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Factores Preditivos de Resposta à Terapêutica Intravesical com BCG

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Em memória dos meus avós...
Para os meus pais e para os amores da minha vida,
a Lena e a nossa bebé que vem a caminho.
Em obediência ao disposto no n° 2 do Artigo 8º do Decreto-lei nº 388/70, o autora declara que participou na concepção e na execução do trabalho experimental, bem como na interpretação dos resultados e na redacção dos seguintes trabalhos publicados e em publicação, que fazem parte integrante desta Dissertação:


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RESUMO

A terapia adjuvante mais eficaz no tratamento do cancro da bexiga não-musculoso invasivo (NMIBC), usada ao longo dos últimos 30 anos, é a instilação intravesical com o Bacilo Calmette–Guérin (BCG). No entanto, apesar da sua eficácia, aproximadamente um terço dos doentes tratados apresentam falência terapêutica, com a possibilidade de progressão da doença.

Neste sentido, o principal objetivo desta tese foi contribuir para a elucidação do mecanismo subjacente à terapia com BCG e a identificação de factores que permitam prever a resposta dos doentes a esta terapêutica.

Foi realizada uma revisão sistemática para caracterizar o estado da arte relativamente a biomarcadores predictivos de resposta à terapêutica com BCG. Entre estes, a presença de macrófagos associados ao tumor (TAMs), em tumores removidos antes desta terapia, revelou ser um dos biomarcadores com resultados mais consistentes na literatura. Assim, um dos objectivos desta tese foi esclarecer a influência dos TAMs, em particular do subtipo M2, em áreas do estroma e do tumor, no contexto da resposta à terapia com BCG. Foi demonstrado que doentes com elevada contagem de macrófagos CD163+, predominantemente localizados no estroma (contagens elevadas no estroma e baixas contagens na área tumoral) apresentam um risco acrescido de recidiva.

Recentemente, o nosso grupo demonstrou que 75 % dos NMIBC de alto grau expressam o carbohidrato associado ao tumor sialil–Tn (sTn). O sTn é uma modificação pós–translacional de proteínas da superfície celular que influencia, tanto a adesão das células, como a resposta imunológica, o que pode influenciar a resposta à imunoterapia com BCG. Nesta tese foi demonstrado, por estudos in vitro, uma maior adesão e internalização do bacilo às células que expressam sTn, promovendo uma morte celular massiva. Observou–se, pela primeira vez, que a expressão do sTn, e do seu antigénio relacionado, o sialil–6–T são
factores predictivos independentes de recidiva após a imunoterapia com BCG.

A revisão sistemática realizada também evidenciou que polimorfismos genéticos em moléculas do sistema imunológico podem ser uma ferramenta útil na previsão da resposta ao tratamento com BCG. Entre estes, moléculas envolvidas em vias como a Fas/FasL podem influenciar a resposta imunológica e a eficácia da imunoterapia com BCG. Neste âmbito, foi realizado um estudo preliminar, numa amostra de 125 doentes tratados com BCG, para avaliar polimorfismos genéticos nos genes FAS e FASL. Observou-se que indivíduos portadores do genótipo CC no polimorfismo FASL-844 apresentam um risco aumentado de falência da terapêutica com BCG. Adicionalmente, tumores de indivíduos portadores deste genótipo possuem uma expressão mais elevada FasL e níveis superiores de FasL estão também associados a um risco acrescido de recorrência após o tratamento BCG.

A amostra inicial entretanto alargada para 204 doentes e nesta foram avaliados 42 polimorfismos em 38 genes de moléculas-chave envolvidas no mecanismo de acção do BCG. Usando análise multivariada de regressão de Cox, propomos o primeiro perfil predictivo de resposta à imunoterapia com BCG, tendo por base polimorfismos genéticos em moléculas do sistema imunológico, em associação com variáveis clínico-patológicas. Este perfil de risco permitiu classificar os doentes em grupos de risco de recidiva pós-terapêutica: doentes que se insiram no grupo de baixo risco têm 90% de probabilidade de responderem ao tratamento, enquanto que aqueles pertencentes ao grupo de alto risco apresentam 75% de probabilidade de recidiva após a terapêutica.

Em conclusão, esta tese permitiu a identificação de novos marcadores predictivos da resposta à imunoterapia com BCG, nomeadamente a infiltração de macrófagos do subtipo M2 e expressão de sTn. Permitiu também estabelecer um perfil genético que poderá estratificar doentes de acordo com o risco de recidiva após a imunoterapia com BCG. Este perfil poderá ser uma ferramenta útil para identificar pacientes com pior prognóstico e melhorar a decisão clínica.
ABSTRACT

The most effective adjuvant therapy of non–muscle invasive bladder cancer (NMIBC) during the last 35 years has been the intravesical instillation with Bacillus Calmette–Guerin (BCG). However, despite its efficacy, approximately one third of the patients present treatment failure, and tumor may progress.

Therefore the main aim of this thesis was to contribute to better elucidate the mechanism behind BCG therapeutic and to identify predictive markers of patient’s response to BCG.

By conducting a systematic review, it was possible to fully characterize the state–of–the–art on predictive biomarkers of BCG treatment outcome. The presence of Tumor–associated macrophages (TAMs) in bladder tumors prior to BCG treatment was one of the most consistent biomarkers in the literature. Therefore one of the aims of this thesis was to clarify the influence of TAMs, in particular of the M2–phenotype in stroma and tumor areas, in BCG treatment outcome. It was demonstrated that patients with high stroma–predominant CD163+ macrophage counts (high stroma but low tumor CD163+ macrophages counts) present an increased risk of recurrence.

Recently, our group has demonstrated that 75% of the high–grade NMIBC expressed tumor–associated carbohydrate antigen sialyl–Tn (sTn). The sTn is a post–translational modification of cell–surface proteins that influences both cell adhesion and immune response, which may influence BCG immunotherapy outcome. In this thesis, it has been demonstrated, by in vitro studies, an higher adhesion and internalization of the bacillus to cells expressing sTn, promoting massive cell death. It was also observed, for the first time, that the expression of tumor–associated carbohydrate sTn, and its related antigen sialyl–6–T, were independent predictive markers of recurrence after BCG immunotherapy.

