (MMM) binary assignment of COX2 expression of a randomly chosen TMA representing approximately 10% of all tissue cores used. Cases were immunohistochemically categorized based on COX2 positivity as described previously (negative, n=334; positive, n=274) (Czachorowski et al., 2012)

5.3.3 Polymorphism selection, genotyping and quality control

SNP selection, genotyping and quality control for all SNPs (with the exception of *TLR9*-rs352139 and *TLR9*-rs352140) used in this study were done as previously described by Lopez et al (*unpublished*). Briefly, three genotyping platforms (two GoldenGate Illumina and one TaqMan) were used to analyze tagSNPs in genes related to the inflammatory response and having an association with cancer were selected. The SYSNPs application (Lorente-Galdos et al., 2012) (using databases dbSNP b25, hg17 and HapMap Release #21) was used to tag SNPs applying Haploview's Tagger algorithm (v. 3.32) with parameters set to default. SNPs were genotyped following manufacturer's instructions (http://www.illumina.com/).

Good agreement (98.2%-99.8%) was observed for SNPs genotyped in all three platforms with any missing genotype data in one platform complemented by SNP data from the remaining platforms. Patients with >95% of successfully genotyped SNPs in each platform were included in the analyses. However, SNPs with >5% missing genotypes, and those with a MAF of <5% and no association with UCB after Fisher's exact test, were excluded from the analyses. The final number of cases and controls included in the analysis was 1,047 and 988, respectively. Missing genotypes were imputed with the *k*-NN method using the package SCRIME in R. To minimize colinearity between variables, pairwise linkage disequilibrium between SNPs was estimated based on r^2 values using the package GENETICS in R; only one of each pair of SNPs was retained for further analyses when r^2 >0.8. Following quality control, the final dataset yielded a total of 911 SNPs from 214 genes.

TLR9-rs352139 and *TLR9*-rs352140, available from a previous genotyping effort using the Illumina Infinium 1M SNP platform (Rothman et al., 2010), were selected for inclusion in

the study *post-hoc*. Thirteen cases and 23 controls had missing genotype information for these two SNPs when compared to the main set of inflammatory SNPs used in this study.

5.3.4 Statistical analysis

Demographic characteristics of controls, and UCB cases defined by the COX2-positivity of their tumors, were compared using the analysis of variance for continuous variables and the Chi-square test for categorical variables. Additionally, Fisher's exact test was used to make comparisons between the two UCB subphenotypes.

Odds ratios (OR) and 95% confidence intervals (CI) were estimated for each of the two molecular UCB subphenotypes using polytomous logistic regression models for each genetic variant and considering codominant and additive modes of inheritance. In each model, the tumor groups were compared to the control group adjusting for the potential confounders: age, gender, region and smoking status (never, occasional, former and current smoker) (Samanic et al., 2006). The global association between variants and risk of any tumor type was assessed using the likelihood ratio test (LRT) comparing models with and without all variant coefficients for the two modes of inheritance examined. The association between variants and risk of distinct subphenotypes was tested with a 2-df LRT in the codominant model and a Wald t-test for each subphenotype in the additive inheritance models. Moreover, heterogeneity of genotype conferred risk was examined in the UCB subphenotypes using the LRT to compare a model in which the variant coefficients were constrained to be equal in both subphenotypes to an unconstrained model for both modes of inheritance.

Recurrence-free and progression-free survival curves were generated using the Kaplan-Meier method, with statistical significance assessed using the log-rank test. Time to each endpoint was calculated from date of primary treatment to the date of event or date of last follow-up. Individuals who did not present any event until the end of the study, those lost to follow-up, or those who died from other causes were censored either at the time of last medical visit or at death. Time to recurrence and progression were defined by applying the "mid-time" between the date of the previous disease-free visit and that when a new event was diagnosed.

Univariable and multivariable Cox-proportional hazards analysis was used to calculate hazard ratios (HR) and 95% CIs. Schoenfeld residual analysis did not suggest any departure from the proportional hazards assumption in multivariable models.

All statistical analyses were done using STATA (version 10.1 SE, StataCorp, College Station, TX, USA). Statistical tests were two-sided and p-values less than 0.05 were considered significant. Bonferroni correction for multiple testing was applied in exploratory risk analyses of the inflammatory gene SNPs.

5.4 RESULTS

5.4.1 Distribution of patient characteristics

The majority of patients were men (88.7%) and either former (38.6%) or current smokers (44.0%). No difference was observed in the distribution of demographic characteristics between patients with negatively or positively COX2 expressing tumors (Table 5.1). However, nearly half (40%) of all COX2 expressing tumors were muscle-invasive as compared to 19% of COX2 negative tumors; a difference that was statistically significant ($p < 0.0001$).

Controls were similar to both subgroups of cases with respect to gender, but were slightly younger (mean age: 64.4 yrs in controls; 66.7 yrs in COX2 negative cases; and 65.7 yrs in COX2-positive cases). The distribution of cases with respect to controls differed by region ($p = 0.004$). As expected, fewer controls (27.1%) than cases were current smokers.

5.4.2 Heterogeneity in genetic susceptibility according to tumor COX2 expression

Overall, 46 SNPs in 37 inflammatory genes were associated with UCB risk in *at least* one of the modes of inheritance considered, and significantly contributed to heterogeneity in genetic susceptibility to the two UCB subgroups examined (Supplementary Table 5.1A-D). None of these 46 SNPs were found to be associated with UCB risk in a logistic regression analysis that pooled cancer subtypes; likely resulting from an attenuation of the risk estimates (data not shown). Two SNPs in *TLR9* (rs187084 and rs352162) maintained these associations in all of the inheritance models examined (Table 5.2) with the strongest evidence of heterogeneity being observed when the additive mode of inheritance was examined (rs187084, p=1.47e-06; rs352162, p=4.50e-04). In the case of *TLR9*-rs187084, this association remained robust after Bonferroni correction for multiple testing (p-value for heterogeneity that was < 6.86e-06). *TLR9*-rs187084 was associated with a 37% reduction in the risk of COX2-negative UCB for every copy of the variant allele (C) (OR=0.63, 95%CI=0.52-0.77, p-value =4.65E-06), but had no association with risk of COX2-positive UCB (OR=1.13, 95%CI=0.93-1.38, p-value =0.218

Table 5.1 Patient and tumor characteristics

Factors (N=1588)	Bladder Cancer Subphenotypes			Group Comparisons	
				All Groups	Case Groups
	Low COX2 (n=331)	High COX2 (n=273)	Controls (n=984)	P value	P value
T stage[b]				-	<0,0001
Ta, T1 (NMIBC)	267 (80,7)	163 (59,7)	-		
T2-T4 (MIBC)	63 (19,0)	110 (40,3)	-		
Age, mean(SD)	66,7 (9,1)	65,7 (10,6)	64,4 (10,0)	0,0011	0,2
Gender				1,0	0,8
Men	293 (88,5)	243 (89,0)	869 (88,3)		
Women	38 (11,5)	30 (11,0)	115 (11,7)		
Region				0,004	0,1
Barcelona	58 (17,5)	28 (10,3)	196 (19,9)		
Valles	62 (18,7)	49 (18,0)	157 (16,0)		
Elche	26 (7,9)	17 (6,2)	79 (8,0)		
Tenerife	62 (18,7)	60 (22,0)	145 (14,7)		
Asturias	123 (37,2)	119 (43,6)	407 (41,4)		
Smoking status[c]				<0,0001	0,7
Never	44 (13,3)	39 (14,3)	277 (28,2)		
Occasional	10 (3,0)	12 (4,4)	79 (8,0)		
Former	133 (40,2)	100 (36,6)	361 (36,7)		
Current	144 (43,5)	122 (44,7)	267 (27,1)		

[a] Chi-square test of independence for categorical variables and one-way analysis of variance for age [b] One case could not be assigned any T stage because the paraffin block could not be retrieved [c] Specification of smoking status variable: never, those who smoked <100 cigarettes in their lifetime; occasional, those who smoked <1 cigarette per day for 6 mo; former, those who smoked at least 1 cigarette per day for 6 mo but did not smoke within 1 yr previous to the interview date; current, those who smoked at least 1 cigarette per day within 1 yr of the interview date.

Table 5.2 Association of common SNPs in TLR9 with UCB characterized by differential expression of COX2 in EPICURO study patients

	N = 1588			1: COX2-negative UCB				2: COX2-negative UCB				LRT p-val	
rs number	**Controls**	**COX2 (-ve)**	**COX2 (+ve)**	**OR**	**L95**	**U95**	**p-val**	**OR**	**L95**	**U95**	**p-val**	**Global[a]**	**Hetero.[b]**
rs187084	984	331	273	0,63	0,52	0,77	4,65E-06[c]	1,13	0,93	1,38	0,218	6,47E-07	1,47E-06
rs352139[d]	965	325	266	1,44	1,19	1,74	1,72E-04	0,81	0,66	0,99	0,039	4,50E-06	1,67E-06
rs352140[d]	965	325	266	1,46	1,21	1,76	9,50E-05	0,81	0,66	0,99	0,042	2,42E-06	1,02E-06
rs352143	984	331	273	0,99	0,79	1,23	9,23E-01	1,27	1,01	1,59	0,038	9,52E-02	6,71E-02
rs352162	984	331	273	1,33	1,10	1,60	2,73E-03	0,88	0,72	1,07	0,197	9,82E-04	4,45E-04
rs4082828	984	331	273	1,15	0,87	1,52	3,16E-01	1,16	0,86	1,55	0,332	4,74E-01	9,79E-01

LRT = likelihood ratio test; OR = odds ratio; rs = reference number for SNPs; SNP = single nucleotide polymorphism

[a] The ‘‘Global’’ LRT tests for a genetic association with any bladder cancer subphenotype.

[b] The ‘‘Hetero’’ LRT tests for heterogeneity in risk estimates between both subphenotypes.

[c] Remained significant after Bonferroni correction for multiple testing (p < 6,86E-06; 911 SNPs * 4 MOIs * 2 UCB subphenotypes)

[d] Included in analysis *post hoc* (see text); missing genotype information for 19 controls, and 6 COX2-negative and 7 COX2-positive cases

(Table 5.2). This association was further examined in strata of several factors/conditions correlated with inflammation to determine whether they could modify the observed association, namely: bladder infection, NSAID/aspirin/paracetamol use, BMI and asthma. The association between *TLR9*-rs187084 and UCB risk remained inversed in nearly all strata examined, and no significant interactions were observed with any of these factors/conditions (Supplementary Table 5.2). Two other SNPs in *TLR9* (*TLR9*-rs352143 and *TLR9*-rs4082828) were also present in our original set of candidate SNPs, but did not associate with the tumor subtypes examined (Table 5.2).

It has been reported that the TLR9 gene variants, rs187084 (-1486T>C) and rs352139 (+1174A>G), concomitantly act to down regulate TLR9 mRNA expression *in vitro* as evidenced by reporter gene assay (Omar et al., 2012; Tao et al., 2007). The potential mechanistic utility of rs352139 led us to subsequently include this SNP in our study, together with another in high linkage disequilibrium (*TLR9*-rs352140; r^2=0.94 in SBC population). The genotype information for both of these SNPs (rs352139 and rs352140) was obtained from a previous genotyping effort using the Illumina Infinium 1M platform. Interestingly, both *TLR9*-rs352139 and *TLR9*-rs352140 were significantly associated with an increased risk of COX2-negative UCB, while an inverse association was observed with COX2-postive UCB (additive MOI, $pLRT_{heterogeneity}$=1.67E-06 for rs352139; $pLRT_{heterogeneity}$=1.02E-06 for rs352140) (Table 5.2).

We proceeded by constructing a categorical variable with sublevels representing patients homozygous for the putative *low* TLR9 mRNA expressing combined genotype (i.e. CC/GG in rs187084/rs352139; homozygous at both loci for variant alleles), patients homozygous for the putative *high* TLR9 mRNA expressing combined genotype (i.e. TT/AA; homozygous at both loci for common alleles) and a reference category representing patients with combined genotypes that did not fall into the two previous categories (Table 5.3a). Patients harboring the CC/GG combined genotype were similarly protected against COX2-negative UCB as observed previously (OR=0.64, 95%CI=0.42-0.98), however those with the TT/AA combined genotype were at an increased risk of the same tumor subtype (OR=1.51, 95%CI=1.11-2.06). Interestingly,

Table 5.3a Association of combined genotypes for TLR9-rs187084 and TLR9-rs352139 with UCB characterized by differential expression of COX2[1]

Combined genotype	N = 1556[a]			1:COX2 (-ve) UCB				2:COX2 (+ve) UCB				LRT p-val	
(rs187084/rs352139)	Controls	COX2 (-ve)	COX2 (+ve)	OR	L95	U95	pLRT	OR	L95	U95	pLRT	Global[b]	Hetero.[c]
others*	621	200	171	1	REF.			1	REF.				
low (CC/GG)	149	31	48	0,64	0,42	0,98	7,68E-04	1,14	0,78	1,67	0,528	6,69E-04	3,51E-04
high (TT/AA)	195	94	47	1,51	1,11	2,06		0,87	0,60	1,27			

[1] see footnotes below Table 3b for superscript explanations

*patients with genotypes that were neither (CC/GG; homozygous for variant alleles) nor (TT/AA; homozygous for common alleles) for (rs187084/rs352139)

Table 5.3b Association of frequent haplotypes of common SNPs in TLR9 with UCB characterized by differential expression of COX2 (additive MOI)

		Haplotype freq.			1:COX2 (-ve) UCB				2:COX2 (+ve) UCB				LRT p-val	
Haplotype*	Haplotype %	Controls	COX2 (-ve)	COX2 (+ve)	OR	95% CI		pval.	OR	95% CI		pval.	Global	Hetero.
WWMWMM	46,0	512	169	133	1,35	1,12	1,63	0,002	0,81	0,66	1,00	0,046	9,93E-05	2,68E-05
MWWWWW	27,6	397	103	111	0,64	0,52	0,80	6,41E-05	0,97	0,79	1,20	0,780	1,58E-04	2,05E-03
WMWMWW	10,7	176	73	56	1,09	0,81	1,46	0,570	1,06	0,77	1,45	0,737	8,38E-01	8,69E-01
MMWWWW	7,0	123	32	49	0,68	0,45	1,01	0,055	1,31	0,92	1,85	0,132	1,52E-02	4,03E-03
WMWWWW	3,1	49	22	19	1,19	0,71	1,98	0,509	1,35	0,80	2,28	0,254	5,05E-01	6,71E-01

* W=common allele, M=variant allele; SNP order: rs187084; rs352139; rs352140; rs352143; rs352162; rs4082828

LRT = likelihood ratio test; OR = odds ratio; rs = reference number for SNPs; SNP = single nucleotide polymorphism

[a] rs352139 had genotype data missing for 19 controls; 6 COX2 negative tumors; 7 COX2 positive tumors

[b] The ''Global'' LRT tests for a genetic association with any bladder cancer subphenotype.

[c] The ''Hetero'' LRT tests for heterogeneity in risk estimates between both subphenotypes.

[d] Remained significant after Bonferroni correction for multiple testing (p < 6,85E-06)

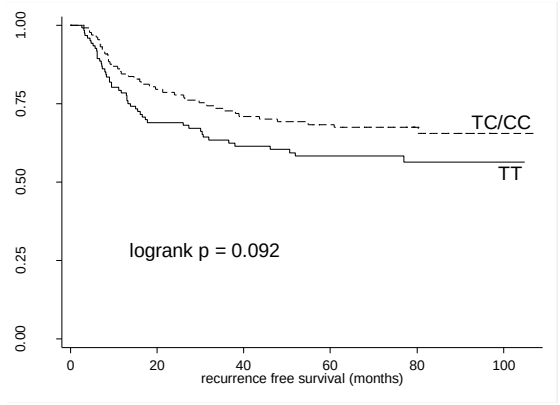
while no significant association between these combined genotypes was observed in patients with COX2-positive tumors ($pLRT_{COX2+}$=0.528), the effect estimates of cancer risk for both combined genotypes were inverse to those observed in COX2-negative tumors ($pLRT_{heterogeneity}$=3.51e-04). These associations between the concomitant effect of rs187084/rs352139 and UCB risk were reiterated when in a haplotype analysis that included the five most frequent haplotypes of the six *TLR9* SNPs identified in this study (Table 5.3b).

TLRs share overlapping downstream signaling cascades and a certain degree of cross-regulation has been reported between TLR9 and other TLRs in various pathologies (Liu et al., 2012; Nickerson et al., 2010; Simmons et al., 2010). To evaluate the potential influence of other TLRs on the UCB risk association observed with *TLR9*-rs187084, the interaction between this SNP and 28 other SNPs in five TLR genes identified in our study (*TLRs 1, 2, 4, 6, 7*) was examined (Supplementary Table 5.3). Of the 28 SNPs evaluated, one SNP, *TLR7*-rs179008, demonstrated a significant interaction with *TLR9*-rs187084 that remained robust after multiple testing correction ($pGlobal_{interaction}$ = 4.64e-04) and increased the risk of COX2-negative UCB (OR=1.63, 95%CI=1.27-2.09, p-value =1.22e-04). However, no association was observed with COX2 positive UCB, and the observed effect estimate (OR) was not significantly heterogeneous between the two UCB subgroups examined ($pHet_{interaction}$ = 0.100).