The systematic review that was conducted also revealed that genetic polymorphisms in immune system molecules may be a helpful
tool in the prediction of BCG treatment outcome. Namely, deregulations in Fas/FasL pathways may lead to immune escape and influence the efficacy of BCG immunotherapy. A preliminary study was performed in a dataset of 125 patients, to evaluate genetic polymorphisms in FAS and FASL genes. It was demonstrated that patients carrying of FASL-844 CC genotype presented an increased risk of BCG treatment failure. Patients carrying this genotype had higher FASL expression in bladder tumors, moreover, higher FASL levels were also associated with an increased risk of recurrence after BCG treatment.

The original dataset was then extended to 204 patients and 42 polymorphisms were evaluated in 38 genes of key molecules involved in BCG mechanism of action. Using stepwise multivariate Cox Regression analysis we propose the first predictive profile of BCG immunotherapy outcome and a risk score based on polymorphisms in immune system molecules in association with clinicopathological variables. This risk score allowed to categorize patients into risk groups: patients within the Low Risk groups have a 90% chance of successful treatment, whereas patients in the High Risk groups present 75% chance of recurrence after BCG treatment.

In conclusion, this thesis deals with the identification of novel tumor–associated predictive markers of BCG immunotherapy outcome, namely M2 macrophages infiltration and tumor–associated carbohydrate sTn. This thesis has allowed the establishment of a genetic risk score that could stratify patients according to the risk of recurrence after BCG immunotherapy. This score may be a helpful tool to identify patients with poor prognosis and to improve clinical decision.
1. INTRODUCTION
1.1 Bladder Cancer

Bladder Cancer (BC) is the most common urinary tract malignancy and is the ninth most common cancer diagnosis worldwide, with more than 330,000 new cases each year and more than 130,000 deaths per year [1]. The estimated male-female ratio is 3.8:1.0, being the seventh most common cancer in men and the 17th in women [1]. The worldwide age standardized incidence rate is 9 per 100,000 for men and 2 per 100,000 for women [2]. In Europe, BC is the fourth most common cancer in men and the eighth most common cause of cancer-specific mortality [2]. In Portugal, BC is the fifth most common cancer in men and the tenth among women [2]. In the North region of Portugal it was observed in 2008 a male incidence rate of 31.4 per 100000 habitants and the female 5.8 [3]. The mortality rate was 4.5 per 100000 habitants, with a relative mortality rate of 28% of the patients diagnosed with BC [2, 3].

Risk factors commonly associated with BC development include smoking, chronic bladder infection, occupational exposure to carcinogenic chemicals, and treatment with chemotherapeutic agents such as cyclophosphamide [4, 5]. Smoking increases about four times the risk of developing BC, and it takes about twenty years to return to basal values after smoking cessation [4, 5]. Conversely, it is thought that exposure to carcinogenic chemicals represent the major cause of BC in men in Europe [4, 5].

Painless haematuria is the most common symptom. Others include urgency, dysuria, increased frequency and, in more advanced tumors, pelvic pain and symptoms related to urinary tract obstruction [5].

To achieve an accurate diagnosis, the American Urological Association (AUA) and the European Association of Urology (EAU) recommend a combined diagnostic procedure including cystoscopy and urinary cytology [6].

Cystoscopy is effective for detecting papillary tumors [5], while the urine cytology is highly specific for the presence of high grade tumors or carcinoma in situ (CIS), however, the sensitivity is low for low grade tumors.
Positive urinary cytology can indicate an urothelial tumor anywhere in the urinary tract, from the calyx to the ureters, bladder and proximal urethra [7, 5].

Approximately 75% to 85% of all BC are non-muscle Invasive Bladder Cancer (NMIBC), carcinoma *in situ* (CIS) and superficial papillary confined to the mucosa or invading the *lamina propria* (Ta/T1). The bladder tumors which invades submucosa, mucosa, muscularis or adjacent organs (stage T2 or higher) are classified as Muscle Invasive Bladder Cancer (MIBC) (Figure 1) [4].

![Figure 1 - Esquematic representation of bladder tumors in different stages [4].](image)

CIS is a poorly differentiated carcinoma confined to the epithelium with an intact basement membrane [5]. It can exist alone, although is usually present in association with other bladder tumors (90%) [5]. Despite, CIS non-invasive nature, it has a poorer prognosis than other superficial tumors. About 60% of the patients progress to muscle-invasive disease over 5 years if untreated [5].

The first-line treatment of BC is transurethral resection of the bladder tumor (TURBT), in which the representation of the *muscularis propria* is mandatory to ensure a proper resection and a more precise staging [7, 6].
The EAU risk classification for NMIBC divides patients into low, intermediate and high risk categories for recurrence and progression [5]. Low risk is defined as single, primary low grade Ta tumors; intermediate risk as multiple or recurrent low grade tumors; and high risk as any T1 and/or CIS [8]. The scoring system is based on the six most significant clinical and pathological factors: tumor stage and grade, multifocality, tumor size, prior recurrence and the presence of concurrent CIS [5]. Patients with low risk disease are kept under cystoscopy surveillance, after tumor resection. However, for patients with recurrent low grade disease or intermediate/high risk disease, adjuvant intravesical therapy is recommended [7]. The gold standard treatment for intermediate/high risk patients is intravesical Bacillus Calmette-Guérin (BCG) instillation, an attenuated strain of *Mycobacterium bovis* [5].

### 1.2 Bacillus Calmette-Guérin (BCG) Immunotherapy

Intravesical BCG immunotherapy is a standard treatment for CIS disease and high-grade or highly recurrent superficial tumors [5]. Meta-analysis studies comparing TURBT alone versus TURBT plus intravesical BCG showed that BCG significantly reduces recurrence and progression [9, 10].

Regarding side effects, BCG immunotherapy is usually well tolerated in the majority of the patients [5, 11]. The most common side effect (90%) include cystitis, hematuria, irritative voiding symptoms and fever [5, 11]. Severe side effects are rare (<5%), and approximately 20% of patients are unable to tolerate BCG therapy [5, 11].

Induction BCG (iBCG) therapeutic scheme generally starts two weeks after TURBT, to allow the healing of the urothelium and to reduce the risk of systemic side effects [12]. iBCG scheme comprises six single courses of intravesical instillations for six weeks. Treatment extension, also designated as maintenance therapy (mBCG), is used to increase efficacy
The suggested maintenance regimen consists of three weekly instillations at 3 months, 6 months, and then every 6 months up to 3 years [13, 14]. The use of maintenance regimen has been a crucial factor for improving BCG efficacy [13].