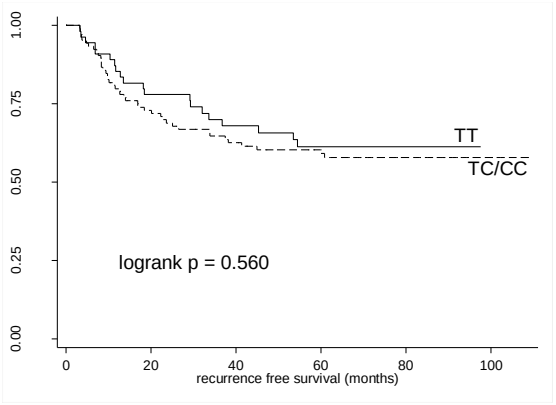
5.4.3 TLR9-rs187084 (-1486T>C) and patient prognosis

Kaplan-Meier analysis was used to evaluate whether the *TLR9*-rs187084 variant allele (C) could affect patient prognosis in tumors characterized by COX2 expression. Wild-type homozygous patients (TT) were compared to patients harboring at least one variant allele (TC/CC) for tumor recurrence and progression in patients with NMIBC, and tumor progression and patient survival in patients with MIBC (Figure 5.1). While no significant prognostic differences were observed for the two genetic backgrounds in patients with COX2-positive tumors (log rank p>0.05), patients with COX2-negative NMIBC and harboring at least one variant allele (TC/CC) in *TLR9*-rs187084 were significantly less likely to observe tumor progression than wild-type homozygous

A)


1.00
0.75
0.50
0.25
0.00
TC/CC
TT
logrank p = 0.092
0
20
40
60
80
100
recurrence free survival (months)

B)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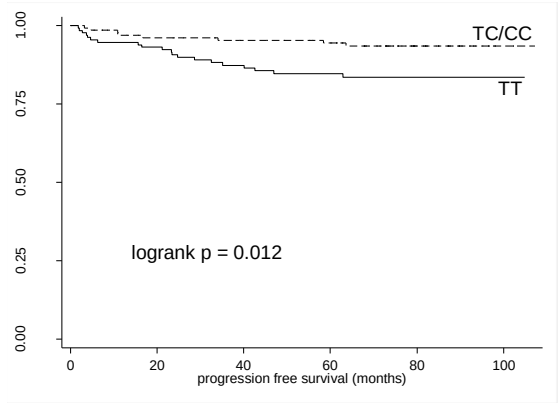
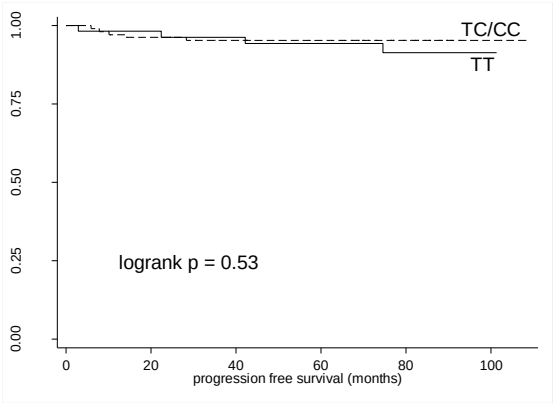
0.75
0.50
0.25
0.00
TT
TC/CC
logrank p = 0.560
0
20
40
60
80
100
recurrence free survival (months)

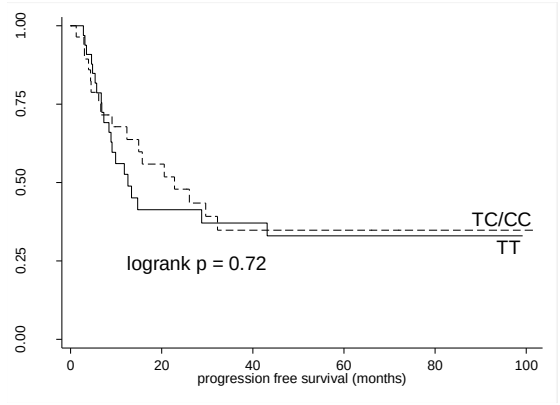
C)


1.00
0.75
0.50
0.25
0.00
TC/CC
TT
logrank p = 0.012
0
20
40
60
80
100
progression free survival (months)

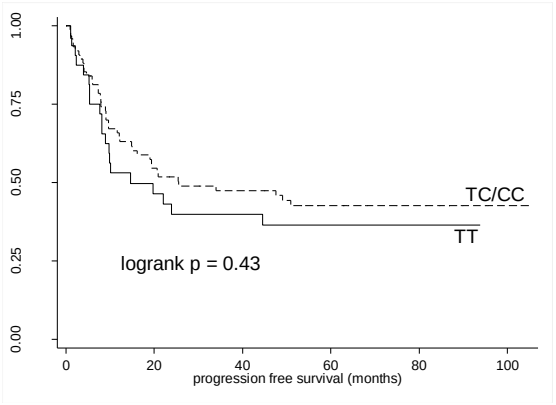
D)


1.00
0.75
0.50
0.25
0.00
TC/CC
TT
logrank p = 0.53
0
20
40
60
80
100
progression free survival (months)

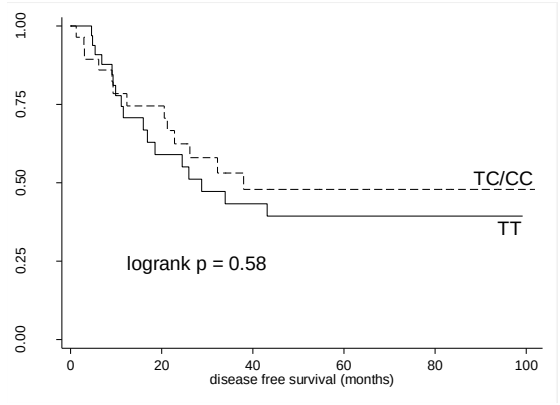
E)


1.00
0.75
0.50
0.25
0.00
TC/CC
TT
logrank p = 0.72
0
20
40
60
80
100
progression free survival (months)

F)


1.00
0.75
0.50
0.25
0.00
TC/CC
TT
logrank p = 0.43
0
20
40
60
80
100
progression free survival (months)

G)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0.75
0.50
0.25
0.00
TC/CC
TT
logrank p = 0.58
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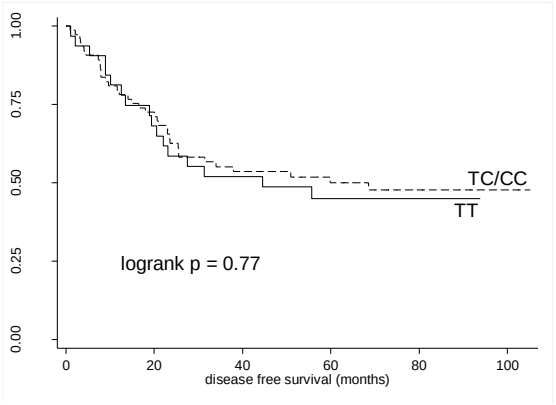
20
40
60
80
100
disease free survival (months)

H)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0.75
0.50
0.25
0.00
TC/CC
TT
logrank p = 0.77
0
20
40
60
80
100
disease free survival (months)

Figure 5.1 (*previous page)* Association of the variant allele -1486T>C in *TLR9* (rs187084) with patient prognosis in the EPICURO study. Patients homozygous for the wild type allele (TT) were compared to individuals harbouring at least one variant allele (TC/CC) for the events indicated along the x-axis of each Kaplan-Meier plot. Patients with: NMIBC (**A-D**); MIBC (**E-H**); COX2-negative tumors (**A, C, E, G**); COX2-positive tumors (**B, D, F, H**).

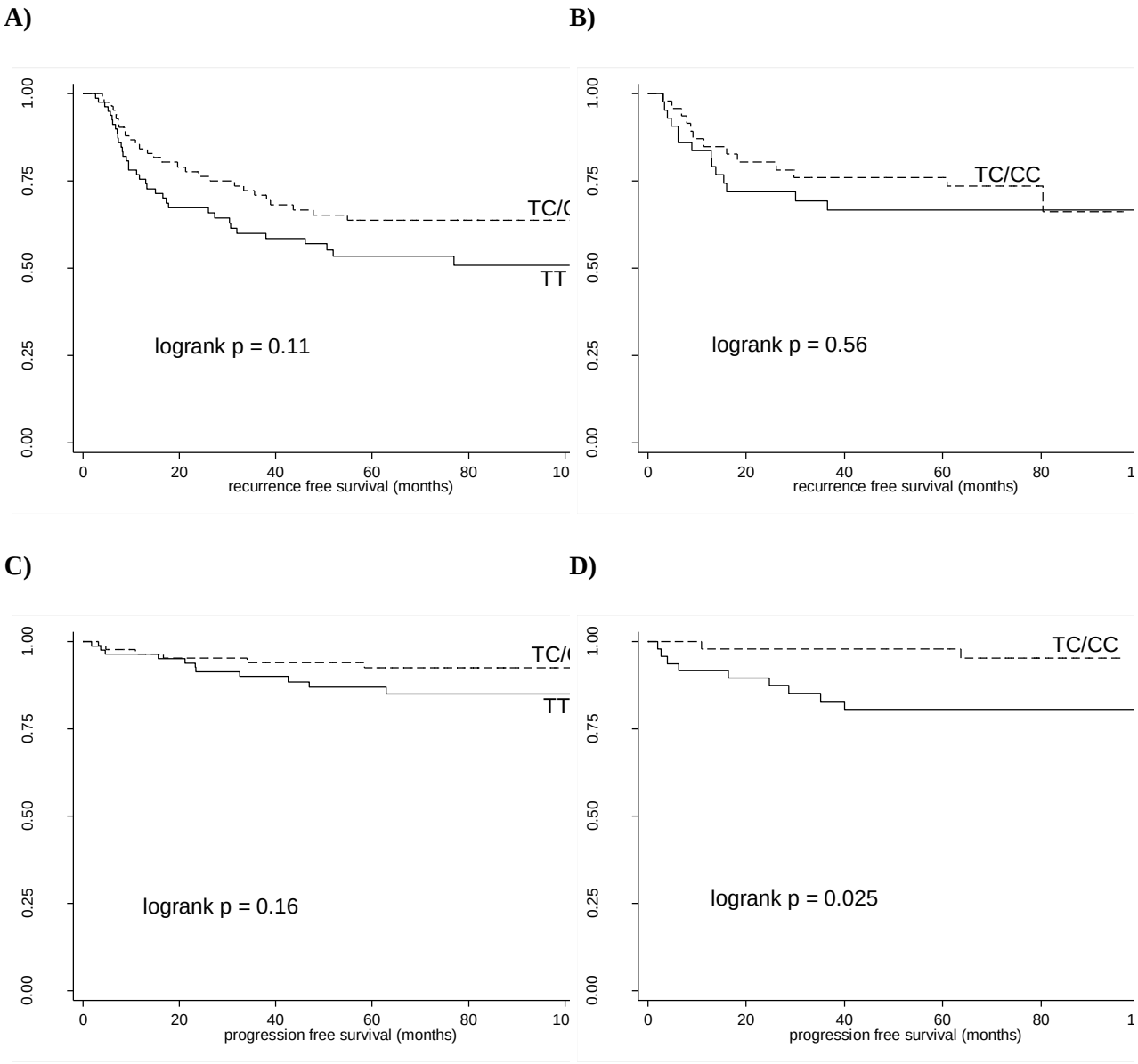

Figure 5.2 The effect of BCG immunotherapy on the association between the variant allele -1486T>C in *TLR9* (rs187084) and patient prognosis in COX2-negative NMIBCs. Patients homozygous for the wild type allele (TT) were compared to individuals harbouring at least one variant allele (TC/CC) for the events indicated along the x-axis of each Kaplan-Meier plot. Patients: not treated with BCG immunotherapy (**A, C**); treated with BCG immunotherapy (**B, D**).

patients (log rank p=0.01). Importantly, the reduction in tumor progression was maintained in multivariate analysis when adjusting for patient age, hospital region, number of tumor recurrences, tumor T-stage and grade, tumor multiplicity and treatment received (HR=0.39, 95%CI=0.16-0.94, p-value =0.036) (Table 5.4).

5.4.4 *TLR9*-rs187084 and response to BCG immunotherapy

We evaluated whether the reduction in tumor progression observed in the patients of this study could be associated with past treatment by BCG instillation therapy. Patients homozygous for the wild type alleles in *TLR9*-rs187084 who did not receive BCG instillation observed no significant differences in risk of recurrence or progression compared to patients harbouring at least one variant allele (TC/CC) (Figure 5.2). However, patients harbouring a variant allele and treated with BCG immunotherapy observed a reduction in the risk of progression compared to wild type homozygous patients (log rank p=0.025). In the multivariate analysis this equated to an 84% reduction in tumor progression of BCG treated carriers of the variant allele in *TLR9*-rs187084 as compared to BCG treated homozygous wild type patients (HR=0.16, 95%CI=0.03-0.82, p-value =0.027) (Table 5.4).

These analyses were repeated for the putative "low" and "high" *TLR9* expressing genotype combinations evaluated earlier in the risk assessment, however no significant associations with prognosis (logrank p>0.05) were identified (data not shown).

Table 5.4 Prognosis in patients with differential COX2 expression in NMIBC or MIBC based on their genotype at postion -1486 in TLR9[a]

	Low COX2 UCB						High COX2 UCB					
Event	**Patients**	**Events**	**HR**	**L95**	**U95**	**p-val[b]**	**Patients**	**Events**	**HR**	**L95**	**U95**	**p-val[b]**
NMIBC[c]												
Recurrence												
All	196	64	0,70	0,41	1,17	0,175	122	53	1,24	0,65	2,34	0,514
BCG treated	68	20	0,65	0,26	1,67	0,375	30	12	2,46	0,59	10,35	0,218
Non-BCG treated	128	44	0,79	0,41	1,50	0,463	92	41	1,73	0,79	3,78	0,170
Progression												
All	258	27	0,39	0,16	0,94	0,036	153	6	4,13	0,32	53,44	0,278
BCG treated	93	11	0,16	0,03	0,82	0,027	38	3	-	-	-	-
Non-BCG treated	165	16	0,64	0,21	1,90	0,417	115	3	-	-	-	-
MIBC												
Progression	52	29	0,88	0,34	2,24	0,786	102	55	0,86	0,48	1,52	0,599
Survival	51	22	1,01	0,29	3,49	0,989	101	46	1,27	0,67	2,42	0,466

BCG = Bacillus Calmette Guerin intravesical instillation therapy; HR = hazard ratio; N/MIBC = Non-/Muscle invasive bladder cancer

[a] Time to event specified was compared between patients homozygous for the wildtype allele (i.e. TT) and those with at least one variant allele (i.e. TC or CC)

[b] Multivariate Cox proportional hazards analysis. Prognostic models adjusted as follows: NMIBC Recurrence adjusted by region, gender, tumour stage and grade, # tumours, size of tumours (≤3 cm, >3 cm), and treatment (transurethral resection [TUR], TUR+BCG, TUR+chemotherapy [chemo], TUR+BCG+chemo, other); NMIBC Progression adjusted by region, # recurrences, age, tumour stage and grade, # tumours, and treatment; MIBC Progression adjusted by region, tumour stage, treatment (cystectomy [cys], cys+chemo, chemo only, radiotherapy +/- chemo, superficial treatment, other) and presence of nodes; MIBC Survival adjusted by region, tumour stage, treatment, presence of nodes, and metastases.

[C] In patients with NMIBC, analysis was also stratified by BCG treatment

5.5 DISCUSSION

In the current study, tagged SNPs in inflammatory genes identified through the literature as relevant to cancer were investigated for heterogeneity in their genetic predisposition to subgroups of UCB characterized by the presence of epithelial COX2 expression. The variant alleles of two SNPs in the gene encoding the innate immune receptor TLR9 (rs187084, rs352139) were observed to contribute to heterogeneity in bladder cancer risk, with the former also showing potential as a predictive marker of BCG treatment response in patients with COX2-negative UCB.

TLR9 is a member of the Toll-like receptor family of integral membrane proteins and is highly expressed in immune cells, particularly plasmocytoid dendritic cells (pDCs), and to a lesser extent in healthy and tumor urothelium (Ayari et al., 2011; Jarrossay et al., 2001). TLR9 receptors become activated and dimerize upon binding to unmethylated CpG-DNA found most prominently in bacteria and viruses. Receptor dimerization induces pDC secretion of T-helper-1 (Th1) polarizing cytokines such as type 1 interferons (IFN-I) and, to a lesser extent, TNF-α (Hemmi et al., 2000). Subsequent pDC maturation and antigen cross-presentation primes $CD4^+$ and $CD8^+$ T-cells thereby targeting the invading pathogen. The same immune response has shown efficacy in mediating tumor rejection leading to the development of artificial TLR9 agonists for use in cancer therapy (Krieg, 2007).

rs187084 is characterized by a T>C transition at position -1486 within the promoter region of TLR9 and has been associated with increased susceptibility to several pathologies with an underlying inflammatory component (Etem et al., 2011; Kastelijn et al., 2010; Lazarus et al., 2003; Liao et al., 2010). Moreover, woman harboring the variant allele are reported as more susceptible to cervical cancer (Roszak et al., 2012) but less likely to develop endometrial cancer (Ashton et al., 2010) (when also carriers of the variant in *TLR9*-rs5743836), while patients with prostate cancer harboring the variant allele observe a better prognosis than homozygous wild-type individuals (Stark et al., 2009). When investigating the functional capacity of rs187084, Chen et al reported an allele-dose dependent increase of the pro-inflammatory cytokine TNF-α

in PBMCs isolated from trauma patients and stimulated with bacterial DNA *ex vivo*; suggesting that rs187084 may contribute to increasing pro-inflammatory cytokine production (Chen et al., 2011). However, Sam-Agudu et al did not observe any rs187084 allele dependent differences in TNF-α or IFN-γ levels in serum drawn from pediatric cerebral malaria patients (Sam-Agudu et al., 2010).

rs352139 is characterized by an A>G transition at position +1174 within intron 1 of *TLR9*. Despite its intronic location, the variant allele of rs352139 has been reported to decrease serum IFN-γ in children with cerebral malaria in an allele-dependent manner, and is associated with susceptibility to several other infectious diseases (Chen et al., 2011; Sam-Agudu et al., 2010; Villani et al., 2010). In contrast to the reduction in COX2-negative tumor risk observed in patients harboring rs187084, the variant allele in rs352139 was associated with an increased risk of COX2-negative UCB and a decreased risk of COX2-positive UCB; with significant heterogeneity between both risk estimates. Using 557 patients of Han Chinese ancestry, Chen et al reported that rs352139 and rs187084 fall within the same haplotype block and are in high linkage disequilibrium (D'=1.00; r^2=1.00). In contrast, the two SNPs were not linked (D'=0.98; r^2=0.54) in our study population which consists of Caucasian patients; a difference probably arising from the ethnic heterogeneity of the two populations. The inversion of UCB risk estimates by these two SNPs for COX2-postive/negative tumors may suggest that each SNP influences the immune response in a distinct molecular manner that should be the focus of future studies.

Using gene reporter assays two independent studies indicated that while having no effect on *TLR9* expression individually, the variant alleles of rs187084 and rs352139 actually decrease *TLR9* expression when the two SNPs are considered jointly (Omar et al., 2012; Tao et al., 2007). By using the combined patient genotypes from rs187084 and rs352139 as a proxy for *TLR9* expression, we investigated whether the UCB risk associations observed herein could be influenced by relative *TLR9* expression. Combined genotypes presumed to decrease *TLR9* gene expression (i.e. CC/GG in rs187084/rs352139) significantly reduced the risk of COX2-negative UCB, while combined genotypes presumed to increase *TLR9* gene expression (TT/AA)

increased risk of the same tumor subtype. No significant associations were observed between these genotype combinations and risk of COX2-positive tumors. By activating the TLR9 signaling pathway, CpG DNA has been shown to up-regulate *COX2* expression through post-transcriptional mRNA stabilization while also inducing the production of PGE_2 in immune cells (Yeo et al., 2003). In this regard, patients harboring the low-*TLR9* expressing genetic signature may produce less COX2 upon TLR9 activation and subsequently generate less PGE_2 to hinder an anti-tumor Th1 immune response - resulting in the reduced UCB risk observed herein. Conversely, high-*TLR9* expressing patients may generate increased levels of PGE_2 and experience immune polarization towards a tolerogenic Th2 response - increasing their risk of UCB.