Several studies were performed to evaluate the efficacy of maintenance regimen following the initial induction cycle [13]. They demonstrated a significantly reduction of tumor recurrence when additional maintenance treatment was used [13]. Furthermore, it was also demonstrated that bladder tumor progression can only be prevented when an additional maintenance BCG regimen is applied [13]. Despite the fact that maintenance therapy is required for optimal efficacy, its use is limited by more pronounced side effects of BCG [11]. Upon maintenance therapy, more than 70% of patients develop local symptoms and up to 37% develop systemic symptoms [11].

Nevertheless, 30–50% of the patients’ present tumor recurrence after this therapy, and 15% display progression to muscle-invasive disease [10, 12, 13]. When BCG immunotherapy fails, radical cystectomy is recommended to reduce the risk of tumor progression [8, 5]. Therefore is important to identify molecules involved in the BCG mechanism of action that could help to identify patients at risk of recurrence after this therapeutic.

### 1.3 BCG Mechanism of action

The key element of the BCG antitumor activity lies in its ability to promote a strong local immune cell response [12]. Still, the exact mechanism remain unknown [15]. Intravesical instillations of BCG induce a massive local immune response, characterized by the secretion of cytokines into urine and bladder wall, as well as, an influx of granulocytes and mononuclear (lymphocytes and macrophages) cells [16, 15]. These
mononuclear cells express activation markers and cellular infiltrates that can persist for 12 months upon instillation [16].

The anticancer immune response may be triggered through the direct interaction of BCG mycobacteria with bladder cancer cells [17]. BCG is able to adhere to, be internalized by, be degraded by and, in general, interact with the bladder neoplastic cells directly in a number of ways (Figure 2) [17, 18]. For example, in response to mycobacterial stimulation, urothelial cells secrete pro-inflammatory cytokines including IL-1, IL-6, IL-8, IL-12 and tumor necrosis factor alpha (TNF-α) [17]. On the other hand, tumor cells may act as professional antigen-presenting cells (this includes expression of MHC class II molecules and adhesion and costimulatory molecules, such ICAM-1), being capable of presenting BCG to T lymphocytes [17, 15]. Moreover, BCG also appears capable of inducing apoptosis and exerting direct potent antiproliferative effects on tumor cells in a dose-and time-dependent manner [19, 15]. Events that may affect bacillus adhesion and internalization, may modulate the anti-tumor effect exerted by this therapy.

Figure 2 – Possible effects exerted by BCG on tumor cells. BCG is internalized and degraded by tumor cells and possibly by benign urothelium. BCG induces expression of ICAM-1 and production of various cytokines including GM-CSF, IL-6, -8, -12, IFN-γ and TNF-α. BCG also exerts antiproliferative and proapoptotic effects on cancer cells. BCG: Bacillus Calmette-Guerin; ICAM: Intracellular adhesion molecule [15].
A few hours after instillation, BCG initiates a complex inflammatory cascade of events. The first cells of innate immune response to be released are neutrophils, followed by monocytes/macrophages that infiltrate the bladder wall producing cytokines and chemokines [16]. The released cytokines and chemokines attract T lymphocytes, Natural Killer (NK) cells and other effector cells that lead to the elimination of the tumor cells [15]. Attracted immunocompetent cells form granuloma-like structures, dominated by CD4+ T-cells, in the bladder wall, which are maintained for several weeks by repeated BCG instillations [16]. Moreover, CD4+ T-cells are activated, leading to a T helper type 1 (Th1) response (Figure 3) [16, 18].

Figure 3 - Suggested cascade of immune responses in bladder mucosa induced by intravesical BCG instillation. Attachment of BCG to urothelial cells including carcinoma cells triggers release of cytokines and chemokines from these cells, resulting in recruitment of various types of immune cells into the bladder wall. Activation of phagocytes and the new cytokine environment lead to the differentiation of naive CD4+ T cells into Th1 and/or Th2 cells that direct immune responses toward cellular or humoral immunity, respectively. The therapeutic effect of BCG depends on a proper induction of Th1 immune responses. IL-10 inhibits Th1 immune responses, whereas IFN-γ inhibits Th2 immune responses [18].
The Th1 response, or cell-mediated immune response, and Th2 response, or humoral immune response, are responsible for different patterns cytokine secretion [20]. Th1 response primarily secret IL-2, IL-12, Interferon-gama and TNF-α; the Th2 response secret IL-4, IL-5, IL-6 and IL-10 [20, 15, 18]. Qualitative analyses of the immune response indicate that the effective establishment of a Th1-cytokine profile is crucial for mounting an effective antitumor response [20]. The importance of the Th1/Th2-dichotomy is further supported by observations that high expression levels of immunoregulatory cytokines, like IL-10-expressing Th2 and regulatory T cells, inhibit Th1 responses and reduce the therapeutic effect of BCG [21]. Therefore the production of Th2 cytokines may interfere with BCG immunotherapy response. In the last years, tumor-associated macrophages (TAMs) have been described as possible modulators of cytokine patterns in tumor stroma [22]. TAMs often assume an immunoregulatory M2 phenotype, characterized by the expression of Th2 cytokines, which may directly interfere with the BCG induced antitumor immune response [22].

At the moment there are no reliable predictive markers of BCG treatment response that may help to identify patients who are at risk of recurrence/progression after BCG treatment [23, 19]. Although studies based on the mechanism of BCG suggest several promising markers, such as IL-2, IL-8, IL-12 and macrophage infiltration, none of these parameters is considered sufficiently consistent to be used in a clinical context [15, 19, 23]. However, it is believed that immunological predictive markers may yield promising clinical value in the context of predicting BCG immunotherapy outcome [15, 19, 23].

In order to elucidate about the predictive markers studied so far and its clinical significance, a systematic review was performed and it is presented in the next chapter of this section.
1.4. Paper I

Predictive biomarkers of bacillus Calmette-Guérin immunotherapy response in bladder cancer: where are we now?

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Review Article

Predictive Biomarkers of Bacillus Calmette-Guérin Immunotherapy Response in Bladder Cancer: Where Are We Now?