In a previous study using the same patient population, we did not observe tumor COX2 levels to influence patient prognosis independently of established UCB prognostic factors (Czachorowski et al., 2012). Similarly, none of the SNPs in *TLR9* identified in the current study were found associated with UCB prognosis in a genome wide analysis using patients from the SBC study (Malats et al, *unpublished*). Thus it would appear that the reduction in risk of progression observed in BCG treated patients in this study depends on *both* a low COX2 tumor microenvironment and, presumably, changes in the innate immune response conferred by the variant allele in *TLR9*-rs187084. A localized Th1cytokine profile, as induced by TLR9 activation by CpG-DNA, is associated with successful BCG therapy while a Th2 profile marks failure of the immunotherapy in patients (Luo et al., 2011). Interestingly, BCG administration has the tendency to induce COX2 expression in dendritic cells and up-regulate PGE_2 production. In a tumor microenvironment where COX2 levels are already high, additional production of COX2 by BCG administration can lead to elevated IL-10 cytokine levels and polarize the immune response in a Th2 direction (Dovedi et al., 2008). Elevated PGE_2 levels in COX2-positive UCB may explain why a positive response to BCG treatment was only observed in COX2-negative tumors in which a Th1 immune profile would have been prominent. In fact, a recent randomized controlled trial reported a marginally significant reduction of metachronous recurrences in UCB patients given celecoxib as an adjuvant to BCG immunotherapy (Sabichi et al., 2011).

No associations with UCB risk were observed when the combined genotypes for *TLR9*-rs187084 and *TLR9*-rs352139 were assessed in the prognostic analyses; suggesting that inherent differences in *TLR9* mRNA levels were not responsible for the reduction in UCB progression observed previously. BCG administration activates not only TLR9 via CpG-DNA but also initiates a complex cytokine response through the activation of other TLRs, particularly TLR2/4 on various immune cells, which may have contributed to the immune response independently of how much *TLR9* was being expressed (Godaly and Young, 2005; Tsuji et al., 2000).

The study benefitted from a large sample size that included uniformly characterized incident tumors spanning a broad spectrum of pathological stages and grades uniformly classified by a panel of expert pathologists. Moreover, genotype information was of high quality showing good agreement among all the genotyping platforms used and meeting the quality control guidelines put forth by Anderson et al (Anderson et al., 2010). We also opted to collect COX2 expression data using an automated scoring system providing a reproducible assessment of expression that correlates well with sample scoring by a pathologist (Czachorowski et al., 2012). Importantly, heterogeneity in genetic susceptibility to UCB remained robust after correction for multiple testing for SNPs in *TLR9*, suggesting the observed associations could be real. Despite these attributes, an automated scoring system may incorporate some immunostained stromal material and thereby increase Type I error within the study. Moreover, despite the large size of the study it was not designed with sufficient power to evaluate heterogeneity by tumor subphenotypes necessitating replication in independent datasets.

To our knowledge this is the first study to evaluate heterogeneity of genetic susceptibility to bladder tumors defined by levels of epithelial COX2 expression. The study implicated a SNP in TLR9 (rs187084) with reduced risk of COX2-negative bladder cancer that also shows potential as a predictive biomarker of BCG treatment response in patients with tumors characterized by low levels of COX2 expression. Moreover, the study emphasizes the utility of sub-grouping bladder cancers by their inherent molecular characteristics as a means of elucidating predictive markers of therapeutic value.

5.6 SUPPLEMENTARY TABLES 5.1 - 5.3

Supplementary Table 5.1 Association of SNPs in inflammatory genes of EPICURO study patients with UCBs characterized by differential expression of COX2 by MOI

A) CODOMINANT			N = 1588				COX2-neg.				COX2-pos.			LRT p-val	
gene_SNP	MAF	Genotype	Control	COX2-neg.	COX2-pos.	OR1	Lower_CI1	Upper_CI1	pLRT1	OR2	Lower_CI2	Upper_CI2	pLRT2	pLRT_Global	pLRT_Het
anpep_rs4932250	0,08	wt	817	276	208	1	REF.		9,47E-01	1	REF.		3,77E-03	2,20E-02	1,36E-02
		hetero	167	55	64	1,01	0,72	1,43		1,50	1,07	2,11			
		variant	0	0	1	0,02	na	na		na	na	na			
bcl10_rs2647396	0,32	wt	452	153	164	1	REF.		9,30E-01	1	REF.		1,21E-04	7,04E-04	2,00E-03
		hetero	445	148	90	0,97	0,74	1,27		0,54	0,40	0,72			
		variant	87	30	19	1,06	0,66	1,69		0,60	0,35	1,03			
bcl10_rs962409	0,44	wt	309	95	61	1	REF.		5,14E-01	1	REF.		3,09E-03	1,39E-02	4,97E-02
		hetero	480	171	141	1,19	0,88	1,60		1,55	1,10	2,18			
		variant	195	65	71	1,08	0,74	1,58		1,94	1,30	2,89			
blnk_rs2861304	0,08	wt	824	277	226	1	REF.		9,69E-03	1	REF.		2,74E-01	4,36E-03	2,58E-03
		hetero	155	46	47	0,86	0,60	1,25		1,08	0,75	1,57			
		variant	5	8	0	5,67	1,72	18,68		0,00	na	na			
blnk_rs7099132	0,07	wt	849	286	234	1	REF.		2,44E-02	1	REF.		5,38E-01	3,16E-02	2,57E-02
		hetero	132	40	39	0,93	0,63	1,38		1,08	0,73	1,61			
		variant	3	5	0	8,37	1,72	40,76		0,00	na	na			
cd80_rs9282638	0,15	wt	716	241	175	1	REF.		7,87E-01	1	REF.		1,80E-02	4,35E-02	2,40E-02
		hetero	239	84	85	1,01	0,75	1,37		1,39	1,02	1,89			
		variant	29	6	13	0,73	0,29	1,84		2,24	1,10	4,56			
il17a_rs1974226	0,17	wt	674	236	197	1	REF.		7,79E-01	1	REF.		2,23E-03	1,18E-02	7,92E-03
		hetero	283	87	76	0,90	0,67	1,21		0,95	0,70	1,29			
		variant	27	8	0	0,91	0,39	2,12		0,00	na	na			
il7_rs11777205	0,08	wt	834	269	237	1	REF.		1,98E-02	1	REF.		5,45E-01	4,14E-02	2,84E-02
		hetero	140	62	34	1,28	0,91	1,81		0,81	0,54	1,23			
		variant	10	0	2	0,00	na	na		0,67	0,14	3,19			
irak2_rs263412	0,09	wt	819	256	226	1	REF.		1,34E-02	1	REF.		2,92E-01	1,13E-02	1,16E-02
		hetero	160	68	47	1,37	0,99	1,91		1,08	0,75	1,57			
		variant	5	7	0	4,31	1,29	14,47		0,00	na	na			
jak1_rs10889513	0,12	wt	769	270	211	1	REF.		1,75E-01	1	REF.		4,50E-02	2,12E-02	4,77E-03
		hetero	202	55	62	0,74	0,53	1,05		1,09	0,78	1,53			
		variant	13	6	0	1,38	0,49	3,88		0,00	na	na			
map2k4_rs12603036	0,10	wt	805	265	239	1	REF.		8,87E-01	1	REF.		3,66E-03	1,51E-02	8,41E-03
		hetero	167	62	34	1,07	0,76	1,49		0,65	0,43	0,98			
		variant	12	4	0	0,84	0,26	2,77		0,00	na	na			
map3k7_rs150126	0,25	wt	556	223	157	1	REF.		1,19E-03	1	REF.		6,21E-01	4,53E-03	1,46E-02

Supplementary Table 5.1 (*cont'd*)

A) CODOMINANT			N = 1588				COX2-neg.				COX2-pos.			LRT p-val	
gene_SNP	MAF	Genotype	Control	COX2-neg.	COX2-pos.	OR1	Lower_CI1	Upper_CI1	pLRT1	OR2	Lower_CI2	Upper_CI2	pLRT2	pLRT_Global	pLRT_Het
		hetero	360	95	94	0,64	0,48	0,84		0,91	0,68	1,22			
		variant	68	13	22	0,49	0,26	0,92		1,17	0,69	2,00			
nfkb1_rs4648127	0,05	wt	882	306	234	1	REF.		1,04E-02	1	REF.		5,99E-02	7,51E-03	3,11E-02
		hetero	99	21	35	0,57	0,35	0,95		1,22	0,80	1,87			
		variant	3	4	4	4,97	0,98	25,20		6,23	1,27	30,64			
nos2a_rs2297518	0,19	wt	643	202	193	1	REF.		4,91E-02	1	REF.		1,71E-01	3,36E-02	3,87E-02
		hetero	299	121	72	1,27	0,97	1,67		0,81	0,59	1,10			
		variant	42	8	8	0,56	0,25	1,23		0,57	0,26	1,26			
ticam1_rs4807653	0,14	wt	729	236	221	1	REF.		3,39E-01	1	REF.		1,26E-02	1,86E-02	1,60E-02
		hetero	237	84	44	1,10	0,81	1,48		0,61	0,42	0,87			
		variant	18	11	8	1,76	0,81	3,86		1,45	0,61	3,43			
tlr7_rs179007	0,27	wt	695	236	211	1	REF.		9,64E-01	1	REF.		1,82E-03	1,13E-02	1,45E-02
		hetero	54	17	4	0,91	0,46	1,82		0,19	0,06	0,56			
		variant	235	78	58	1,01	0,74	1,37		0,86	0,61	1,20			
tlr9_rs187084	0,40	wt	360	168	89	1	REF.		1,77E-05	1	REF.		4,49E-01	8,45E-06	8,65E-06
		hetero	467	132	134	0,61	0,46	0,80		1,17	0,86	1,60			
		variant	157	31	50	0,42	0,27	0,65		1,27	0,84	1,90			
tlr9_rs352162	0,47	wt	262	70	87	1	REF.		8,04E-03	1	REF.		3,06E-01	5,13E-03	2,53E-03
		hetero	508	163	131	1,20	0,87	1,67		0,79	0,57	1,09			
		variant	214	98	55	1,76	1,21	2,54		0,79	0,53	1,17			
tnfrsf7_rs2286598	0,42	wt	331	87	106	1	REF.		2,43E-02	1	REF.		3,37E-01	1,32E-02	2,98E-03
		hetero	468	187	120	1,51	1,12	2,04		0,80	0,59	1,08			
		variant	185	57	47	1,22	0,83	1,81		0,83	0,56	1,24			
ulbp3_rs2010259	0,47	wt	332	104	81	1	REF.		8,40E-01	1	REF.		5,74E-03	1,57E-02	9,03E-03
		hetero	384	124	133	1,01	0,74	1,38		1,44	1,04	1,98			
		variant	268	103	59	1,10	0,79	1,52		0,84	0,57	1,23			

Supplementary Table 5.1 (*cont'd*)

B) ADDITIVE		N = 1588			COX2-neg.				COX2-pos.				LRT p-val	
gene_SNP	MAF	Control	COX2-neg.	COX2-pos.	OR1	Lower_CI1	Upper_CI1	pTREND1	OR2	Lower_CI2	Upper_CI2	pTREND2	pLRT_Global	pLRT_Het
akr1c3_rs6601899	0,14	984	331	273	1,33	1,03	1,70	2,73E-02	0,90	0,67	1,21	4,82E-01	3,73E-02	2,05E-02
anpep_rs4932250	0,08	984	331	273	1,01	0,71	1,42	9,70E-01	1,56	1,12	2,17	9,25E-03	2,92E-02	3,26E-02
bcl10_rs2647396	0,32	984	331	273	1,00	0,82	1,23	9,70E-01	0,65	0,52	0,82	2,26E-04	4,54E-04	1,06E-03
bcl10_rs962409	0,44	984	331	273	1,05	0,88	1,27	5,70E-01	1,40	1,15	1,70	8,83E-04	3,57E-03	1,59E-02
card4_rs11536450	0,14	984	331	273	1,11	0,86	1,44	4,16E-01	0,72	0,53	0,99	4,33E-02	4,30E-02	1,51E-02
casp8_rs2349070	0,28	984	331	273	0,81	0,66	1,00	4,50E-02	1,14	0,92	1,41	2,17E-01	2,40E-02	7,70E-03
casp8_rs10931934	0,40	984	331	273	0,82	0,68	0,97	2,46E-02	1,09	0,91	1,32	3,39E-01	2,06E-02	8,64E-03
casp9_rs4646075	0,23	984	331	273	0,77	0,61	0,96	2,03E-02	1,02	0,82	1,29	8,39E-01	4,27E-02	3,82E-02
cd80_rs9282638	0,15	984	331	273	0,97	0,75	1,25	7,85E-01	1,44	1,12	1,84	4,20E-03	1,03E-02	9,21E-03
icam1_rs3093032	0,13	984	331	273	0,69	0,52	0,93	1,60E-02	1,05	0,79	1,39	7,49E-01	2,86E-02	2,33E-02
ikbkb_rs17875671	0,08	984	331	273	0,80	0,56	1,15	2,24E-01	1,41	1,01	1,96	4,19E-02	2,43E-02	7,18E-03
il21r_rs8060368	0,38	984	331	273	0,82	0,68	0,99	4,10E-02	1,11	0,91	1,35	3,17E-01	3,22E-02	1,22E-02
irak2_rs263412	0,09	984	331	273	1,51	1,12	2,03	6,26E-03	1,02	0,72	1,45	9,19E-01	2,08E-02	4,25E-02
map2k4_rs12603036	0,10	984	331	273	1,03	0,77	1,39	8,31E-01	0,58	0,40	0,86	6,29E-03	9,57E-03	7,25E-03
map3k7_rs13208824	0,11	984	331	273	1,13	0,86	1,50	3,72E-01	0,65	0,46	0,93	1,73E-02	1,34E-02	4,62E-03
map3k7_rs150126	0,25	984	331	273	0,67	0,53	0,84	4,27E-04	1,00	0,80	1,25	9,85E-01	8,81E-04	3,33E-03
ncf2_rs2296164	0,47	984	331	273	1,11	0,92	1,33	2,77E-01	0,81	0,67	0,99	4,22E-02	2,97E-02	9,09E-03
scarb1_rs4765621	0,42	984	331	273	0,92	0,76	1,11	3,65E-01	0,72	0,59	0,89	1,88E-03	7,18E-03	4,82E-02
tlr9_rs187084	0,40	984	331	273	0,63	0,52	0,77	4,65E-06	1,13	0,93	1,38	2,18E-01	6,47E-07	1,47E-06
tlr9_rs352162	0,47	984	331	273	1,33	1,10	1,60	2,73E-03	0,88	0,72	1,07	1,97E-01	9,82E-04	4,45E-04
ulbp3_rs12202737	0,26	984	331	273	0,91	0,73	1,14	4,12E-01	1,27	1,02	1,58	3,48E-02	3,88E-02	1,46E-02

C) DOMINANT			N = 1588			COX2-neg.					COX2-pos.					LRT p-val	
gene_SNP	MAF	Genotype	Control	COX2-neg.	COX2-pos.	OR1	Lower_CI1	Upper_CI1	pval1	pLRT1	OR2	Lower_CI2	Upper_CI2	pval2	pLRT2	pLRT_Global	pLRT_Het
akr1c3_rs6601899	0,14	wt	731	227	211	1	REF.				1	REF.					
		het.+hom.	253	104	62	1,36	1,02	1,80	3,42E-02	3,55E-02	0,88	0,64	1,23	4,61E-01	4,58E-01	4,32E-02	2,15E-02
anpep_rs4932250	0,08	wt	817	276	208	1	REF.				1	REF.					
		het.+hom.	167	55	65	1,01	0,71	1,43	9,59E-01	9,59E-01	1,53	1,09	2,14	1,30E-02	1,45E-02	4,05E-02	4,29E-02
arhgdib_rs2075267	0,41	wt	354	124	80	1	REF.				1	REF.					
		het.+hom.	630	207	193	0,98	0,75	1,28	8,84E-01	8,84E-01	1,45	1,08	1,96	1,47E-02	1,34E-02	3,28E-02	2,48E-02
bcl10_rs2647396	0,32	wt	452	153	164	1	REF.				1	REF.					
		het.+hom.	532	178	109	0,98	0,76	1,27	8,97E-01	8,97E-01	0,55	0,41	0,73	2,75E-05	2,32E-05	7,35E-05	4,26E-04
casp8_rs2349070	0,28	wt	514	198	133	1	REF.				1	REF.					
		het.+hom.	470	133	140	0,74	0,57	0,96	2,25E-02	2,20E-02	1,17	0,89	1,55	2,53E-01	2,53E-01	1,34E-02	4,90E-03
casp8_rs10931934	0,40	wt	386	153	99	1	REF.				1	REF.					
		het.+hom.	598	178	174	0,75	0,58	0,97	3,11E-02	3,14E-02	1,14	0,86	1,52	3,56E-01	3,54E-01	2,85E-02	1,20E-02
casp9_rs4646075	0,23	wt	583	218	156	1	REF.				1	REF.					
		het.+hom.	401	113	117	0,73	0,55	0,95	1,97E-02	1,88E-02	1,08	0,81	1,43	6,05E-01	6,05E-01	3,07E-02	1,98E-02
cd80_rs9282638	0,15	wt	716	241	175	1	REF.				1	REF.					