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The most effective therapeutic option for managing nonmuscle invasive bladder cancer (NMIBC), over the last 30 years, consists of intravesical instillations with the attenuated strain Bacillus Calmette-Guérin (the BCG vaccine). This has been performed as an adjuvant therapeutic to transurethral resection of bladder tumour (TURBT) and mostly directed towards patients with high-grade tumours, T1 tumours, and in situ carcinomas. However, from 20% to 40% of the patients do not respond and frequently present tumour progression. Since BCG effectiveness is unpredictable, it is important to find consistent biomarkers that can aid either in the prediction of the outcome and/or side effects development. Accordingly, we conducted a systematic critical review to identify the most preeminent predictive molecular markers associated with BCG response. To the best of our knowledge, this is the first review exclusively focusing on predictive biomarkers for BCG treatment outcome. Using a specific query, 1324 abstracts were gathered, then inclusion/exclusion criteria were applied, and finally 87 manuscripts were included. Several molecules, including CD68 and genetic polymorphisms, have been identified as promising surrogate biomarkers. Combinatory analysis of the candidate predictive markers is a crucial step to create a predictive profile of treatment response.

1. Introduction

Thirty years have passed, and intravesical instillations with the attenuated strain bacillus Calmette-Guérin (BCG) are still considered the most adjuvant treatment for non-muscle invasive bladder cancer (NMIBC). Generally this treatment is performed adjuvant to transurethral resection of bladder tumour (TURBT) in intermediate and especially high-risk NMIBC, such as, patients with high-grade tumours, T1 tumours, carcinoma in situ (CIS), multiple tumours, large volume tumours, and high rate of prior recurrence tumours [1].

Recent systematic reviews and meta-analysis have shown that BCG therapy contributes to a significant reduction of recurrence and disease progression for high-risk patients and CIS when compared to TURBT alone or intravesical chemotherapy [2–4]. However, several studies demonstrated that from 20% to 40% of the patients fail to respond to
this therapeutic, which may result in tumour progression [5–9]. Other important fact related with BCG treatment is that 90% of patients will experience some sort of side effects (local cystitis symptoms such dysuria, frequency alteration, and occasional haematuria) [10, 11] and, for this reason, an elevated number of patients did not complete the treatment schedule [12, 13] although a significant higher withdrawal rate of patients treated with BCG could not be demonstrated [12–14].

Since the response to BCG is unpredictable, it is important to find a reliable predictive biomarker and/or a marker that could identify elevated risk groups of treatment failure and side effects development. Currently, no markers are available to predict BCG response (neither clinicopathologic, immunological, inflammatory nor genetic markers).

Biomarkers are defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological process, pathogenic process, or pharmacological responses to a therapeutic intervention.” Predictive biomarkers will foretell how the patient is going to respond to a given therapy. A predictive marker predicts response or resistance to a specific therapy, whereas a prognostic marker, as described above, predicts relapse or progression independently of future treatment effects. Many markers may have both a prognostic and a predictive value [15].

There is some controversial among studies regarding clinical and histopathological predictive factors; therefore, up-to-date none of these markers have demonstrated a reliable predictive role in BCG response, possibly because the NMIBC population candidate for BCG therapy was already selected for its aggressive potential.

Despite intensive research, the exact mechanisms involved in BCG therapy remain elusive. One of the major goals for the next years is the identification of a reliable set of immunological predictive factors, which would allow the identification of responders and nonresponders prior to or at the beginning of immunotherapy. In particular, this may permit the early identification of those patients who suffer the more unpleasant and potentially hazardous side effects associated with BCG therapy, enabling them to be offered alternative treatment [16].

Therefore, the purpose of this systematic review is to conduct a critical analysis of the available literature in order to assess molecular markers (predictive biomarkers) found to be related with BCG treatment recurrence and progression. To the best of our knowledge, this is the first systematic reviews focusing only on molecular predictive biomarkers of BCG treatment outcome.

2. Material and Methods


Through this search 1324 abstracts were gathered and then read. Inclusion/exclusion criteria were created to retrieve only papers focusing molecular markers and BCG immunotherapy response published before 1995. Finally, the reference list of all selected publications and review articles excluded was also checked for additional studies missed on the PubMed search; therefore, two studies were included. Currently, 87 manuscripts were included. Selected studies were then characterized in a structured sheet, the quality assessed, and the pooled data analyzed.

The quality of papers was also independently assessed by two researchers (LL and LS). The quality of the studies was assessed using an eight-item quality assessment scale, based on STROBE Statement [17]. Each item had a score of 1, and the mean quality score of all 87 manuscripts was 5.26/8.

Predictive factors (biomarkers) found were divided in three major categories, such as “Tumour molecular characteristics” with 34 papers that analysed a total of 40 tumour molecular characteristics (mean quality score was 5.13/8), “Urinary markers” 18 which were evaluated in a total of 21 published papers (mean quality score was 4.62/8), and “Genetic Polymorphisms” with 17 papers published studying 65 genetic polymorphisms in 36 genes (mean quality score was 6.33). The outcomes evaluated were recurrence, recurrence-free survival (RFS), progression, and progression free Survival (PFS).

3. Results

Using the criteria defined in the material and methods section several biomarkers related with BCG treatment have been identified and organized according to their biological nature. This information has been comprehensively summarized in Tables 1, 2, and 3. In particular, Table 1 refers to molecular characteristics evaluated in the tumour prior to treatment, Table 2 refers to urinary markers measured during treatment, and Table 3 compiles information about genetic polymorphism evaluated in the context of BCG treatment response. The most promising biomarkers are presented in more detail the following sections.

3.1. Tumour Molecular Characteristics

3.1.1. p53. p53 is a well-known protein involved in cell cycle and apoptosis regulation, its expression was evaluated in 18 studies, making it the most studied molecular tumour marker. p53 expression showed no correlation with recurrence rate after BCG treatment in none of the studies [19, 20, 22–35]. Although higher protein expression seems to be associated with reduced time to recurrence [18, 21, 27] or progression [18, 19, 22, 26, 27, 29], but
Table 1: Tumour-associated markers predicting BCG treatment outcome. The markers are ordered from the most studied to the less, and, within each marker, the studies are ordered by quality score.

<table>
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<th>Marker</th>
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<th>Rec(P)</th>
<th>RFS (P/HR (95% CI))</th>
<th>Prog(P)</th>
<th>PFS (P/HR (95% CI))</th>
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<td>NS/NS*</td>
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−: negative impact, marker associated with a poor BCG response.
+ : positive impact, marker associated to a better BCG response.
Rec: recurrence; P value for recurrence.
RFS: recurrence-free survival; P value for log-rank test/HR: hazard ratio from Cox regression; (95% CI): 95% confidence interval.
Prog: progression; P value for progression.
PFS: progression-free survival; P value for log-rank test/HR: hazard ratio from Cox regression; (95% CI): 95% confidence interval.
iBCG: induction BCG scheme only.
mBCG: maintenance BCG scheme.
NS: no statistical significance.
X: not evaluated.
* all analysed variables (independent prognostic factor).
a adjusted for grade and stage.
b adjusted for age, gender, T stage and number of mBCG instillations.
c adjusted for age and gender.
da adjusted for age, gender.
1 only CIS patients.
Table 2: Urinary markers predicting BCG treatment outcome. The markers are ordered from the most studied to the less, and, within each marker, the studies are ordered by quality score.

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this association could not be demonstrated by several other authors (Table 1) [19, 20, 23, 24, 28, 30, 35]. Only Saint et al. (2004) [27] and Lee (1997) [21] found that p53 could be an independent prognostic factor, but with opposite results. TP53 gene mutation was also associated with higher recurrence rate [91]. It seems that p53 could not be a suitable predictive marker, since the majority of the studies could not corroborate these findings.

3.1.2. Ki-67. Ki-67 is a nuclear protein for cellular proliferation, used as a marker of cell proliferation index. Higher Ki-67 expression seems to be associated with recurrence after BCG [21, 23, 25, 37] and with lower time to recurrence [18, 20, 21]. Still, multivariate analysis failed to prove its value as an independent predictive marker [18, 20, 21]. Furthermore, Lopez-Beltran et al. [18] and Blanchet et al. [36] found that the Ki-67 expression could be associated with lower PFS in univariate analysis and multivariate analysis, respectively. At the moment, Ki-67 could not be used as predictive marker of BCG response, due to the fact that half of the studies regarding this marker did not find any association with BCG treatment response.

3.1.3. (Retinoblastoma Protein) pRB. Only three studies evaluated the tumor suppressor protein, pRB; namely, Cormio and colleagues in 2010 [38] assessed pRB-altered expression in only 27 patients treated with a full maintenance BCG treatment schedule (mBCG) and found it associated with RFS and PFS. Park et al. [19] and Esvuvaranathan et al. [24] evaluate pRB in patients subjected only to induction schedule with BCG (iBCG) and did not find any relationship with protein-positive staining and recurrence, RFS or PFS. These findings suggest that this marker could be a possible indicator of BCG response in patients treated with mBCG although more studies need to be performed in order to clarify this association.

3.1.4. CD68 (Marker of TAMs Presence). Tumour-associated Macrophages (TAMs) may have a dual role in cancer. They could be involved in tumor-cell elimination or can stimulate tumor-cell proliferation, promote angiogenesis, and favour invasion and metastasis [92]. CD68 is a glycoprotein, and its expression allows identifying macrophages. In 2009 Ayari et al. [39] found that a higher TAM count in peritumoural region was associated with lower RFS and with a high risk of BCG treatment failure. The same was reported for CIS tumors treated with BCG by Takayama [40]. This marker could be a suitable biomarker for predicting BCG treatment response although more studies are necessary to confirm these findings and to prove TAMs influence in BCG immunotherapy response.

3.1.5. Other Intracellular Markers. c-erB2 is a proto-oncogene, member of the epidermal growth factor receptor (EGFR/ErbB) family. Janane et al. (2011) [42] found that c-erB2 expression was associated with lower RFS after BCG treatment. Apoptosis regulator protein, bcl-2, was also studied, but doubts persist about its predictive value of BCG treatment. Apoptosis regulator protein, bcl-2, was also studied, but doubts persist about its predictive value of BCG treatment outcome due to conflicting results found by Okamura et al. [31] and Lee et al. [21]. Some authors evaluated the role cyclin-dependent kinase inhibitors, p21 and 27, as predictors of BCG response. Zlotta et al. [20] found that higher p21 expression was associated with decreased RFS in univariate analysis, and Lopez-Beltran and colleagues [18] found that higher expression of p27 was associated with decreased RFS and PFS. These markers are regarded unsuitable candidates to predict BCG treatment response, due to the lack of consistency of the so far presented results (see Table 1). Proteins involved in cell cycle regulation, such as Cyclin D1 and D3, were found to be slightly associated to reduced RFS and PFS [18] although these results were limited to one study, thus needing further investigation. On the other

### Table 2: Continued.

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<th>Marker</th>
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<th>Quality</th>
<th>n</th>
<th>Treatment scheme</th>
<th>Impact</th>
<th>Rec(P)</th>
<th>RFS (P/HR (95% CI))</th>
<th>Prog(P)</th>
<th>PFS (P/HR (95% CI))</th>
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<td>23</td>
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</table>

iBCG: induction BCG scheme only.
mBCG: maintenance BCG scheme.
−: negative impact, marker associated with a poor BCG response.
+: positive impact, marker associated with a better BCG response.
Rec: recurrence; P value for recurrence.
RFS: recurrence-free survival; P value for log-rank test/HR: hazard ratio from Cox regression; (95% CI): 95% confidence interval.
Prog: progression; P value for progression.
PFS: progression-free survival; P value for log-rank test/HR: hazard ratio from Cox regression; (95% CI): 95% confidence interval.
NS: no statistical significance.
X: not evaluated.
*all analysed variables (independent prognostic factor).
*adjusted for BCG-related complications, tumour stage, and grade.
Table 3: Genetic polymorphisms associated to BCG outcome. The markers are ordered from the most studied to the least, and, within each marker, the studies are ordered by quality score.

<table>
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<th>Quality</th>
<th>n</th>
<th>Treatment scheme</th>
<th>Impact</th>
<th>RFS (P/HR (95% CI))</th>
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<td>6/8</td>
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### Table 3: Continued.