Supplementary Table 5.1 (*cont'd*)

C) DOMINANT			N = 1588			COX2-neg.					COX2-pos.					LRT p-val	
gene_SNP	MAF	Genotype	Control	COX2-neg.	COX2-pos.	OR1	Lower_CI1	Upper_CI1	pval1	pLRT1	OR2	Lower_CI2	Upper_CI2	pval2	pLRT2	pLRT_Global	pLRT_Het
		het.+hom.	268	90	98	0,99	0,74	1,32	9,36E-01	9,36E-01	1,47	1,09	1,97	1,04E-02	1,11E-02	2,84E-02	2,59E-02
gata3_rs10752126	0,41	wt	345	107	116	1	REF.				1	REF.					
		het.+hom.	639	224	157	1,13	0,86	1,49	3,72E-01	3,70E-01	0,74	0,56	0,98	3,77E-02	3,85E-02	3,68E-02	1,29E-02
icam1_rs3093032	0,13	wt	737	268	200	1	REF.				1	REF.					
		het.+hom.	247	63	73	0,68	0,49	0,94	1,79E-02	1,58E-02	1,07	0,78	1,46	6,71E-01	6,72E-01	2,97E-02	2,12E-02
irak2_rs263408	0,10	wt	807	250	225	1	REF.				1	REF.					
		het.+hom.	177	81	48	1,47	1,08	2,00	1,56E-02	1,69E-02	0,97	0,67	1,39	8,61E-01	8,61E-01	3,91E-02	4,11E-02
jak3_rs2286662	0,39	wt	374	101	108	1	REF.				1	REF.					
		het.+hom.	610	230	165	1,38	1,05	1,82	2,19E-02	2,07E-02	0,94	0,71	1,25	6,66E-01	6,66E-01	3,76E-02	2,57E-02
lepr_rs1137100	0,23	wt	579	173	167	1	REF.				1	REF.					
		het.+hom.	405	158	106	1,36	1,05	1,76	2,17E-02	2,17E-02	0,91	0,69	1,21	5,24E-01	5,23E-01	3,03E-02	1,74E-02
map2k4_rs12603036	0,10	wt	805	265	239	1	REF.				1	REF.					
		het.+hom.	179	66	34	1,05	0,76	1,46	7,53E-01	7,53E-01	0,60	0,40	0,90	1,37E-02	1,06E-02	2,20E-02	1,32E-02
map3k7_rs13208824	0,11	wt	774	250	232	1	REF.				1	REF.					
		het.+hom.	210	81	41	1,14	0,84	1,55	3,96E-01	3,99E-01	0,63	0,44	0,92	1,70E-02	1,39E-02	1,43E-02	5,08E-03
map3k7_rs150126	0,25	wt	556	223	157	1	REF.				1	REF.					
		het.+hom.	428	108	116	0,61	0,47	0,81	4,16E-04	3,50E-04	0,95	0,72	1,25	7,14E-01	7,14E-01	1,38E-03	1,05E-02
socs6_rs713129	0,14	wt	731	243	181	1	REF.				1	REF.					
		het.+hom.	253	88	92	1,04	0,77	1,39	7,98E-01	7,98E-01	1,50	1,11	2,03	7,71E-03	8,35E-03	2,72E-02	4,10E-02
ticam1_rs4807653	0,14	wt	729	236	221	1	REF.				1	REF.					
		het.+hom.	255	95	52	1,15	0,86	1,53	3,41E-01	3,43E-01	0,67	0,48	0,94	2,13E-02	1,85E-02	1,64E-02	5,43E-03
tlr9_rs187084	0,40	wt	360	168	89	1	REF.				1	REF.					
		het.+hom.	624	163	184	0,56	0,43	0,73	1,29E-05	1,28E-05	1,20	0,89	1,60	2,32E-01	2,30E-01	3,26E-06	6,80E-06
tlr9_rs352162	0,47	wt	262	70	87	1	REF.				1	REF.					
		het.+hom.	722	261	186	1,37	1,00	1,86	4,75E-02	4,45E-02	0,79	0,58	1,07	1,22E-01	1,24E-01	1,27E-02	3,37E-03
tnfrsf7_rs2267966	0,30	wt	473	148	154	1	REF.				1	REF.					
		het.+hom.	511	183	119	1,17	0,91	1,52	2,25E-01	2,25E-01	0,75	0,57	0,99	4,02E-02	3,97E-02	2,20E-02	6,28E-03
tnfrsf7_rs2286598	0,42	wt	331	87	106	1	REF.				1	REF.					
		het.+hom.	653	244	167	1,43	1,07	1,90	1,49E-02	1,36E-02	0,81	0,61	1,07	1,41E-01	1,43E-01	4,09E-03	1,21E-03

Supplementary Table 5.1 (*cont'd*)

D) RECESSIVE			N = 1588			COX2-neg.					COX2-pos.					LRT p-val	
gene_SNP	**MAF**	**Genotype**	**Control**	**COX2-neg.**	**COX2-pos.**	**OR1**	**Lower_CI1**	**Upper_CI1**	**pval1**	**pLRT1**	**OR2**	**Lower_CI2**	**Upper_CI2**	**pval2**	**pLRT2**	**pLRT_Global**	**pLRT_Het**
aicda_rs2580874	0,38	wt+het.	844	284	216	1	REF.				1	REF.					
		hom.	140	47	57	0,98	0,68	1,42	9,21E-01	9,21E-01	1,55	1,09	2,20	1,55E-02	1,75E-02	4,30E-02	3,64E-02
blnk_rs2861304	0,08	wt+het.	979	323	273	1	REF.				1	REF.					
		hom.	5	8	0	5,80	1,76	19,09	3,84E-03	3,26E-03	0,00	na	na	1,00E+00	1,21E-01	8,60E-04	9,46E-04
blnk_rs7099132	0,07	wt+het.	981	326	273	1	REF.				1	REF.					
		hom.	3	5	0	8,45	1,74	41,09	8,18E-03	6,86E-03	0,00	na	na	1,00E+00	2,97E-01	6,03E-03	8,35E-03
casp8_rs11674814	0,38	wt+het.	845	266	238	1	REF.				1	REF.					
		hom.	139	65	35	1,46	1,04	2,05	2,81E-02	3,02E-02	0,88	0,59	1,33	5,58E-01	5,54E-01	4,39E-02	2,65E-02
il21r_rs2189521	0,46	wt+het.	784	272	199	1	REF.				1	REF.					
		hom.	200	59	74	0,80	0,57	1,12	1,93E-01	1,88E-01	1,42	1,03	1,96	3,11E-02	3,32E-02	1,42E-02	3,94E-03
il2ra_rs12722588	0,19	wt+het.	940	324	258	1	REF.				1	REF.					
		hom.	44	7	15	0,39	0,17	0,89	2,57E-02	1,44E-02	1,14	0,61	2,13	6,86E-01	6,89E-01	2,69E-02	1,72E-02
irak2_rs263412	0,09	wt+het.	979	324	273	1	REF.				1	REF.					
		hom.	5	7	0	4,05	1,21	13,57	2,32E-02	2,28E-02	0,00	na	na	1,00E+00	1,31E-01	8,42E-03	5,64E-03
ly96_rs11786591	0,28	wt+het.	906	310	239	1	REF.				1	REF.					
		hom.	78	21	34	0,80	0,48	1,33	3,91E-01	3,84E-01	1,66	1,07	2,58	2,47E-02	2,84E-02	2,77E-02	1,10E-02
oscar_rs11669029	0,43	wt+het.	799	269	202	1	REF.				1	REF.					
		hom.	185	62	71	1,00	0,72	1,38	9,78E-01	9,78E-01	1,49	1,07	2,06	1,67E-02	1,82E-02	4,76E-02	4,30E-02
tlr9_rs187084	0,40	wt+het.	827	300	223	1	REF.				1	REF.					
		hom.	157	31	50	0,54	0,35	0,82	3,71E-03	2,37E-03	1,16	0,81	1,66	4,31E-01	4,34E-01	2,56E-03	1,63E-03
tlr9_rs352162	0,47	wt+het.	770	233	218	1	REF.				1	REF.					
		hom.	214	98	55	1,55	1,16	2,08	3,45E-03	3,79E-03	0,91	0,65	1,29	6,09E-01	6,07E-01	5,94E-03	6,33E-03
tmem189_rs6125888	0,12	wt+het.	972	320	270	1	REF.				1	REF.					
		hom.	12	11	3	2,78	1,16	6,67	2,23E-02	2,47E-02	0,83	0,23	3,06	7,80E-01	7,76E-01	4,67E-02	4,58E-02
ulbp3_rs2010259	0,47	wt+het.	716	228	214	1	REF.				1	REF.					
		hom.	268	103	59	1,09	0,82	1,44	5,57E-01	5,58E-01	0,68	0,49	0,95	2,23E-02	1,98E-02	2,96E-02	1,30E-02

Supplementary Table 5.2 Association of TLR9 (-1486T>C) and bladder cancer risk based on level of COX2 expression and stratified by patient characteristics

			Cases		Low COX2 Cases				High COX2 Cases				LRT		
ADDITIVE MOI	**N**	**Controls**	**Low COX2**	**High COX2**	**OR**	**Lower_CI**	**Upper_CI**	**pTREND**	**OR**	**Lower_CI**	**Upper_CI**	**pTREND**	**Global[a]**	**Hetero[b]**	**Interaction[c]**
Overall															
	1588	984	331	273	0,63	0,52	0,77	4,65E-06	1,13	0,93	1,38	2,18E-01	6,47E-07	1,47E-06	
Gender															
Men	1405	869	293	243	0,65	0,53	0,80	5,26E-05	1,10	0,89	1,36	3,78E-01	2,35E-05	4,53E-05	
Women	183	115	38	30	0,53	0,27	1,04	6,56E-02	1,28	0,72	2,29	4,03E-01	7,31E-02	2,79E-02	
Age															
<=60	439	299	69	71	0,57	0,37	0,89	1,27E-02	0,87	0,59	1,29	4,93E-01	3,84E-02	1,17E-01	
>60 & <=70	617	386	134	97	0,60	0,44	0,83	2,24E-03	1,28	0,91	1,80	1,50E-01	4,50E-04	2,15E-04	
>70	532	299	128	105	0,66	0,48	0,90	9,09E-03	1,20	0,87	1,66	2,66E-01	4,10E-03	1,74E-03	
Smoking															
Non-smoker	360	277	44	39	0,61	0,36	1,01	5,68E-02	1,13	0,69	1,85	6,31E-01	1,09E-01	6,42E-02	
Occasional	101	79	10	12	0,86	0,30	2,44	7,80E-01	1,23	0,49	3,11	6,61E-01	8,47E-01	5,76E-01	
Former	594	361	133	100	0,66	0,49	0,91	9,65E-03	1,16	0,84	1,59	3,62E-01	8,25E-03	4,43E-03	
Current	533	267	144	122	0,56	0,41	0,78	4,42E-04	1,03	0,76	1,42	8,31E-01	5,31E-04	1,14E-03	
Region															
Barcelona	282	196	58	28	0,81	0,51	1,29	3,74E-01	1,44	0,80	2,62	2,27E-01	2,42E-01	9,24E-02	
Valles	268	157	62	49	0,31	0,18	0,54	3,32E-05	0,90	0,54	1,49	6,71E-01	3,15E-05	7,53E-04	
Elche	122	79	26	17	0,53	0,25	1,11	9,33E-02	2,17	0,95	4,94	6,56E-02	1,29E-02	3,25E-03	
Tenerife	267	145	62	60	1,05	0,68	1,63	8,27E-01	1,51	0,97	2,34	6,84E-02	1,73E-01	1,62E-01	
Asturias	649	407	123	119	0,57	0,42	0,79	7,07E-04	0,91	0,67	1,22	5,10E-01	2,27E-03	1,82E-02	
Bladder infection															0,307
No	1267	777	270	220	0,64	0,51	0,80	6,16E-05	1,04	0,83	1,30	7,34E-01	7,54E-05	3,31E-04	
Yes	299	202	52	45	0,60	0,36	1,01	5,23E-02	1,54	0,96	2,46	7,44E-02	1,19E-02	2,93E-03	
Enlarged prostate															0,523
No	911	564	189	158	0,68	0,52	0,87	2,59E-03	0,89	0,68	1,15	3,71E-01	9,29E-03	8,76E-02	
Yes	195	139	28	28	0,69	0,35	1,34	2,71E-01	1,23	0,67	2,25	4,99E-01	3,52E-01	1,54E-01	
NSAIDs															0,395
Never	1134	701	236	197	0,65	0,52	0,82	2,58E-04	0,98	0,78	1,24	8,68E-01	6,88E-04	4,21E-03	
Ever	78	61	11	6	0,35	0,05	2,40	2,88E-01	2,60	0,21	32,64	4,60E-01	1,80E-01	5,43E-01	
Aspirin															0,287
Never	564	349	111	104	0,53	0,37	0,74	2,78E-04	0,99	0,72	1,37	9,55E-01	5,25E-04	2,07E-03	
Ever	648	413	136	99	0,74	0,55	1,00	4,88E-02	0,97	0,70	1,34	8,52E-01	1,30E-01	1,66E-01	
Paracetamol															0,041[d]
Never	864	529	177	158	0,60	0,46	0,78	1,54E-04	0,85	0,66	1,11	2,31E-01	5,48E-04	3,00E-02	
Ever	348	233	70	45	0,79	0,51	1,22	2,80E-01	1,70	1,03	2,79	3,73E-02	3,30E-02	9,80E-03	
BMI															0,296
<25	656	398	138	120	0,54	0,39	0,74	1,72E-04	0,90	0,66	1,24	5,30E-01	4,91E-04	7,00E-03	

Supplementary Table 5.2 (*cont'd)*

			Cases		Low COX2 Cases				High COX2 Cases				LRT		
ADDITIVE MOI	**N**	**Controls**	**Low COX2**	**High COX2**	**OR**	**Lower_CI**	**Upper_CI**	**pTREND**	**OR**	**Lower_CI**	**Upper_CI**	**pTREND**	**Global[a]**	**Hetero[b]**	**Interaction[c]**
>=25 & <30	456	293	97	66	0,86	0,61	1,23	4,19E-01	1,39	0,95	2,05	9,32E-02	1,10E-01	3,93E-02	
>=30	88	60	14	14	0,79	0,32	1,96	6,09E-01	0,91	0,34	2,42	8,44E-01	8,72E-01	8,22E-01	
Asthma															0,970
Yes	105	73	17	15	0,59	0,24	1,42	2,38E-01	1,20	0,48	3,00	6,97E-01	4,04E-01	2,27E-01	
No	1170	736	235	199	0,67	0,53	0,84	5,11E-04	1,00	0,79	1,26	9,95E-01	1,22E-03	4,39E-03	
Cruciferous veg. (quart.)															0,545
Q1	328	180	90	58	0,72	0,49	1,07	1,09E-01	1,23	0,79	1,90	3,62E-01	7,81E-02	3,02E-02	
Q2	346	215	67	64	0,47	0,30	0,75	1,40E-03	0,88	0,57	1,37	5,79E-01	3,74E-03	2,38E-02	
Q3	364	237	67	60	0,90	0,60	1,36	6,23E-01	1,14	0,76	1,72	5,28E-01	6,55E-01	3,59E-01	
Q4	107	63	18	26	0,93	0,38	2,29	8,81E-01	1,08	0,49	2,37	8,51E-01	9,57E-01	7,70E-01	

BMI = body mass index; LRT = likelihood ratio test; OR = odds ratio; rs = reference number for SNPs; SNP = single nucleotide polymorphism

[a] The ''Global'' LRT tests for a genetic association with any bladder cancer subphenotype.

[b] The ''Hetero'' LRT tests for heterogeneity in risk estimates between both subphenotypes.

[c] The "Interaction" LRT tests for modification of UCB risk in strata of the factors examined

[d] Did not remain significant after Bonferroni correctin for multiple testing (p=0,328)

Supplementary Table 5.3 Interaction between TLR9 rs187084 and other SNPs in TLR genes in tumors subphenotyped by level of COX2 expression

					Main Effects TLR9 rs187084								Main Effects SNPs in other TLR genes							
Additive MOI	N = 1588				low COX2 tumors				high COX2 tumors				low COX2 tumors				high COX2 tumors			
Gene : rs no.	Total	Controls	low COX2	high COX2	OR	L95	U95	pTREND	OR	L95	U95	pTREND	OR	L95	U95	pTREND	OR	L95	U95	pTREND
tlr1 : rs4833095	1588	984	331	273	0,70	0,53	0,94	1,597E-02	1,22	0,92	1,63	0,172	1,15	0,88	1,51	0,309	1,18	0,86	1,62	0,310
tlr1 : rs5743594	1588	984	331	273	0,62	0,49	0,78	3,160E-05	1,17	0,94	1,47	0,156	0,93	0,65	1,32	0,687	0,97	0,64	1,48	0,902
tlr1 : rs5743611	1588	984	331	273	0,60	0,49	0,74	2,200E-06	1,12	0,91	1,39	0,281	0,59	0,30	1,17	0,130	1,24	0,64	2,40	0,528
tlr2 : rs4696480	1588	984	331	273	0,60	0,44	0,83	1,878E-03	1,31	0,96	1,78	0,087	1,00	0,77	1,29	0,981	1,13	0,83	1,53	0,450
tlr2 : rs11938228	1588	984	331	273	0,62	0,47	0,82	6,558E-04	1,15	0,88	1,51	0,293	0,98	0,75	1,27	0,865	0,92	0,67	1,26	0,608
tlr2 : rs3804099	1588	984	331	273	0,62	0,45	0,85	2,678E-03	1,17	0,84	1,62	0,349	1,02	0,78	1,33	0,885	1,27	0,93	1,74	0,128
tlr2 : rs3804100	1588	984	331	273	0,65	0,52	0,80	5,360E-05	1,08	0,88	1,34	0,454	1,09	0,68	1,74	0,714	0,79	0,44	1,43	0,432
tlr2 : rs7656411	1588	984	331	273	0,60	0,45	0,80	4,185E-04	1,08	0,81	1,43	0,608	0,98	0,74	1,29	0,878	1,01	0,73	1,40	0,929
tlr4 : rs4986790	1588	984	331	273	0,61	0,50	0,76	4,860E-06	1,11	0,90	1,37	0,348	0,97	0,54	1,76	0,925	1,25	0,65	2,40	0,500
tlr4 : rs11536889	1588	984	331	273	0,61	0,49	0,77	3,380E-05	1,20	0,95	1,51	0,122	0,71	0,49	1,04	0,082	0,97	0,63	1,49	0,885
tlr4 : rs11536897	1588	984	331	273	0,67	0,54	0,82	1,384E-04	1,11	0,90	1,37	0,339	1,29	0,78	2,12	0,319	1,13	0,62	2,05	0,693
tlr4 : rs11536898	1588	984	331	273	0,64	0,51	0,80	8,120E-05	1,13	0,90	1,41	0,299	1,03	0,69	1,52	0,901	1,07	0,67	1,69	0,784
tlr4 : rs1554973	1588	984	331	273	0,65	0,51	0,84	9,603E-04	1,09	0,84	1,40	0,525	1,07	0,80	1,43	0,652	0,97	0,68	1,38	0,863
tlr4 : rs1927906	1588	984	331	273	0,65	0,52	0,80	7,370E-05	1,10	0,88	1,37	0,396	1,22	0,79	1,90	0,368	1,03	0,61	1,76	0,902
tlr4 : rs2737191	1588	984	331	273	0,60	0,46	0,78	1,468E-04	1,18	0,90	1,54	0,229	1,07	0,81	1,42	0,618	1,27	0,92	1,76	0,149
tlr4 : rs5030717	1588	984	331	273	0,61	0,49	0,76	9,110E-06	1,10	0,89	1,36	0,376	0,80	0,53	1,22	0,308	0,59	0,34	1,02	0,059
tlr6 : rs3775073	1588	984	331	273	0,59	0,45	0,78	2,323E-04	1,28	0,98	1,67	0,073	0,91	0,69	1,19	0,471	0,95	0,69	1,30	0,732
tlr6 : rs5743810	1588	984	331	273	0,63	0,47	0,84	1,622E-03	0,94	0,70	1,26	0,676	0,95	0,73	1,25	0,723	0,80	0,58	1,10	0,171
tlr7 : rs1634319	1588	984	331	273	0,64	0,52	0,79	2,470E-05	1,12	0,91	1,38	0,285	0,95	0,70	1,29	0,746	0,78	0,52	1,17	0,233
tlr7 : rs1634320	1588	984	331	273	0,64	0,52	0,79	2,890E-05	1,13	0,91	1,39	0,267	1,07	0,77	1,49	0,683	1,07	0,73	1,57	0,730
tlr7 : rs1634322	1588	984	331	273	0,66	0,53	0,83	3,072E-04	1,17	0,93	1,47	0,171	1,04	0,83	1,30	0,719	1,05	0,80	1,36	0,744
tlr7 : rs179007	1588	984	331	273	0,61	0,49	0,78	4,230E-05	1,10	0,88	1,38	0,408	0,97	0,78	1,20	0,772	0,84	0,64	1,11	0,218
tlr7 : rs179008	1588	984	331	273	0,51	0,41	0,64	1,050E-08	1,02	0,82	1,28	0,842	0,72	0,56	0,92	0,009	0,92	0,70	1,22	0,561
tlr7 : rs179010	1588	984	331	273	0,72	0,57	0,91	6,406E-03	1,21	0,95	1,54	0,116	1,17	0,95	1,43	0,139	1,22	0,96	1,54	0,107
tlr7 : rs179012	1588	984	331	273	0,51	0,40	0,65	5,930E-08	1,00	0,79	1,27	0,974	0,81	0,65	1,00	0,052	0,90	0,70	1,15	0,394
tlr7 : rs179014	1588	984	331	273	0,70	0,56	0,87	1,405E-03	1,18	0,94	1,49	0,143	1,08	0,86	1,35	0,521	1,18	0,91	1,53	0,203
tlr7 : rs5741880	1588	984	331	273	0,67	0,54	0,83	2,314E-04	1,22	0,98	1,51	0,077	1,02	0,79	1,32	0,882	1,11	0,83	1,49	0,490
tlr7 : rs864058	1588	984	331	273	0,64	0,51	0,79	2,990E-05	1,16	0,94	1,43	0,178	1,03	0,78	1,35	0,849	0,94	0,67	1,33	0,747
tlr9 : rs352139*	1556	965	325	266	0,75	0,54	1,04	8,079E-02	1,04	0,76	1,44	0,794	1,17	0,88	1,57	0,280	0,84	0,61	1,17	0,301
tlr9 : rs352140*	1556	965	325	266	0,75	0,53	1,04	8,737E-02	1,06	0,77	1,47	0,705	1,20	0,90	1,61	0,223	0,86	0,62	1,20	0,369
tlr9 : rs352143	1588	984	331	273	0,58	0,46	0,75	2,290E-05	1,04	0,81	1,35	0,742	0,86	0,63	1,17	0,341	1,10	0,78	1,56	0,591
tlr9 : rs352162	1588	984	331	273	0,69	0,50	0,95	2,117E-02	1,04	0,76	1,44	0,794	1,05	0,80	1,39	0,721	0,90	0,65	1,23	0,499
tlr9 : rs4082828	1588	984	331	273	0,60	0,49	0,75	6,590E-06	1,10	0,88	1,37	0,410	0,87	0,62	1,23	0,438	1,00	0,66	1,50	0,989