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<td>2.54 (1.47–4.39) *</td>
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</table>

iBCG: induction BCG scheme only.
mBCG: maintenance BCG scheme.
−: negative impact, marker associated with a poor BCG response.
+: positive impact, marker associated to a better BCG response.
RFS: recurrence-free survival; P value for log-rank test/HR: hazard ratio from Cox regression; (95% CI): 95% confidence interval.
NS: no statistical significance.
*all analysed variables (independent prognostic factor).
1adjusted for age, gender, ethnicity, tumour stage and grade, smoking history, and BCG vaccination status.
2adjusted for Cis background, multifocality, and mBCG treatment.
3adjusted for age, gender, and smoking history.
4adjusted for age and gender.
hand, Cyclin D3 gene amplification was also associated with decreased RFS as shown by Lopez-Beltran et al. [93].

3.1.6. Other Protein Markers. Other 30 different markers were also studied, as shown in Table 1. All of them were evaluated only in one single study. One of the most promising markers is ezrin, a cytoplasmic peripheral membrane protein involved in cell surface structure adhesion, migration, and organization. Palou et al. [23] never shown that this protein was associated to higher recurrence rate, reduced RFS and PFS. Other markers have shown some potential as predictive marker. Cox-2, which promotes the conversion of arachidonic acid to prostaglandins, could also help to predict early recurrence and progression [48]. Yutkin et al. [45] studied natural killer cells cytotoxic receptors and described that expression of Nkp family proteins, 30, 44, and 46, were associated with less recurrence after treatment. Heat shock protein 90 (HSP90) loss of expression was associated to higher recurrence and progression rates [49]. These may therefore be candidate markers to predict recurrence after BCG treatment.

All of these markers, and others [39, 41] need further investigation once they were only evaluated in one study and with samples rounding 30 or 50 patients, almost only treated with iBCG schedule.

3.1.7. Genetic Markers Evaluated on Tumour

Gene Expression. Other markers have been studied in tumour biopsies, such as genetic markers (not shown in Table 1). Gazzaniga et al. (2009) [94] evaluated α5β1 integrin gene expression (the integrin involved in BCG attachment and internalization into cells) in the tumours of 11 patients treated with BCG and found that lower α5β1 expression was associated with recurrence [94]. Videira et al. [95] evaluated the expression of 10 immunological genes involved in antigen presentation (CD1 and MHC-I) and chemokines (MIP-1, MCP-1, IP10 and MIG). This study showed higher mRNA levels of MHC-I for tumours that will not relapse after treatment and tumours that will recur have lower expression of CD1c, CD1e and MCP-1. They also found higher expression of CD1a, CD1b, CD1c, CD1e, MHC-I, MIG, and IP10 in biopsies after treatment in the group of patient without recurrence when compared with the recurrence group [95].

Kim and colleagues [96] performed a microarray analysis in tumours from 80 patients treated with BCG, and they could identify a subset of genes that individually are associated with reduced RFS and PFS. When evaluated together, the “poor predictive signature” presented a 3.38 higher risk of recurrence or 10.49 higher risk of progression after BCG treatment [96].

These findings demonstrate that evaluation of gene expression patterns in tumours prior to treatment has the potential to disclose a new subset of biomarkers capable predicting BCG treatment response. More studies are needed to validate these markers and possible find new ones.

Gene Methylation. Alvarez-Múgica et al. [97] studied the methylation status of myopodin gene (involved in actin-bundling activity) and found that this event is associated with reduced RFS [97]. Recently, Agundez and colleagues [98] evaluated methylation status in 25 tumour suppressor genes. It was found that differential methylation for several genes had an impact BCG treatment outcome. Therefore, methylation of PAX6 gene is associated with lower RFS [98]. However, unmethylated MSH6, RB1, THBS1, PYCARD, TP73, ESR1, and GATA5 genes are associated with higher PFS [98]. This new approach could contribute to establish new candidate predictive biomarkers of BCG treatment response.

3.2. Urinary Markers

3.2.1. IL-8 (Major Mediators of the Inflammatory Response). Urinary levels of the chemokine IL-8, a potent chemoattractant of neutrophils and macrophages, could be a potential biomarker of BCG treatment response. Several authors found that higher IL-8 levels are significantly associated with a better treatment outcome [51–59]. Only Sagnak et al. (2009) [51] and Watanabe et al. (2003) [57] found that lower levels of IL-8 are a slightly associated with reduced RFS. These studies presented levels measured in different time points of BCG treatment and its predictive value was accessed with different cutoff values; therefore, it is imperative to evaluate the same cutoff values in larger sets of samples.

3.2.2. Interleukin 2 (IL-2). IL-2 is a Th1 subset cytokine, involved in cytotoxic T lymphocyte expansion (cytotoxic T lymphocytes and natural killer cells) and macrophage activation. IL-2 urinary levels were extensively studied [53, 54, 57, 60–63], and higher IL-2 urinary levels were appointed to be a good predictive marker of recurrence [53, 54, 57, 61, 63] and higher RFS [57, 60, 62]. Saint also found that lower or absent levels of IL-2 were associated with shorter PFS in mBCG-treated patients but not in iBCG [60, 62]. IL-2 urinary levels are the most promising predictive biomarker of BCG treatment response; however, it could only be measured during treatment and could not be used in treatment definition. These results highlight the key role of IL-2 in BCG treatment response; therefore, it is important to evaluate why nonresponders have lower IL-2 levels, in order to establish IL-2-related biomarkers that could predict BCG response prior to treatment.

3.2.3. Other Urinary Cytokines. Other urinary cytokines have demonstrated to have potential as predictive biomarkers, yet some need further investigation. Tumour necrosis factor α (TNF-α), whose primary role is the regulation of immune cells, and its urinary levels have been evaluated during the course of BCG treatment in several studies. It was found that higher TNF-α levels are associated with a higher response rate [53, 54, 56, 57, 61]. Watanabe et al. (2003) [57], also demonstrated that higher levels of this molecule are associated with better RFS.

IL-6 is an interleukin that acts as both a proinflammatory and anti-inflammatory cytokine. It is secreted by T cells and...
macrophages to stimulate immune response. Higher IL-6 urinary levels during BCG treatment were associated with lower recurrence rates and higher RFS [53, 54, 57, 61].

IL-18 is a proinflammatory cytokine, produced by macrophages, and induces cell-mediated immunity. Lower urinary levels of this protein have been found within the first 12 h after BCG in nonresponders to BCG treatment [58]. Although this cytokine was only evaluated in 17 patients, others authors suggest that IL-18 has a key role in the mechanism of intravesical immunotherapy with BCG [99].

IFN-γ is involved in macrophage activation and Th1 differentiation, and higher urinary levels were associated with a good treatment response in a first course of iBCG [63], yet other authors could not confirm this association [54, 56, 57, 60].

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine that functions as a white blood cell growth factor. GM-CSF stimulates stem cells to produce granulocytes (neutrophils, eosinophils, and basophils) and monocytes. GM-CSF levels were evaluated in 2 papers [54, 56]; only Jackson et al. (1998) [54] found that higher levels of these molecule were associated with reduced recurrence rate.

Somehow, all of these cytokine are associated with treatment response; however, their predictive value fails to be consistent among the studies. Once more, important molecules involved in BCG mechanism of action have been highlighted; hence, it is essential to explore other biomarkers related to these cytokine urinary levels variability.

3.2.4. Other Markers. Other 7 markers were evaluated in 4 papers, only regarding recurrence rate [54, 69, 70]. Higher levels of survivin (member of the inhibitor of apoptosis family) and soluble CD14 (acts as a coreceptor in recognize pathogen-associated molecular patterns) were present in the recurrence group [54, 70]. The soluble intercellular adhesion molecule 1 (ICAM-1), which facilitates transmigration of leukocytes across vascular endothelia in processes such as extravasation and the inflammatory response, was associated with recurrence in multivariate analysis [54]. The biomarker value of these molecules warrants further studies in order to evaluate its role in BCG immunotherapy response.

Efforts were made in order to find serological predictive markers of BCG treatment outcome. Molecules such as purified protein derivative (PPD), HSP65/70, major secreted antigen complex (Ag85), immunogenic, and skin-reactive protein, p64, have been explored [100–102]. Still, the serological levels of these proteins were not able to predict BCG treatment failure [100–102]. Also, several immunological mediators were evaluated in blood of BCG-treated patients, but none was associated with recurrence after BCG treatment with the exception that lower levels of IL-2 appear to be associated with recurrence [100, 101]. Therefore, with the exception of IL-2, molecules found in the peripheral circulation may not be a suitable approach to find predictive biomarkers of BCG response.

3.3. Genetic Polymorphisms

3.3.1. NRAMP1(SLC11A1) Gene. Natural resistance-associated macrophage protein 1 (NRAMP1) gene regulates intracellular pathogen proliferation and macrophage inflammatory responses. NRAMP1 is one of the most studied genes, with 5 polymorphisms analyzed in 2 papers [71, 72]. Chiong et al. (2010) [71] found that (GT)n repeat and D543N GA genotype were associated with reduced RFS; this author also studied hGPX1 gene, and an association was found [71]. On the other hand, Decobert in 2006 [72] found that D543N GG genotype is also associated with reduced time to recurrence.

3.3.2. DNA Repair Genes. Gu et al. (2005) [73] analyzed several polymorphisms in XPA, XPC, XPD, XPG, ERCCI, and ERCC6 genes and found that XPA 5′UTR AA was correlated with higher RFS when compared with AG and GG genotypes, and ERCC6 Met1097Val GG genotype was associated with reduced RFS after BCG treatment [73]. However, Gangwar and colleagues (2010) [74] have also studied XPC gene polymorphisms and found that patients carrying AC or CC genotypes of XPC Lys939Gln have reduced RFS [74]. The same author published other paper in 2010 regarding polymorphisms in APEX1 and ERCC2 genes and found that ERCC2 Asp312Asn AA was also associated with reduced RFS [82]. Polymorphisms in XRCC1/3/4 genes were also studied, only XRCC1 codon11 AA genotype was associated with reduced RFS after BCG treatment [88, 89].

3.3.3. Inflammation-associated Genes. Rama Mittal group has published several studies [75, 83–85] regarding several polymorphisms in inflammatory genes such as IFNG, TNFA, TGFBI, COX2, PPARG, IL1B, IL1RN, IL4, IL6, and IL8 [75, 77, 78, 83, 85]. They found that IL8-251 AA, TNFA-1031 CC, IL6-174 CC, and TGFBI+28 TT genotypes were associated with higher RFS after BCG treatment [75, 77, 78, 85]. On the other hand, they found that patients carrying COX2-765 CC genotype or NFKB ATTG Del/Del genotypes or IFNA LOH or IFNG+874 A allele have a decreased RFS after treatment. Considering the IL6-174 G/C, Leibovici et al. (2005) [76] found conflicting results in which CC genotype was associated with a reduced RFS after BCG. Other paper (not shown in Table 3) evaluated the influence 22 polymorphisms in 13 inflammatory genes on recurrence after BCG treatment [103]; patients carrying the TGFB codon 10 T allele, TGFB codon 25 G allele, IL4-1098 GG genotype, and IL10-1082 GG genotype are at higher risk of recurrence after BCG treatment [103].

3.3.4. Cell Cycle and Apoptosis Genes. The role of genetic polymorphisms on genes such as MMP1/2/3/7/8/9, FAS, CASP8/9, MDM2, and CCDN1 on BCG treatment outcome was addressed by some authors [79–81, 86, 87]. It was found that patients carrying MMP2-1306 T allele or MMP3-1171 5A/6A have a reduced RFS after treatment [80, 81] and patients carrying MMP1-1607 1G/2G or CASP9-1263
the evaluation of these markers in the context of BCG progression, p53 and ki67 are the most well studied. Still, it has been suggested that a higher number of TAMs (TAMs), when detected at tumour core and surrounding dictate biomarker. Indeed, tumour-associated macrophages as predictive biomarkers. 

4. Discussion

Several studies were conducted to personalize and improve the NMIBC treatment with BCG. A plethora of exciting data has emerged recently, which represents a potential tool to define differences in BCG treatment response.

Among the proteins associated with bladder cancer progression, p53 and ki67 are the most well studied. Still, the evaluation of these markers in the context of BCG treatment did not offer strong evidences regarding their role as predictive biomarkers.

Conversely, CD68 has shown a huge potential as a predictive biomarker. Indeed, tumour-associated macrophages (TAMs), when detected at tumour core and surrounding tissue, strongly correlated with tumour treatment response [39, 40]. It has been suggested that a higher number of TAMs can promote a more efficient phagocytosis and elimination of BCG, preventing BCG from inducing a long-term local inflammation [39]. Although the results regarding this marker are consistent, complementary information are still necessary to confirm the predictive value of these marker and the influence of TAMs presence in the treatment outcome. Namely, it will be important to verify the phenotypic nature of this TAMs, as only the M2 macrophages are known to produce protumor factors such as inflammatory cytokines that could inhibit BCG treatment response [92].