LRT = likelihood ratio test; OR = odds ratio; rs = reference number for SNPs; SNP = single nucleotide polymorphism

* From Illumin Infinium 1M genotyping platform (selected *a posteriori*)

[a] The ''Global'' LRT tests for a genetic association with any bladder cancer subphenotype.

[b] The ''Hetero'' LRT tests for heterogeneity in risk estimates between both subphenotypes.

Supplementary Table 5.3 (cont'd)

					Interaction: TLR9 rs187084 and SNPs in other TLR genes									
Additive MOI	N = 1588				low COX2 tumors				high COX2 tumors				LRT	
Gene : rs no.	Total	Controls	low COX2	high COX2	OR	L95	U95	pTREND	OR	L95	U95	pTREND	Global[a]	Hetero.[b]
tlr1 : rs4833095	1588	984	331	273	0,86	0,65	1,15	0,308	0,90	0,68	1,20	0,464	0,528	0,806
tlr1 : rs5743594	1588	984	331	273	1,06	0,73	1,54	0,744	0,87	0,59	1,28	0,471	0,674	0,393
tlr1 : rs5743611	1588	984	331	273	1,56	0,85	2,88	0,153	1,03	0,58	1,84	0,910	0,352	0,259
tlr2 : rs4696480	1588	984	331	273	1,05	0,79	1,39	0,732	0,84	0,64	1,11	0,226	0,379	0,201
tlr2 : rs11938228	1588	984	331	273	1,03	0,77	1,36	0,858	0,97	0,73	1,28	0,809	0,942	0,731
tlr2 : rs3804099	1588	984	331	273	1,03	0,78	1,36	0,847	0,97	0,73	1,28	0,808	0,938	0,721
tlr2 : rs3804100	1588	984	331	273	0,85	0,50	1,45	0,547	1,33	0,78	2,26	0,297	0,381	0,173
tlr2 : rs7656411	1588	984	331	273	1,08	0,80	1,46	0,621	1,08	0,80	1,46	0,628	0,827	0,996
tlr4 : rs4986790	1588	984	331	273	1,28	0,69	2,37	0,433	1,21	0,67	2,20	0,527	0,676	0,881
tlr4 : rs11536889	1588	984	331	273	1,11	0,75	1,64	0,612	0,83	0,56	1,23	0,353	0,491	0,247
tlr4 : rs11536897	1588	984	331	273	0,62	0,34	1,15	0,128	1,14	0,67	1,96	0,625	0,195	0,087
tlr4 : rs11536898	1588	984	331	273	0,95	0,62	1,45	0,795	1,02	0,67	1,54	0,941	0,956	0,782
tlr4 : rs1554973	1588	984	331	273	0,93	0,67	1,29	0,676	1,09	0,78	1,50	0,623	0,754	0,453
tlr4 : rs1927906	1588	984	331	273	0,89	0,54	1,45	0,636	1,16	0,72	1,89	0,536	0,659	0,363
tlr4 : rs2737191	1588	984	331	273	1,10	0,82	1,49	0,521	0,94	0,70	1,27	0,709	0,695	0,402
tlr4 : rs5030717	1588	984	331	273	1,18	0,75	1,88	0,473	1,16	0,70	1,94	0,557	0,707	0,957
tlr6 : rs3775073	1588	984	331	273	1,10	0,82	1,47	0,511	0,82	0,61	1,12	0,211	0,276	0,117
tlr6 : rs5743810	1588	984	331	273	1,00	0,74	1,34	0,989	1,29	0,96	1,74	0,091	0,216	0,164
tlr7 : rs1634319	1588	984	331	273	0,94	0,67	1,32	0,707	1,06	0,74	1,52	0,765	0,862	0,589
tlr7 : rs1634320	1588	984	331	273	0,89	0,62	1,28	0,540	1,02	0,73	1,43	0,898	0,790	0,533
tlr7 : rs1634322	1588	984	331	273	0,91	0,71	1,15	0,424	0,93	0,73	1,18	0,555	0,668	0,862
tlr7 : rs179007	1588	984	331	273	1,05	0,84	1,32	0,668	1,06	0,84	1,35	0,629	0,846	0,951
tlr7 : rs179008	1588	984	331	273	1,63	1,27	2,09	1,221E-04	1,27	0,99	1,63	0,061	4,642E-04	0,100
tlr7 : rs179010	1588	984	331	273	0,79	0,63	0,99	0,040	0,89	0,72	1,11	0,304	0,100	0,365
tlr7 : rs179012	1588	984	331	273	1,42	1,14	1,77	0,002	1,23	0,98	1,53	0,075	0,005	0,282
tlr7 : rs179014	1588	984	331	273	0,74	0,56	0,97	0,030	0,91	0,72	1,16	0,443	0,082	0,191
tlr7 : rs5741880	1588	984	331	273	0,84	0,63	1,12	0,230	0,80	0,61	1,06	0,126	0,209	0,806
tlr7 : rs864058	1588	984	331	273	0,97	0,72	1,31	0,857	0,91	0,66	1,26	0,572	0,851	0,740
tlr9 : rs352139*	1556	965	325	266	0,87	0,63	1,19	0,368	0,92	0,67	1,26	0,599	0,637	0,768
tlr9 : rs352140*	1556	965	325	266	0,92	0,68	1,25	0,587	0,91	0,67	1,25	0,567	0,781	0,973
tlr9 : rs352143	1588	984	331	273	1,18	0,85	1,65	0,325	1,20	0,88	1,66	0,254	0,408	0,929
tlr9 : rs352162	1588	984	331	273	0,86	0,63	1,16	0,318	1,04	0,77	1,41	0,791	0,527	0,300
tlr9 : rs4082828	1588	984	331	273	1,29	0,76	2,22	0,348	1,56	0,93	2,61	0,092	0,215	0,560

LRT = likelihood ratio test; OR = odds ratio; rs = reference number for SNPs; SNP = single nucleotide polymorphism

* From Illumin Infinium 1M genotyping platform (selected *a posteriori*)

[a] The ''Global'' LRT tests for a genetic association with any bladder cancer subphenotype.

[b] The ''Hetero'' LRT tests for heterogeneity in risk estimates between both subphenotypes.

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CHAPTER 6. SUMMARY AND GENERAL DISCUSSION

6.1. Introduction

UCB is a complex disease with both environmental and genetic factors contributing to its etiology. Similarly, induction and resolution of the inflammatory response is dependent on the interplay of an assortment of immunoregulatory factors in the host and, in the case of infection, in the pathogen as well. Consequently, studies investigating the complex relationship between bladder carcinogenesis and chronic inflammation should undertake a multi-faceted approach in which environmental, molecular and genetic factors are all considered. It was within the aim of this thesis to further clarify and broaden the current body of knowledge linking chronic inflammation and UCB by evaluating environmental, molecular and genetic factors associated, to various degrees, with both of these conditions.

The experiments described in chapters 3 and 4 of this thesis have attempted to reevaluate long-standing questions regarding the association between UCB risk and UTIs, and the prognostic utility of COX2 tumor expression in the disease, respectively. While numerous studies have tackled these issues to various extents, the larger patient sample size together with more detailed medical and epidemiologic data afforded by the Spanish Bladder Cancer/EPICURO Study enabled for a more in-depth and precise evaluation than has been reported in the literature to date. In contrast, the studies undertaken in chapter 5 analyzed an as of yet unexamined relationship between germline genetic variation in inflammatory genes and susceptibility to UCB subgroups delimited by their level of COX2 expression; with particular emphasis placed on how this information could improve patient responsiveness to BCG immunotherapy. The following section briefly summarizes these studies, their main findings and how they compared to the original hypotheses outlined in chapter 2.

6.2. Summary of presented work

In chapter 3 a case-control analysis was used to evaluate the widely studied yet inconclusive notion that inflammation resulting from uropathogensis contributes to elevating UCB risk. Self-

reported patient information regarding the frequency of lifetime bladder infections was collected along with information regarding patient use of UTI medications and the urinary tract analgesic phenazopyridine. Nearly a 50% reduction in UCB risk was observed in patients who reported using medication to treat episodes of UTI compared to uninfected individuals (OR=0.51; 95% CI=0.37-0.70); while non-medicating patients exhibited a non-significant increase in UCB risk (OR=1.3; 95% CI=0.61-2.79). Interestingly, the reduced UCB risk was most prominent in patients who reported using phenazopyridine (OR= 0.35; 95% CI=0.20-0.62). A reduction in UCB risk in patients who medicated to treat episodes of UTI is consistent with the experimentally-based hypothesis that some commonly administered UTI medications have antitumorigenic properties (Kamat and Lamm, 2004), as is the increased risk seen in non-medicating patients. That phenazopyridine, an azo-based compound, would protect against UCB risk falls in contrast to our hypothesis based on the uro-carcinogenic properties of this class of molecules (Gonzales et al., 1988).

In chapter 4 the largest and most comprehensive evaluation to date of the independent prognostic utility of COX2 tumor expression in UCB was undertaken. Moreover, an attempt was made to homogenize and compare the array of data already available on the topic through a systematic review of the literature and a meta-analysis. Using follow-up information collected over a period of nearly ten years for almost 800 UCB patients with available tumor tissue, COX2 expression was evaluated for its association with recurrence and progression of NMIBC, and progression and survival of patients with MIBC. COX2 expression was not a significant independent prognostic marker of any of the endpoints considered; a finding that was corroborated by the results from the meta-analysis. This finding contrasts the original hypothesis that COX2 expression is a biomarker of poor prognosis in UCB given its overexpression in an array premalignant lesions and tumors (Subbaramaiah and Dannenberg, 2003) and correlation with tumor invasiveness and high tumor grade in UCB.

In chapter 5, the association between genetic aberrations in inflammatory genes and the risk of UCB subtypes defined by their level of COX2 expression was evaluated. Two SNPs, rs184087 and rs352139, found within the promoter and first intron, respectively, of the gene encoding the innate immune response receptor TLR9, exhibited heterogeneity in genetic susceptibility for the two cancer subtypes (rs184087, P_{het}= 1.47E-06; rs312139, P_{het}= 1.67E-06) . Patients harboring the variant allele of

rs187084 were at a reduced risk of COX2-negative UCB (OR=0.63, 95% CI 0.52-0.77); the reverse was true of patients harboring variants of rs352139 who observed an increased risk of COX2-negative UCB (OR=1.44, 95% CI 1.19-1.74) and a decreased risk of COX2-positive UCB (OR=0.81, 95% CI 0.66-0.99). A history of bladder infections was not observed to influence these associations. The variant allele of rs187084 also conferred a reduced rate of tumor progression in patients with COX2-negative tumors, but only in those treated with BCG instillation therapy (HR=0.39, 95% CI=0.16-0.94), suggesting it may be an independent predictive marker of BCG response for these patients. While, *a priori* we expected to identify some variants in inflammatory genes that conferred differential susceptibility to tumor subtypes defined by COX2 expression given the role of COX2 and PGE2 in inflammation, cancer and immunity (Greenhough *et al.*, 2009), it was unexpected that one gene (*TLR9*) would factor so prominently in both the UCB risk and prognostic assessments.

6.3 Clinical and public health implications of presented work

6.3.1 Antibiotics and UCB prophylaxis

Mounting experimental evidence indicates that commonly administered antibiotics used to treat UTIs also confer cytotoxicity against UCB cells by means that, although not completely understood, reduce cellular proliferation and block apoptosis (Kamat and Lamm, 2004). The analyses described in chapter 3 of this thesis are the first to corroborate this experimental evidence in a large study. Together with data from experimental studies, these results provide a strong basis for the introduction of certain antibiotics in the prophylactic arsenal against UCB (Gurtowska et al., 2010). Antibiotic prophylaxis is already used (discretionary) at the start of various urologic surgical interventions including TUR to minimize post-operative infectious complications (Bootsma *et al.*, 2008). However, prolonged antibiotic regimens can be associated with various side-effects that range from minor gastrointestinal discomfort to serious cardiotoxicity (De Sarro and De Sarro, 2001), and the potential of developing microbial resistance to certain pathogens is also a very real concern. Consequently, these risks must be weighed against the benefits of a potential reduction in UCB risk. Therefore, antibiotic prophylaxis may be best suited for high-risk patients such as smokers with the *NAT2*-slow acetylator phenotype who have a high potential of developing UCB and would benefit

most from an antibiotic regimen as a means of primary prevention despite the risks associated with prolonged usage. Moreover, patients already diagnosed with low-grade NMIBC could also benefit from such a drug regimen as a means of reducing their risk of tumor progression.

The finding that phenazopyridine use was associated with a further reduction in UCB risk went contrary to our original hypothesis given the uro-carcinogenic nature of azo-dyes. Long term local exposure (40 or 52 weeks) of phenazopyridine in mouse bladders has been reported to induce tumors in a small proportion of mice (Allen *et al.*, 1957). While long-term exposure to phenazopyridine in humans may similarly be tumorigenic, phenazopyridine use in UTI treatment is generally short-term and not recommended for periods of more than two days (Zelenitsky and Zhanel, 1996). To date, no association between phenazopyridine use and neoplasia has been reported despite side-effects that may include myo- and nephrotoxicity (Lin, 2008). As the nature of the potential protective mechanism proposed for this association in chapter 3 is highly speculative and the association could arise from uncontrolled confounding, extreme caution is advised along with replication and corroboration of this finding in experimental and large observational studies before any prophylactic measures are considered.

6.3.2 *TLR9*, COX2 and UCB risk and therapy

The highest levels of COX2 protein expression in UCB are generally noted in MIBCs which are characterized by poor overall prognosis. Therefore, the availability of biomarkers identifying individuals based on their susceptibility to COX2-positive UCB would provide a means to take early preventive measures for this high-risk group. This could entail changes in lifestyle to reduce potential exposure to UCB risk factors (e.g. ceasing smoking or leaving a high-risk occupation) and/or initiation of a long-term chemotherapeutic regimen, such as low-dose aspirin or NSAIDs, to reduce UCB risk and keep COX2 activity in check (Baris *et al.*, 2013). In chapter 5 of this thesis, *TLR9* was observed to harbor several SNPs (*TLR*-rs197084, -rs352139, -rs352140) shown to predict heterogeneity in genetic susceptibility to COX2-positive or -negative UCB. Variants of *TLR9*-rs352139 (in linkage disequilibrium with *TLR9*-rs352140) conferred increased susceptibility for COX2-negative tumors and reduced the risk of COX2-positive tumors. Thus, WT homozygous patients could be considered for

prolonged NSAID therapy, while those harboring the variant alleles may consider not initiating such a regimen given the cardiovascular complications associated with prolonged NSAID use (Marnett, 2009).

BCG instillation is the most effective adjuvant therapy for high-grade NMIBC, but 30-50% of patients do not respond to treatment and 15% suffer from tumor progression to MIBC (Zuiverloon *et al.*, 2012). No reliable biomarkers are currently available that can predict patient response to BCG instillation but a urinary cytokine profile favoring a Th1 immune response (e.g. IL2, IL12, IFN-g, TNF-B) is generally associated with successful therapy. *TLR9*-rs187084 has the potential to fill to this void in BCG predictive markers when considered together with COX2 expression levels in NMIBCs; both factors predict positive BCG response characterized by decreased tumor progression independently of conventional clinicopathological parameters. Although it is unclear how *TLR9*-rs187084 may affect TLR9 activity, reduced tumor progression following BCG instillation was only observed for COX2-negative tumors suggestive of an immunoregulatory role for tumor COX2, or more likely PGE_2 (Greenhough et al., 2009). This observation brings with it the clinically relevant implication that tumor COX2 levels may confer a tolerogenic effect that influences the BCG induced cytotoxic response. Therefore, patients with COX2-positive tumors may derive benefit from taking NSAIDs before BCG instillation and into the maintenance period. While a recent randomized, double-blind, placebo-controlled clinical trial did not observe a significant difference in recurrence rates when examining BCG response in patients given long-term COXIBs or a placebo, it would be interesting to examine recurrence rates when taking *TLR9*-rs187084 genotype status into consideration in the same trial (Sabichi *et al.*, 2011).

6.4 Considerations

6.4.1 UTIs and chronic inflammation of the bladder

There is strong evidence implicating a role for chronic inflammation in bladder cancer (Michaud, 2007). From an observational study point of view, distinguishing chronic from acute inflammation or from other tumor initiating/promoting factors is not an elementary undertaking. Squamous cell carcinoma of the bladder arises in spinal cord injury patients outfitted with indwelling

catheters and more prevalently in cases of *S. haematobium* infestation. In both of these instances prolonged and continuous irritation of the bladder urothelium, resulting from physical contact with the catheter or *S. haematobium* eggs, is sufficient to induce a chronic inflammatory state (Michaud, 2007). While a slight increase in UCB risk was observed in patients who did not use UTI medication (chapter 3), the results were not significant; potentially due to the small sample size in this stratum. Another explanation may be that the inflammation produced by common bladder pathogens like *E. coli*, even after multiple recurrences, is not *prolonged* nor *continuous* enough (i.e. acute inflammation) to precipitate tumorigenesis as observed during *S. haematobium* infestation or indwelling catheter use (i.e. chronic inflammation). This is iterated by the observation that bladder cancer risk factors that produce prolonged and continuous urothelial irritation, such as *S. haematobium* infestation, indwelling catheter use and certain urogenital pathologies (e.g. calculi and BPH), also have a tendency to increase the risk of concurrent chronic bacterial infections in the bladder which in turn contribute to tumorigenesis (Michaud, 2007). Thus, the determining factor in the association between UTI and UCB risk may be the length of time uropathogens are continuously exposed to the urothelium, and whether or not they were treated with UTI medication.

Alternatively, chronic irritation of the bladder in humans may only lead to SCC, and not UCB. In an analysis of patients with UCB, SCC and adenocarcinomas of the bladder, Kantor et al reported that individuals with a high frequency of urinary tract infections had a significantly elevated risk of SCC (relative risk=5.7) (Kantor *et al.*, 1988). Given that no SCC patients were included in the analyses presented in this thesis, it would lend an explanation as to why the study in chapter 3 did not observe any meaningful association between UCB risk and infection in individuals who did *not* use UTI medication.

6.4.2 Linking chronic inflammation, COX2 expression and bladder cancer

Under normal physiological conditions the prostaglandin products of COX2 expression are involved in mediating the inflammatory response induced by both endogenous and exogenous stimuli such as proinflammatory cytokines, pathogens and growth factors. However, aberrant overexpression of COX2 can maintain persistent inflammation in both premalignant and malignant lesions and promote

tumorigenesis by blocking apoptosis and accelerating cell proliferation and angiogenesis (Kundu and Surh, 2008). While COX2 appears to play an instrumental role in bladder carcinogenesis, not all facets of how it functions to link chronic inflammation and tumor development, particularly in UCB, are clear.

In histological variants of bladder cancer with a well established chronic inflammatory etiology such as squamous cell carcinoma, tumor COX2 expression is substantially elevated in comparison to UCB (Shirahama and Sakakura, 2001). Moreover, factors that elicit an inflammatory response in the bladder, such as cyclophosphamide induced cystitis, also increase urothelial COX2 expression; but similar levels of expression are obtained regardless of whether the exposure induces acute or chronic cystitis (Klinger et al., 2007). These findings suggest that COX2 expression in UCB may not be solely related to the presence of chronic inflammation in the bladder. This was shown to be the case in Barrett's esophagus, a premalignant condition of the esophageal cells in which COX2 levels are increased, but independently of the degree of inflammation (Abdalla et al., 2005).

Alternatively, aside from inflammatory stimuli, COX2 expression in UCB may be dependent on tumor-specific factors. When considering tumor lymphocyte infiltration as a marker of inflammation, Cai et al did not observe a significant difference ($p>0.05$) in the degree of inflammation between NMIBC and MIBC (Cai *et al.*, 2006). However, as reported in chapter 4 of this thesis, COX2 protein expression is significantly elevated in MIBC compared to NMIBC. In contrast to NMIBC, MIBC are characterized by a high degree of genomic instability and genetic aberrations in tumor suppressor genes like *TP53* (Netto, 2012). Genomic instability could affect the molecular players involved in COX2 signalling pathways, resulting in *COX2* upregulation observed in MIBC. In fact mutations in *TP53* have been reported to result in a protein product incapable of binding to the TATA motif of COX2 mRNA resulting in stabilization and increased expression of COX2 (Subbaramaiah *et al.*, 1999). Thus, in a pathological state like UCB, factors intrinsic to the tumor microenvironment may have a supporting or even prominent role in shaping the influence of COX2 in contrast to tumor with a more prominent inflammatory etiology such as SCC

6.5 Strengths and limitations of the study

The patient data used in most of the analyses detailed in this thesis was collected as part of the SBC/EPICURO study, a hospital-based case-control study. This study benefitted from a large patient sample size and extensive and thoroughly collected patient information that was further strengthened by very high response rates of 84% in cases and 88% in controls. The clinical data collected from patients was of high quality and all tumor samples were uniformly classified by expert pathologists to reduce the possibility of tumor misclassification. Good agreement was observed between all platforms used to genotype patient DNA ensuring the accuracy of the genetic data. While COX2 expression was assessed using quantitative image analysis software instead of a pathologist, this approach was highly correlated with a pathologist's assessment on a small set of tissue cores and, as a result of its automated nature, is very reproducible.

A case-control approach is ideal for studying associations in rare multifactorial diseases like cancer and allows for the inclusion of a large number of cases in a relatively small patient sample (relative to cohort studies), while providing the opportunity to study a variety of different exposures (important for multifactorial diseases like UCB). Although a convenient means of selecting cases and controls, hospital-based studies have the limitation that not all factors assessed can be generalized to the whole population; with one study in Spain indicating that hospital controls have elevated alcohol consumption in comparison to the general population (Ruano-Ravina et al., 2008) – a factor not evaluated in this thesis. Moreover, as information is collected retrospectively by patient interview, case-control studies (like other observational studies) are susceptible to bias.

Recall bias occurs when cases, as a result of their disease status, consistently over or under report exposure in comparison to controls. This type of bias can lead to differential misclassification in which the true effect estimate for an association is amplified away from unity. Recall bias may have influenced the urinary tract exposures evaluated in chapter 3; however its effect was probably marginal given that an inverse association was observed between cystitis and UCB risk. As the history of urinary tract conditions was self-reported, a greater concern is the misclassification of symptoms of cystitis with the early manifestation of UCB; especially given the overlap of symptoms between both

pathologies. To reduce the effects of this type of misclassification, time of last infection relative to tumor diagnosis was taken into account with patients who reported presenting with symptoms of cystitis within a year of diagnosis excluded from the analyses (chapter 3).

Confounding can occur when a factor related to the disease of interest and associated with another exposure, distorts the magnitude of the association between that exposure and the disease. Although the possibility of uncontrolled confounding is always an issue in observational studies, potential confounders were included in multivariable models or examined by stratification.

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CONCLUSIONS

1.) These results do not support an association between bladder infections or other urinary tract conditions and increased UCB risk.

    a. Use of UTI medication to treat episodes of infection is associated with a reduced risk of UCB that correlates with the frequency of infection.

    b. Use of the azo-based urinary tract analgesic phenazopyridine is associated with reduced UCB risk; a reduction in risk that is not associated with the frequency of treatment with this drug.

2.) Tumor COX2 expression is associated with UCB T-stage and G-grade in low- and high-risk NMIBC/MIBC tumors.

3.) Tumor COX2 expression is not independently associated with tumor recurrence and progression in NMIBC patients, nor with progression and disease-free survival in MIBC patients.

4.) Germline variants in the innate immune response gene, *TLR9*, confer heterogeneity in susceptibility to UCB subgroups defined by levels of COX2 expression in tumors.

    a. *TLR9*-rs187084, located in the 5' promoter region, is associated with a reduced risk of COX2-negative UCB.

    b. *TLR9*-rs352139, located within the first intron, is associated with an increased risk of COX2-negative UCB and a reduced risk of COX2-positive UCB.

5.) The variant allele of *TLR9*-rs187084 is an independent predictor of improved progression-free survival time in patients with COX2-negative NMIBC who undergo BCG immunotherapy.

CONCLUSIONES

1.) Los resultados de esta tesis no apoyan la asociación entre las infecciones del tracto urinario (ITU), u otras enfermedades del tracto urinario, y un aumento de riesgo de CUV.

   a. El uso de medicamentos para tratar la ITU se asocia con un riesgo reducido de CUV que se correlaciona con la frecuencia de las infecciones.

   b. El uso de fenazopiridina, un analgésico del tracto urinario, se asocia con una reducción de riesgo de CUV; esta asociación no se correlaciona con la frecuencia del tratamiento.

2.) La expresión tumoral de COX2 se asocia con los valores histológicos de T-estadio y G-grado en tumores no músculo invasivos de bajo riesgo, y en tumores no músculo invasivo de alto riesgo/tumores invasivos.

3.) La expresión tumoral de COX2 no se asoció independientemente con la recurrencia y progresión en en tumores no músculo invasivos ni con progresión y la supervivencia de los pacientes con tumores invasivos.

4.) Variantes en el gen que codifica el receptor de la inmunidad innata, TLR9, confirieron heterogeneidad en la susceptibilidad a CUV.

   a. *TLR9*-rs187084, localizado en la región promotora, se asoció con un riesgo reducido de los tumores negativos para COX2.

   b. *TLR9*-rs352139, ubicado en el primer intrón, se asoció con un riesgo aumentado de los tumores negativos para COX2 pero con un riesgo reducido de los tumores positivos para COX2.

5.) El alelo variante de TLR9-rs187084 confirió, de manera independiente, una tasa reducida de progresión tumoral en pacientes con tumores negativos para COX2 en los pacientes tratados con inmunoterapia de BCG.

APPENDIX

SPANISH BLADDER CANCER/EPICURO STUDY INVESTIGATORS

Institut Municipal d'Investigació Mèdica, Universitat Pompeu Fabra, Barcelona – Coordinating 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).

PUBLICATIONS RELEVANT TO THE THESIS

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Cyclooxygenase-2 Expression in Bladder Cancer and Patient Prognosis: Results from a Large Clinical Cohort and Meta-Analysis

Maciej J. Czachorowski[1], André F. S. Amaral[1], Santiago Montes-Moreno[2], Josep Lloreta[3,4,5], Alfredo Carrato[6,7], Adonina Tardón[8], Manuel M. Morente[9], Manolis Kogevinas[10], Francisco X. Real[5,11], Núria Malats[1]*, for the SBC/EPICURO investigators[¶]

1 Genetic and Molecular Epidemiology Group, Spanish National Cancer Research Centre (CNIO), Madrid, Spain, **2** Servicio de Anatomía Patológica, Hospital Universitario Marqués de Valdecilla, Santander, Spain, **3** Institut Municipal d'Investigació Mèdica – Hospital del Mar, Barcelona, Spain, **4** Departament de Patologia, Hospital del Mar – Parc de Salut Mar, Barcelona, Spain, **5** Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Barcelona, Spain, **6** Hospital General Universitario de Elche, Elche, Spain, **7** Hospital Ramon y Cajal, Madrid, Spain, **8** Universidad de Oviedo, Oviedo, Spain, **9** Tumor Bank Unit, Spanish National Cancer Research Centre (CNIO), Madrid, Spain, **10** Centre de Recerca en Epidemiologia Ambiental (CREAL), Barcelona, Spain, **11** Epithelial Carcinogenesis Group, Spanish National Cancer Research Centre (CNIO), Madrid, Spain

Abstract

Aberrant overexpression of cyclooxygenase-2 (COX2) is observed in urothelial carcinoma of the bladder (UCB). Studies evaluating COX2 as a prognostic marker in UCB report contradictory results. We determined the prognostic potential of COX2 expression in UCB and quantitatively summarize the results with those of the literature through a meta-analysis. Newly diagnosed UCB patients recruited between 1998–2001 in 18 Spanish hospitals were prospectively included in the study and followed-up (median, 70.7 months). Diagnostic slides were reviewed and uniformly classified by expert pathologists. Clinical data was retrieved from hospital charts. Tissue microarrays containing non-muscle invasive (n = 557) and muscle invasive (n = 216) tumours were analyzed by immunohistochemistry using quantitative image analysis. Expression was evaluated in Cox regression models to assess the risk of recurrence, progression and disease-specific mortality. Meta-hazard ratios were estimated using our results and those from 11 additional evaluable studies. COX2 expression was observed in 38% (211/557) of non-muscle invasive and 63% (137/216) of muscle invasive tumors. Expression was associated with advanced pathological stage and grade ($p<0.0001$). In the univariable analyses, COX2 expression - as a categorical variable - was not associated with any of the outcomes analyzed. As a continuous variable, a weak association with recurrence in non-muscle invasive tumors was observed (p-value = 0.048). In the multivariable analyses, COX2 expression did not independently predict any of the considered outcomes. The meta-analysis confirmed these results. We did not find evidence that COX2 expression is an independent prognostic marker of recurrence, progression or survival in patients with UCB.

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* E-mail: nmalats@cnio.es

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Introduction

Urothelial carcinoma of the bladder (UCB) is the most common bladder cancer type in developed nations [1]. UCB predominantly manifests (70–80% of patients) as a non-muscle invasive tumor (NMIBC: pTa-pT1) characterized by an overall good prognosis following transurethral resection in patients with low-grade tumors (pTaG1/2), and intravesical chemotherapy and/or Bacillus Calmette Guerin (BCG) instillation in patients with high-grade tumors (pTaG3 or pT1G2/3) [2]. Approximately 70% of NMIBC patients suffer a recurrence following treatment and a further 15% progress, developing new tumors exhibiting muscle invasion (MIBC: pT2-pT4); the risk of progression being higher among patients with high-grade tumors [2]. Due to a high rate of recurrence and the need for close follow-up over a patient's lifetime, UCB remains one of the most expensive tumors to treat on a per patient basis [3]. A lower proportion (20–30%) of UCB patients are diagnosed with muscle invasive tumors (MIBC; pT2-pT4) characterized by poor prognosis: 50% of these patients die from their cancer [2]. Genomic profiling and gene expression analyses indicate a strong correlation between these pathologic classifications and the underlying molecular architecture of UCB [4].

Growing evidence indicates that chronic inflammation may increase the risk of UCB [5]. Studies investigating the prolonged use of cyclooxygenase-2 (COX2) inhibiting non-steroidal anti-inflammatory drugs (NSAIDs) have reported a decrease in UCB risk [6,7]. COX2 is a prostaglandin endoperoxide synthetase that catalyzes the production of prostanoids upon induction by proinflammatory cytokines, growth factors, tumor promoters and other external stimuli [8]. COX2 activation mediates cellular processes also implicated in carcinogenesis such as angiogenesis, cell survival/proliferation and apoptosis [9]. Moreover, studies have shown that bladder tissue from patients with cystitis or UCB exhibits elevated COX2 levels in contrast to benign bladder tissue [10,11].

While numerous groups have investigated the prognostic potential of COX2 expression in UCB [12–31], there is no clear consensus on its utility. The objective of this study was to assess whether COX2 protein expression in UCB cells is associated with prognosis using a large and standardized cohort of newly diagnosed bladder cancer patients. A meta-analysis was also done to summarize these results together with those from other studies published on the topic.

Materials and Methods

Study Population

A total of 773 newly diagnosed UCB cases aged 22–80 years (mean ± SD = 66±10 yrs) with a median follow-up of 70.7 months (range 0.7–117.7 months) and available tumor tissue were used in the current analysis. All cases were recruited between 1998 and 2001 from 18 hospitals in five regions of Spain as part of the Spanish Bladder Cancer (SBC)/EPIdemiology of Cancer of the UROthelium (EPICURO) study, a hospital-based case-control study described previously [32]. A pathologist review panel uniformly classified the T stage and grade (G) of each tumor biopsy according to the criteria of the TNM classification and the WHO-ISUP [33], using the three grade redefinition provided by the WHO [34,35]. All bladder tumor samples used in the study were collected prior to the administration of any intravesical or systemic therapy. Clinical information related to diagnostic procedures, tumor characteristics and treatment was collected from medical records, and a computerized questionnaire was used for the collection of sociodemographic data. NMIBCs were removed by transurethral resection and patients received intravesical chemo- or immunotherapy (i.e. BCG) as appropriate. The majority of patients presenting with MIBCs were treated by radical cystectomy; in cases where surgery was not possible, radiotherapy or systemic chemotherapy were administered. Follow-up information was collected annually from hospital records and through direct telephone interviews by trained monitors using structured questionnaires. Among NMIBCs, recurrence was defined as the appearance of a new NMIBC following a previous negative follow-up cystoscopy, and progression, as the development of a MIBC. In patients initially presenting with MIBCs, any tumor reappearance after treatment was considered progression, regardless of whether the tumor relapse was local or distal. Tumour-specific survival was assessed only for patients with MIBCs. Informed written consent was obtained from study participants in accordance with the Ethics Committees of each participating hospital.

Immunohistochemistry

Tissue blocks of formalin-fixed, paraffin-embedded primary bladder tumors were used to construct tissue microarrays (TMA) containing tumor cores of 0.6-mm in diameter represented in duplicate and selected from the most representative regions of the tumor on which T and G were based. After deparaffinisation and heat-induced antigen retrieval, all slides were stained simultaneously at the Histology and Immunohistochemistry Core Unit of the CNIO using the PT LINK system as per manufacturer's instructions (Dako Inc., Glostrup, Denmark). Briefly, tissue sections were incubated with anti-COX2 rabbit monoclonal antibody (ThermoFisher Scientific, Fremont, CA, USA; #RM-9121-R7; pre-diluted, ready-to-use) at room temperature, followed by visualization using the EnVision Flex Visualization system (Dako Inc., Glostrup, Denmark) and exposure to diaminobenzedine. Tissues were then counterstained with haematoxylin, dehydrated and mounted. A section of colon tissue was used as a positive control.

Evaluation of COX2 Immunostaining

COX2 expression was quantified using the Ariol SL-50 (version 3.1.2, Applied Imaging Corp., San Jose, CA, USA) high-throughput slide imaging scanner. All cores were imaged and processed using a light microscope and the accompanying TMA Multistain Imaging software. The program was trained by a pathologist (SM) to maximize the inclusion of positively stained tumor epithelium while minimizing stromal material, as described previously [36]. COX2 expression score was calculated as the product of the mean intensity of staining (by defining the background and saturation limits of the antibody and imaging sensor, respectively) and the proportion of cellular antibody-positive area divided by total cellular area. Values from replicate cores were averaged to provide a final expression score for each patient. Furthermore, one randomly selected TMA (representing 10% of all cores) was analyzed by direct visual microscopic inspection by an independent pathologist (MMM) to enable comparison with the automated scoring approach. The pathologist-derived score was calculated as the product of COX2 staining intensity (1 = weak, 2 = intermediate, 3 = strong) and a quartile of the percentage of epithelial tumor cells stained (0–4; with 0 representing 0% staining), providing a final categorical score in the range of 0–12. There was a high and significant correlation between the machine and pathologist derived scores (Spearman rho = 0.85; 95%CI = 0.79–0.90; p-value$<$0.00001). COX2 expression was analyzed as both a continuous variable and categorical variables partitioned at the median and extreme tertiles. Additionally, expression was examined as a categorical variable dichotomized at a threshold (0.340 arbitrary units [au]) above which COX2 expression was considered to be *positive*. This expression threshold was derived by comparing the pathologist's (MMM) binary assignment of positive expression (i.e. score of 0 vs. score $\geq$1, as described above) to the machine-derived continuous score using receiver operating characteristic (ROC) curve analysis (area under the curve = 0.95; 86% sensitivity and 92% specificity) [37].

Meta-analysis

The meta-analysis included COX2 expression results from our own series (using the ROC-derived categorical expression variable) and relevant studies published before 1 January 2012 identified by searching PubMed and ISI Web of Knowledge. The search string used was: (cox2 OR cox-2 OR cyclooxygenase-2 OR "cyclooxygenase 2" OR ptgs2) AND (prognos* OR survival OR mortality OR recurrence OR relapse OR progression) AND ("bladder cancer"). Studies were considered eligible if: (i) they reported the effect measure (as HRs, survival curves or log-rank p-values) of COX2 protein expression on recurrence, progression or disease-specific survival; (ii) COX2 was assessed in primary tumors

exhibiting homogeneity in tumor histology (≥75% UCB), and subphenotype (≥75% NMIBC *or* MIBC); (iii) they were written in English or Spanish (Table S1). Reviews, abstracts, non-clinical studies, and duplicate publications were excluded. HRs and 95%CIs were directly extracted from the publications whenever available. For those reporting only the log-rank p-value or the Kaplan–Meier survival curves, the HRs and 95%CIs were independently calculated by two of the co-authors (MJC, AFSA) using the spreadsheet prepared by Sydes and Tierney with any discrepancies resolved by discussion [38]. In a few indicated cases, authors were directly contacted for clarification or provision of data not shown in the published manuscripts (Table S1). The level of heterogeneity among studies was calculated by means of the I^2 statistic [39], and publication bias was assessed by analyzing funnel plots and Egger's asymmetry test [40].

Statistical Analysis

Associations between demographic and clinico-pathological parameters and COX2 expression were assessed using Fisher's exact test. In NMIBCs, expression was also assessed distinctly in low-grade/risk (pTaG1/G2) and high-grade/risk (pTa/pT2G3) tumors, based on our previous evidence suggesting differential prognostic, genetic and molecular profiles between these subgroups [41,42]. Recurrence-free, progression-free, and overall disease-specific survival curves were generated using the Kaplan-Meier method, with statistical significance assessed using the log-rank test. Time to each endpoint was calculated from date of primary treatment to the date of event, date of last follow-up, or date of patient's death. Individuals who did not present any event until the end of the study, those lost to follow-up, or those who died from other causes were censored either at the time of last medical visit or at death. Time to recurrence and progression were defined by applying the "mid-time" between the date of the previous disease-free visit and that when a new event was diagnosed. Survival time was measured as the time from initial treatment to death resulting from cancer. Univariable and multivariable Cox-proportional hazards analysis was used to calculate hazard ratios (HR) and 95% confidence intervals (CI). Schoenfeld residual analysis did not suggest any departure from the proportional hazards assumption in multivariable models.

All statistical analyses were done using STATA (version 10.1 SE, StataCorp, College Station, TX, USA). Statistical tests were two-sided and p-values less than 0.05 were considered significant. The REMARK [43] guidelines for prognostic studies as well as the PRISMA [44] guidelines for systematic reviews and meta-analyses were adhered to in the preparation of the manuscript.

Results

Patients and COX2 Expression in Bladder Cancer TMAs

COX2 expression was assessed in 557 patients with NMIBCs and 216 individuals with MIBCs. Median COX2 expression was 0.121 au (range 0–42.590; interquartile range 1.382) in NMIBCs, and 0.760 au (0–30.806; 3.600) in MIBCs (p-value = 4×10^{-12}). Representative COX2 immunostaining patterns in UCBs are shown in Figure S1. Of patients with NMIBCs, 41% (230/557) were treated only by transurethral resection, with the remainder (56%) receiving endovesical BCG immunotherapy and/or chemotherapy following transurethral resection, or other treatment (3%; Table 1). Nearly half (46%) of patients with MIBCs were treated by cystectomy, with the remainder receiving systemic chemotherapy, radiotherapy, superficial or other treatment, or some combination thereof (Table 2).

Table 1. Distribution of characteristics of patients with NMIBCs by COX2 expression.

		COX2 expression*		
Patient characteristics	**Total, N**	**negative, n**	**positive, n**	**P value†**
	557	346	211	
Area				0,506
Barcelona	98	68	30	
Valles	105	66	39	
Elche	51	32	19	
Tenerife	122	71	51	
Asturias	181	109	72	
Age (yrs.)				0,385
≤60	140	81	59	
>60 and ≤70	210	130	80	
>70	207	135	72	
Gender				0,891
Men	494	306	188	
Women	63	40	23	
Tumor Invasion				<0,0001
Ta	477	277	200	
T1	80	69	11	
Grade				<0,0001
GI	200	131	69	
GII	219	95	124	
GIII	138	120	18	
Low/High Grade				<0,0001
Low (TaG1/TaG2)	408	221	187	
High (TaG3/T1G2/T1G3)	149	125	24	
Number of tumors				0,106
1	348	209	139	
>1	178	120	58	
missing	31	17	14	
Tumour Size				0,564
≤3 cm	294	188	106	
>3 cm	111	67	44	
missing	152	91	61	
Number of Recurrences				0,409
none	366	232	134	
at least 1	191	114	77	
Treatment‡				0,393
TUR	230	133	97	
TUR+BCG	158	105	53	
TUR+Chem.	132	83	49	
TUR+BCG+Chem.	19	14	5	
Other	18	11	7	

*COX2 expression score dichotomised at the threshold of positivitiy (0,340 au).
†Fisher's exact test comparing distribution of COX-2 negative versus positive patients; missing values excluded from analysis where applicable.
‡TUR: transurethral resection; BCG: Bacillus Calmette-Guerin instillation; Chem.: chemotherapy via endovesical instillation.
doi:10.1371/journal.pone.0045025.t001

Table 2. Distribution of characteristics of patients with MIBCs by COX2 expression.

		COX2 expression*		
Patient Characteristics	**Total, N**	**negative, N**	**positive, N**	**P value†**
	216	79	137	
Area				0,207
Barcelona	39	16	23	
Valles	36	9	27	
Elche	15	9	6	
Tenerife	39	14	25	
Asturias	87	31	56	
Gender				0,816
Men	194	72	122	
Women	22	7	15	
Age (yrs.)				0,426
≤60	45	14	31	
>60 and ≤70	84	35	49	
>70	87	30	57	
Tumor invasion				0,896
T2	114	42	72	
T3	55	21	34	
T4	47	16	31	
Grade				0,326
GII	19	9	10	
GIII	197	70	127	
Metastases				0,296
M0	168	57	111	
M1	29	13	16	
Mx	19	9	10	
Lymphatic invasion				0,862
N0	141	50	91	
N1, N3	49	16	33	
Nx	26	13	13	
Number of tumors				0,008
1	146	63	83	
>1	54	12	42	
missing	16	4	12	
Tumour size				0,572
≤3 cm	53	19	34	
>3 cm	66	28	38	
missing	97	32	65	
Treatment‡				0,417
Cystectomy	67	19	48	
Cystectomy+Chem.	32	15	17	
Chem. only	23	9	14	
RT +/− Chem.	19	7	12	
Superficial Treatment	13	3	10	
Others	61	25	36	
missing	1	1	0	

*COX2 expression score dichotomised at the threshold of positivity (0,340 au).
†Fisher's exact test comparing distribution of patients with negative or positive COX-2 expression; missing, Nx and Mx values excluded from analysis where applicable.
‡Chem.: Systemic chemotherapy; RT: Radiation therapy.
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COX2 Expression and Clinicopathological Features

Two-hundred eleven (38%) NMIBCs and 137 (58%) MIBCs expressed COX2 (Tables 1 and 2, respectively), with positive expression defined as a score equal to or greater than the ROC-derived threshold of 0.340 au. Patient and tumor characteristics in the analyzed sample did not differ significantly from the initial SBC/EPICURO study population with the exception of geographic region and tumor size in NMIBC patients (data not shown). The distribution of COX2 positivity was assessed according to established bladder cancer prognosticators including tumor invasion and grade, tumor multiplicity, tumor size and treatment, among others. Demographic factors like age, gender and region were not associated with COX2 expression, nor was the type of primary treatment received by patients (Tables 1 and 2). In NMIBCs, COX2 expression was significantly associated only with T and G; being more prominent in low-grade/risk pTaG1/2 tumors than in high-grade/risk pTa/pT1G3 tumors (p-value$<$0.0001; Table 1). Further assessment of COX2 distribution in relevant molecular subtypes of UCB [4], revealed a greater proportion of pTaG2 than pTaG1 tumors positively expressing COX2 in low-grade NMIBCs ($p<0.0001$, subtype 1; Figure S2). COX2 expression did not differ among high-grade/risk NMIBCs ($p = 0.075$), but a greater proportion of MIBCs positively expressed COX2 than did all high-grade/risk NMIBCs combined ($p<0.0001$, subtype 2; Figure S2). Only tumor multiplicity was associated with positive COX2 expression in MIBC patients (p-value = 0.008; Table 2).

COX2 Expression and Prognosis in Bladder Cancer Patients

We analyzed the association of COX2 expression with tumor recurrence and progression in patients with NMIBCs and with progression and disease-specific survival in patients with MIBCs (Table 3; Figure 1). When considered as a continuous variable in the univariable analysis, COX2 expression was marginally associated with an increased risk of recurrence in NMIBCs (HR = 1.02, 95%CI = 1.00–1.04, p-value = 0.048; Table 3). However, this association disappeared upon multivariable analysis when adjusting for region, gender, tumor stage and grade, multiplicity, tumor size, and treatment. Moreover, COX2 expression was not significantly associated with recurrence in NMIBCs when considered as a categorical variable, neither in the univariable nor multivariable analyses (Figure 1A; Figure S3AC; Table 3). Lastly, no significant association between COX2 expression and progression or survival was observed in patients with NMIBCs or MIBCs, regardless of whether expression was considered as a continuous or categorical variable in non-adjusted or adjusted analyses (Figure 1B–D; Figure S3B, 3D–H; Table 3).

Meta-analysis of COX2 Expression and Bladder Cancer Prognosis

Twenty publications on COX2 expression and bladder cancer prognosis were identified through the literature review (Table S1) [12–31]. Three of them lacked prognostic data, two overlapped with other larger studies and four included patient cohorts that did not meet the eligibility criteria outlined earlier, leaving 11 evaluable publications [12–14,19,21–25,28,29] plus the current study for the meta-analysis (Figure S4). Studies were classified by the tumor subtype(s) they reported on (i.e. NMIBC or MIBC), and whether adjustment for covariates was considered for each prognostic endpoint examined (i.e. univariable or multivariable; Figures 2 and 3). Of the four meta-analyses conducted with univariable data, only the metaHR of the association between COX2 expression and recurrence in NMIBCs showed marginal significance (metaHR = 1.35, 95%CI = 1.00–1.83; Figure 2). This result was not affected by study heterogeneity (I^2 p-value = 0.13) but exhibited significant publication bias, as evidenced by Egger's test (p-value = 0.019). The remaining meta-analyses considering univariable data suggested increased, albeit non-significant, risks of tumor progression in patients with NMIBCs (metaHR = 2.07, 95%CI = 0.76–5.64) and MIBCs (metaHR = 1.45, 95%CI = 0.77–2.74), and death in patients with MIBCs (metaHR = 1.13, 95%CI = 0.8–1.59; Figure 2). Notably, the summary effect for progression in NMIBCs and that observed for survival in MIBCs were both significantly affected by study heterogeneity (I^2 p-values: 0.006 and 0.004, respectively), with the former also significantly influenced by publication bias (Egger's test p-value = 0.001).

Due to a paucity of published prognostic studies performing multivariable analysis on patients with NMIBCs, we could only address the multivariable meta-association with progression and survival in patients with MIBCs (Figure 3). A small, non-significant increased summary risk of progression (metaHR = 1.12, 95%CI = 0.53–2.35; Figure 3) was observed in COX2 expressing MIBCs that was unaffected by study heterogeneity (I^2 p-value = 0.139). Similarly, a null summary effect was observed for survival (metaHR = 0.97, 95%CI = 0.69–1.36; Figure 3). This effect was influenced neither by study heterogeneity (I^2 p-value = 0.114) nor by publication bias (Egger's test p-value = 0.108.

Discussion

Despite many published studies, contradictory findings prevail on COX2 expression as an independent prognostic marker in patients with UCB. The current study suggests that COX2 expression is not an independent marker associated with recurrence, progression or survival in patients with UCB.

Using the largest cohort of patients with NMIBCs evaluated for COX2 expression to date, we observed that 38% of these tumors expressed the protein. Other groups have reported frequencies ranging from 53–88%; however, these studies used different COX2 antibodies and expression evaluation techniques and had smaller sample sizes [16,28,29,45,46]. In accordance with reported results [11,18,45] we observed significantly higher COX2 expression in MIBCs (58%) than in NMIBCs. This frequency is similar to that observed in other large, histologically homogeneous studies [21,28], while groups using heterogeneous cohorts of squamous and transitional cell carcinomas report frequencies different from our own [12,29]. Collectively, these findings reiterate the importance of homogeneity, or stratification, in tumor marker studies.

The association between COX2 and clinico-pathological characteristics remains a contentious issue in the literature. The majority of studies report an association between COX2 overexpression and advanced tumor invasion and grade, but use heterogeneous populations of NMIBCs *and* MIBCs in their assessments [21,25,26,28,47]. Given the known disparity in COX2 expression between NMIBCs and MIBCs, an association of this type would be expected in a mixed tumor population. After pooling NMIBCs and MIBCs in our study we also observe a strong significant association between COX2 overexpression and advanced tumor invasion ($p>0.0001$) and grade ($p>0.0001$). Notably, several groups report no association between COX2 expression and T and G [14,29,45]; especially those working strictly with homogeneous cohorts of MIBCs [12,23]. Similarly, in our study, COX2 expression did not differ significantly among pT2, pT3 and pT4 tumors ($p = 0.896$). Interestingly, we observed

Table 3. Analysis of COX2 expression in NMIBCs and MIBCs; univariable and multivariable analyses.

	Univariate COX-regression					Multivariate COX-regression[†]				
Score Categorization*	**Patients, n**	**Events, n**	**HR**	**(95% CI)**	**P value[‡]**	**Patients, n**	**Failures, n**	**HR**	**(95% CI)**	**P value[‡]**
Non-muscle invasive tumors										
Recurrence [§]										
Continuous	556	191	1,02	1,00–1,04	0,048	401	141	1,02	1,00–1,04	0,140
Negative vs. Positive	556	191	1,08	0,81–1,44	0,612	401	141	1,11	0,78–1,59	0,555
Median	556	191	1,08	0,82–1,44	0,583	401	141	1,17	0,82–1,67	0,390
Extreme tertiles	370	127	1,06	0,89–1,27	0,483	268	94	1,08	0,86–1,37	0,510
Progression										
Continuous	557	48	0,92	0,84–1,01	0,094	526	43	0,96	0,87–1,05	0,350
Negative vs. Positive	557	48	0,72	0,39–1,33	0,302	526	43	1,38	0,61–3,11	0,434
Median	557	48	0,67	0,38–1,20	0,181	526	43	1,11	0,53–2,33	0,780
Extreme tertiles	371	33	0,71	0,49–1,01	0,059	351	29	0,92	0,54–1,56	0,750
Muscle invasive tumors										
Progression										
Continuous	216	131	0,99	0,96–1,03	0,617	189	110	0,99	0,96–1,03	0,750
Negative vs. Positive	216	131	0,94	0,66–1,34	0,734	189	110	0,85	0,56–1,29	0,448
Median	216	131	0,97	0,69–1,37	0,869	189	110	0,89	0,60–1,32	0,560
Extreme tertiles	144	85	0,92	0,75–1,14	0,464	128	75	0,90	0,70–1,15	0,410
Disease specific survival										
Continuous	216	110	1,00	0,97–1,04	0,908	187	89	1,01	0,97–1,05	0,730
Negative vs. Positive	216	110	0,91	0,61–1,34	0,627	187	89	0,77	0,48–1,23	0,267
Median	216	110	0,94	0,64–1,36	0,726	187	89	0,78	0,50–1,23	0,290
Extreme tertiles	144	68	0,90	0,71–1,15	0,407	126	57	0,78	0,58–1,04	0,090

*Expression cut-points used for categorical variables: "Neg. vs. Pos." - NMIBC/MIBC: 0.340; "Median" - NMIBC: 0.121, MIBC: 0.760; "Extreme tertiles" - NMIBC: (<0.0239, >0.586), MIBC: (<0.270, >2.149).

[†]Multivariate models adjusted for established bladder cancer prognostic factors as follows: NMIBC Recurrence adjusted by region, gender, tumour stage and grade, # tumours, size of tumours, and treatment; NMIBC Progression adjusted by region, # recurrences, age, tumour stage and grade, # tumours, and treatment; MIBC Progression adjusted by region, tumour stage, treatment, and presence of nodes; MIBC Survival adjusted by region, tumour stage, treatment, presence of nodes, and metastases.

[‡]Cox proportional hazards analysis.

[§]One patient excluded due to incomplete follow-up record.

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lower COX2 positivity in pT1 and high-grade/risk NMIBCs, than in pTa and low-grade/risk NMIBCs tumors. This result may seem counterintuitive if grade progression is considered a linear trait and COX2 expression is deemed to increase linearly with T and G. However, there is strong evidence indicating that UCB exists as two molecularly distinct subtypes, with high-grade/risk NMIBCs having a molecular signature more similar to MIBCs than to low-grade/risk NMIBCs [4,42]. In this respect, we observed that COX2 positivity increased significantly with increasing T and G within each molecular tumor subtype (Figure S2). Shirahama et al. [26] reported a COX2 distribution similar to ours, observing 8% positivity in pT1 tumors and 50% in MIBCs when using whole section staining and a 5% expression threshold. Collectively, these results reiterate the disparity in COX2 expression between NMIBCs and MIBCs first reported by Komhoff et al. [47], and highlight the importance of considering expression within the proper molecular context.

To minimize the effects resulting from selecting an arbitrary expression threshold, we investigated COX2 protein expression as a continuous variable and three categorical variables. Only when considered as a continuous variable in the univariable analysis was COX2 expression found to be associated with a slight increase in the risk of recurrence. The meta-analysis, consisting of five other univariable studies, reiterated this association and showed a 35% increased risk of recurrence in patients with COX2 expressing NMIBCs. However, both effect estimates exhibit only marginal significance, suggesting that the observed associations may be due to chance. Moreover, the association observed in the univariable analysis did not hold after adjustment for conventional prognostic factors of recurrence in the multivariable analysis. Lastly, the summary effect observed in the meta-analysis may have been skewed by two small studies which selected only high risk NMIBCs (T1G3 [19] and Cis [24]). When a sensitivity analysis was performed removing these two studies from the meta-analysis, the association between recurrence and COX2 expression was no longer maintained (metaHR = 1.14, 95%CI = 0.94–1.38). The observed disparity between effect estimates of progression in the present study and the meta-analysis could also be attributed to the inclusion of these two studies. Upon their exclusion, the summary HR showed no association with progression (metaHR = 0.98, 95%CI = 0.47–2.03). These results do not support a role for COX2 expression in NMIBCs as an independent prognostic marker of recurrence or progression.

Several groups have investigated the ability of COX2 expression to predict outcome in patients with MIBCs. Despite wide inter-

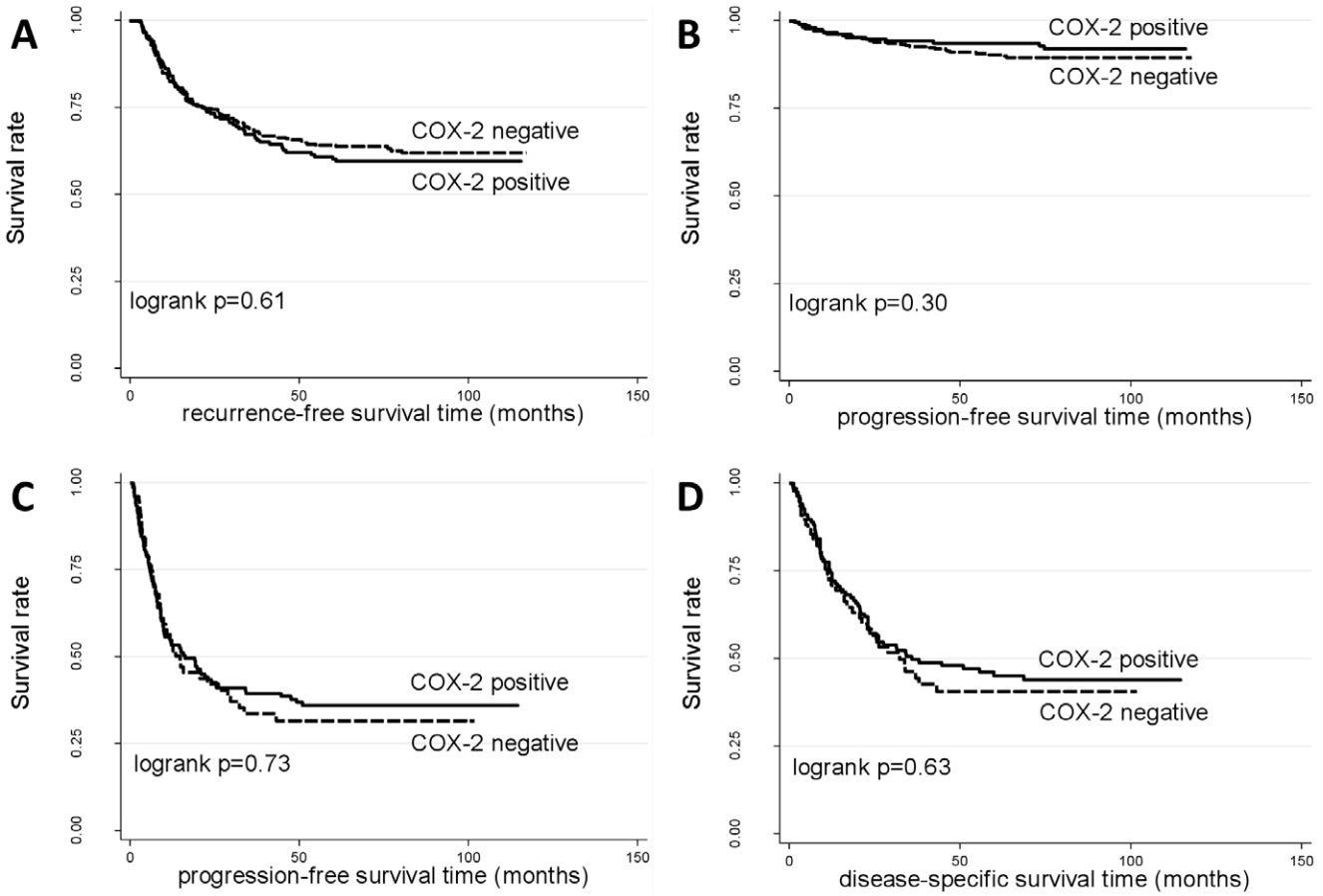

Figure 1. Kaplan-Meier survival curves corresponding to failures in superficial (A, B) and invasive (C, D) tumors for specified prognostic endpoints. Dashed curves: patients with tumors positive for COX2 protein staining; solid curves: patients with tumors negative for COX2 protein staining. Significance values from two-sided logrank test.
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study variation in methodology, antibodies used, sample size, and adjustment parameters in the case of multivariable analyses, the majority of these studies did not identify any significant association between COX2 expression and progression or survival, consistent with our findings [13,14,23,28,29]. Shariat and Margulis and their colleagues observed a negative association between high COX2 expression and tumor progression and mortality [21,25]. However, both studies relied on heterogeneous sample populations which included a small proportion of patients with NMIBCs; potentially accounting for the observed associations given the disparity in COX2 expression between superficial and advanced bladder tumors [45]. In another study, Wulfing et al. reported that high COX2 expression was an independent predictor of poor overall survival in a subgroup of 62 patients with MIBC treated with cisplatin-based chemotherapy [29]. We did not identify any meaningful interaction between COX2 expression and treatment (data not shown), and were unable to replicate their findings in a smaller subset of 39 patients treated with cisplatin (HR = 1.47, 95%CI = 0.48–4.51, p-value = 0.497). Aziz et al. reported a 36% survival advantage associated with increased COX2 levels in a cohort of 266 patients with MIBCs (221 with UCB) that was independent of lymph node status and neo/adjuvant chemotherapy [12]. While we also observed improved survival among patients with COX2 overexpressing MIBCs, this association did not reach significance, consistent with other univariable [23,28] and multivariable [26] analyses.

Our study had a large sample size, included only incident cases and relied on extensive and accurately acquired follow-up information spanning ten years. Additionally, we used automated scoring of immunostained TMAs, a strategy providing a reproducible assessment of expression that correlated highly with the independent evaluation of a subset of samples by an independent pathologist. COX2 staining was done in one laboratory to avoid heterogeneity in immunohistochemical staining and scoring, and evaluated as a continuous variable in the prognostic analyses to avoid potential bias related to selection of an expression threshold. Moreover, the sample population provides an accurate representation of bladder cancer in the general population as no inclusion criteria were applied in the recruitment process which included a good mix of referral centers and county hospitals. Lastly, the recommendations of the REMARK and PRISMA studies were followed in all of the reported analyses.

Despite these considerations and attempts to accurately quantify COX2 expression only in epithelial cells, the pathologist-trained automated imaging system may have incorporated some immunostained stromal material found on the tissue core, thereby increasing type I error. To reduce potential error we averaged the expression scores from duplicate cores and also explored a method investigated by Henriksen et al. [48] in which the higher score was used (data not shown). Both methods produced similar material associations between COX2 expression and clinico-pathological parameters or HRs. Moreover, adjusted analyses for progression

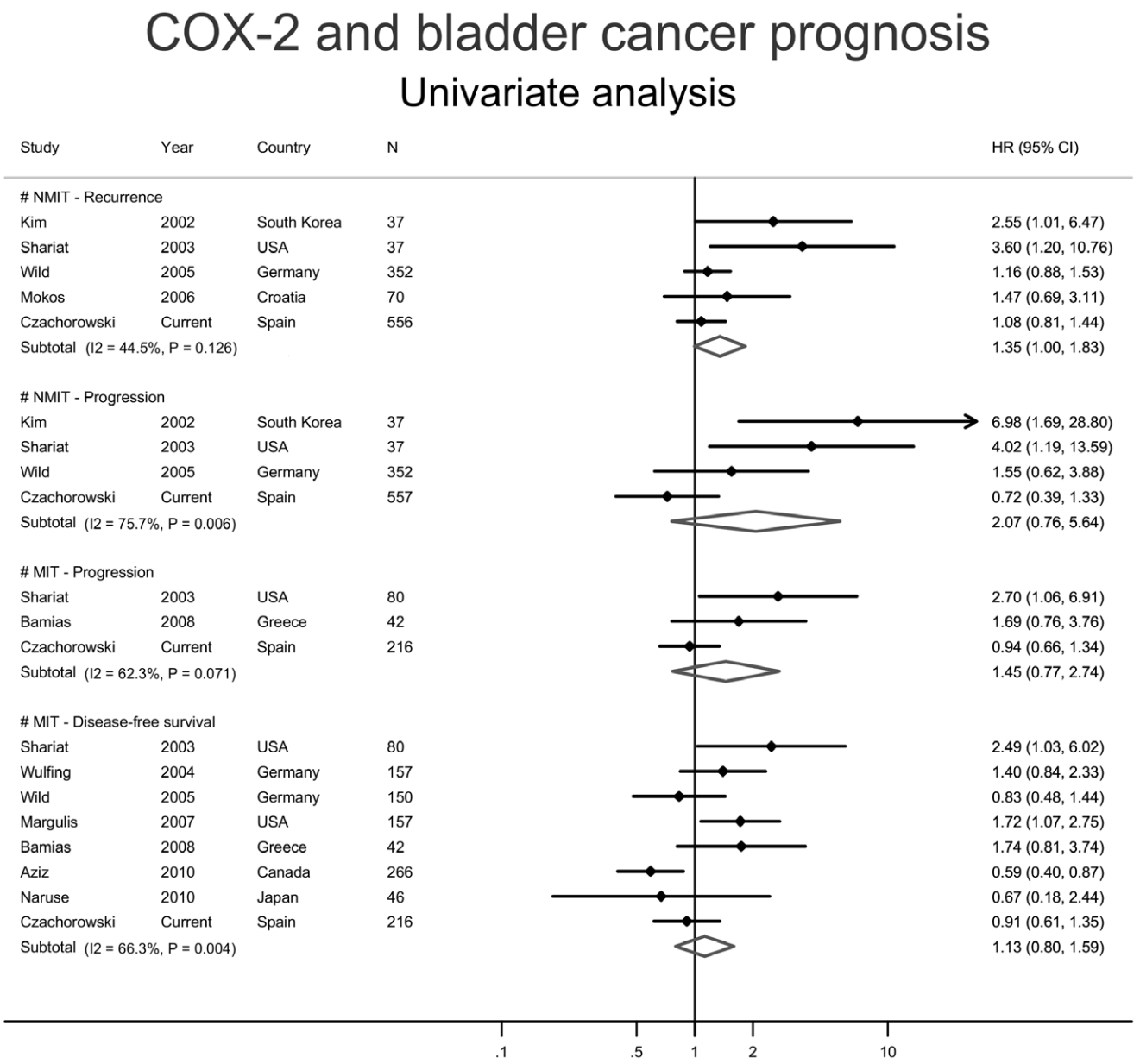

Figure 2. Forest plots from selected univariable studies indicating the risk of reaching the indicated prognostic endpoints in non-muscle invasive (NMIBC; two upper panels), and muscle invasive (MIBC; two lower panels) UCBs in the presence of urothelial COX2 expression.

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in NMIBCs should be interpreted cautiously given the low number of events in relation to covariates. Also, different patient management practices across recruitment hospitals could increase sample heterogeneity, necessitating the inclusion of both recruitment area and treatment regimen in our multivariable analyses.

The results presented herein focus on COX2 expression levels measured in tumor epithelial cells – only one aspect of the complex interplay between the tumor and the host immune/inflammatory response [49]. The prognostic potential of COX2 (if any) may only be revealed when considered together with other tumoral markers. When investigating several potential prognostic parameters in UCB, Hilmy et al. concluded that systemic factors of the inflammatory response such as levels of C-reactive protein were superior to tumor-based factors such as grade, COX2 expression or T-lymphocytic infiltration [18]. Moreover, in models of cervical cancer, Ferrandina et al. observed that while COX2 expression was mutually exclusive in the tumor and stromal inflammatory cells, high expression in both cell types could be used as an independent marker of poor survival [50]. Future studies investigating the prognostic value of COX2 expression in UCB should take into consideration the multi-factorial and multi-dimensional context of the inflammatory response during carcinogenesis.

The current study is the largest to investigate COX2 expression as an independent marker of outcome in a prospective cohort of UCB patients. These findings, supported by a meta-analysis that included our own data and that from other relevant studies, do not

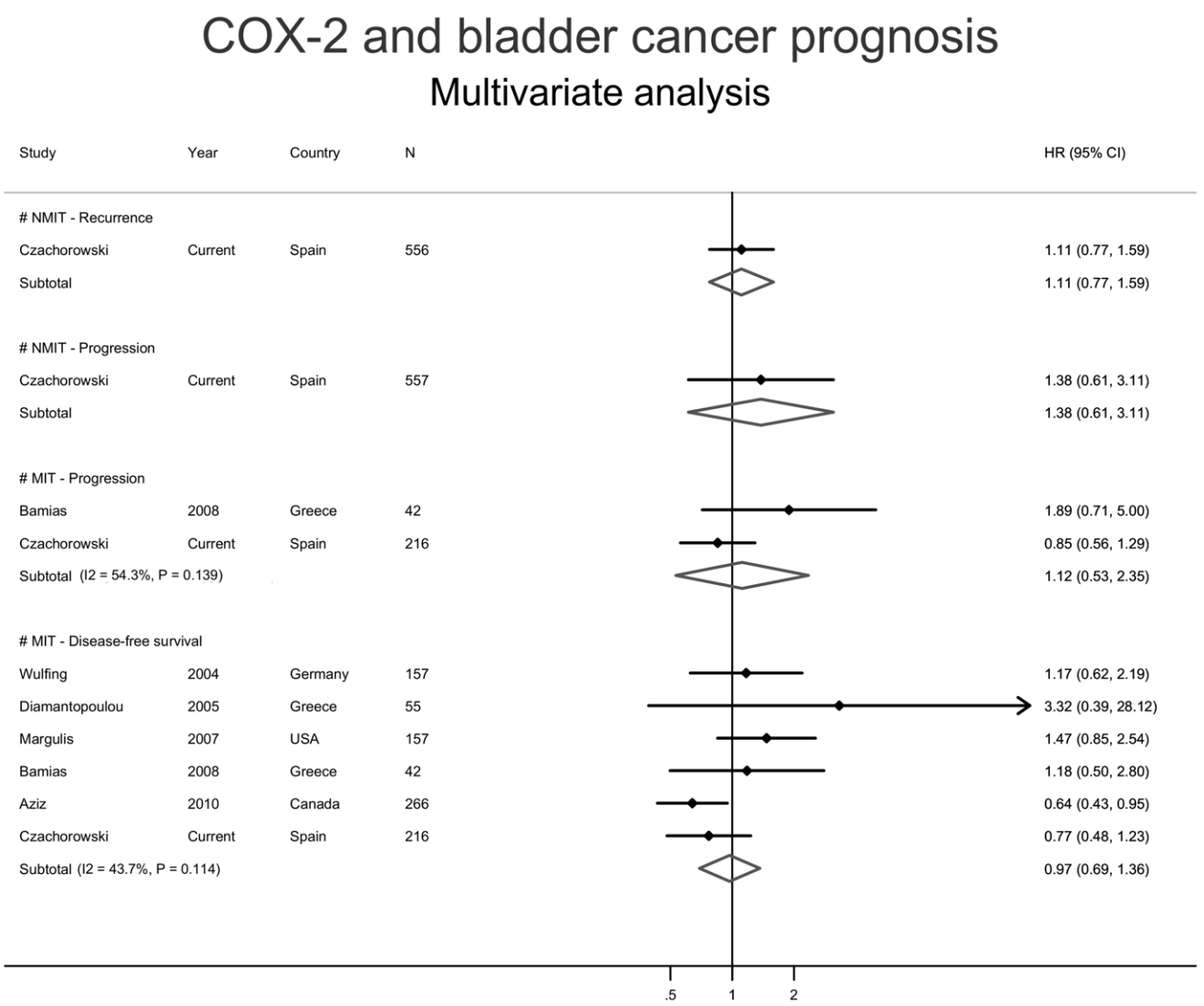

Figure 3. Forest plots from selected multivariable studies indicating the risk of reaching the indicated prognostic endpoints in non-muscle invasive (NMIBC; two upper panels), and muscle invasive (MIBC; two lower panels) UCBs in the presence of urothelial COX2 expression.
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support COX2 tumor cell expression being an independent prognosticator of UCB.

Supporting Information

Figure S1 Immunohistochemical staining of COX2 in primary UCBs on TMAs. Expression was scored as a product of the percentage of epithelial area stained and the staining intensity using automated imaging analysis. A score of <0.340 au was considered negative for COX2 expression, while a score of ≥ 0.340 was considered positive. Representative sections of a pTaG1 UCB lacking COX2 expression (**A and D**) and a pT2G3 UCB expressing COX2 (**B and E**) are shown. Normal colon tissue was used as a positive control (**C and F**). Upper panels show sections under 100x magnification (**A-C**); lower panels show sections under 200x magnification (**D–F**).
(PDF)

Figure S2 Distribution of positive COX2 expression in urothelial carcionomas of the bladder classified by their molecular and pathological stage-grade subtypes. Positive COX2 expression assessed as described in Figure S1. Statistical significance assessed using Fisher's exact test with a 0.05 significance level. pT1G2 tumors excluded due to low sample size in the current study (n = 11), and a reported tendency to overlap both molecular subtypes.
(PDF)

Figure S3 Kaplan-Meier survival curves corresponding to failures in superficial (A, B, C, D) and invasive (E, F, G, H) tumors for specified prognostic endpoints and quantiles of COX2 expression. Dashed curves: patients with tumors expressing COX2 at lower specified quantiles; solid curves: patients with tumors expressing COX2 at upper specified quantiles. Significance values from two-sided logrank test.
(PDF)

Figure S4 Flow diagram of study selection and inclusion in meta-analysis.
(PDF)

Table S1 Main characteristics of eligible studies used in meta-analysis.
(PDF)

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Spanish Bladder Cancer/EPICURO Study investigators

Institut Municipal d'Investigació Mèdica, Universitat Pompeu Fabra, Barcelona – Coordinating 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).

Author Contributions

Conceived and designed the experiments: NM FXR MK JL AC AT. Performed the experiments: MJC NM FXR MK JL AC AT SMM MMM. Analyzed the data: MJC AFSA. Contributed reagents/materials/analysis tools: NM FXR MK AC AT. Wrote the paper: MJC AFSA NM. Revised the manuscript: SMM MMM.

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