Tumour markers like ezrin, HSP90, CD83, and others also reveal a potential as biomarkers of BCG treatment response. However, only one paper addresses these biomarkers in the context of BCG treatment outcome. In this sense, more studies are needed to validate if these markers are suitable candidates to predict BCG treatment outcome.

Urinary markers are widely studied worldwide, and several molecules, such as IL-8, IL-2, and, in a lesser extent, TNF-α and IL-18, are currently believed to play a role on BCG immunotherapy mechanism of action. More importantly, their levels have appeared to be associated with treatment failure. However, as state by Zuiverloon et al. [104], these markers are “during BCG markers,” only present in urine during the course of treatment, thus failing to provide insight on the outcome prior to that. pm Nonetheless, the role of urine in noninvasive approaches to monitor response has been demonstrated.

Pharmacogenomic investigation has also demonstrated to be a powerful tool in the identification of predictive biomarkers. Regarding BCG immunotherapy, several polymorphisms in a large set of genes have demonstrated the potential to predict treatment outcome. Polymorphism in inflammatory genes such as IL8, TNFA, IL6, TGFB1, COX2, and IFNG are examples of putative predictive markers [75, 77, 78, 83, 85].

However most of them were studied in the same Indian population which was small in number (80 patients). Moreover, the majority of these patients have been subjected only to a induction schedule with BCG (iBCG) [74, 75, 77–83, 85–89]. In order to be used as a predictive markers, it is still necessary to evaluate these polymorphism in larger sets of patients, with a representative number of patients treated with a full maintenance BCG treatment schedule (mBCG) and from other ethnicities. Furthermore, there are several other molecules involved in BCG immunotherapy mechanism of action and potentially involved in the treatment response that may be subjected to polymorphism analysis. In a recent review, Alexandrof and colleagues suggest that molecules such as IL-2, IL-17, IL-23, soluble CD40L, and TRAIL may be important key targets and may serve as putative markers [16]. A careful evaluation of such candidates should be undertaken in order to access their biomarker value.

Recently, the studies by Kim and colleagues [96] using a microarray analysis allowed to identify a “poor predictive signature” of BCG treatment response. This work is suggesting that a combinatory analysis involving all predictive markers may permit to create a useful score or a predictive profile. The combination of several markers will allow explaining and consequently predicting all recurrences after BCG treatment. Other current approaches, such as microRNAs profiling and Genome wide association studies (GWAS) can be important features in the context of BCG immunotherapy research and treatment response prediction.

5. Conclusion

Regarding the tumour molecular characteristics studied, three major conclusions can be drawn, p53 and ki67 are not suitable predictive biomarkers, markers such as TAMs and other molecules (ezrin, HSP90, CD83, and Cox2) require validation, and different approaches such as gene expression and epigenetic alterations of the tumour prior to treatment may bring new insights in the search for predictive biomarkers of BCG immunotherapy.

Concerning urinary markers, the monitoring of IL-2 levels during treatment seems a consistent noninvasive approach to determine treatment response; hence, other cytokines could have the same predictive power. The only drawback is the fact that these markers are unable to predictive treatment response prior to therapy.

In relation to genetic polymorphisms, those in the genes IL8, TNFA, IL6, TGFB1, COX2, and IFNG were found to be among the most informative. Nevertheless, it is important to validate the findings in larger samples from different ethnicities and evaluate other genetic polymorphism in molecules that have shown to have a important role in BCG.
immunotherapy mechanism of action (e.g., IL-2, TRAIL, and Th17 cytokines).

It is our belief that only the introduction of an array of biomarkers can improve the accuracy of current status on the prediction of BCG treatment outcome and thus improve the management of high-risk NMIBC. Future studies combining the most promising putative biomarkers are warranted if not mandatory.

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2. AIMS and STUDY OUTLINE
Based on the systematic review, three groups of biomarkers with potential to influence BCG immunotherapy outcome were identified. These include tumor-associated markers, urinary markers and host genetic markers (genetic polymorphisms). Although several molecules associated with the BCG mechanism of action may be found in urine, they can only be measured during the course of treatment, which precludes its use as predictive markers. Regarding tumor-associated markers, some molecules, may have potential to be used as predictive biomarkers of recurrence after BCG treatment; however more studies are needed to confirm their predictive value.

Recently, the presence of TAMs in bladder tumors prior to BCG treatment was associated with recurrence after treatment [24-26, 19, 23]. These studies have assessed the expression of CD68, a lineage marker found in both M1 and M2 macrophages [27]. Several authors showed that, in order to accurately determine TAMs influence in prognosis and treatment outcome, M2-specific markers, such as CD163, should be used [28-31]. Therefore, is important to disclose the role of differential macrophage polarization on BCG treatment, using these specific markers.

Moreover, our group has been devoted to comprehending the role of tumor-associated carbohydrate antigen sialyl-Tn (STn) expression, in bladder cancer [32]. We reported that 75% of the high-grade bladder tumors, presenting elevated proliferation rates and high risk of recurrence/progression, expressed sTn (in Appendix section) [32]. Since these high risk patients are treated with BCG immunotherapy and this antigen is known to influence both cell-cell interactions and immune response it was important to evaluate the influence and the predictive value of this antigen in the context of BCG immunotherapy.

Concerning genetic polymorphisms, some have been associated with BCG treatment outcome. Nevertheless, it was important to validate these findings in larger sample number, gathered from patients with different ethnicities and to integrate several genetic polymorphisms in molecules with a potential role in BCG mechanism of action (e.g., IL-2, TRAIL, and Th17 cytokines).
Based on these evidences, the main goal of this thesis, was to establish a panel of biomarkers that can improve the prediction of BCG treatment outcome and thus improve the management of high-risk NMIBC. For that in this thesis is proposed to evaluate tumor-associated markers and genetic polymorphisms as biomarkers of BCG immunotherapy outcome.

The specific aims of this thesis are:

- To clarify the correlation of tumor-associated macrophages (TAMs), in particular M2-phenotype in stroma and tumor areas, with BCG treatment outcome.

- To evaluate the role of a tumor-associated carbohydrate antigen, the sTn, that is frequently expressed in high grade NMIBC tumors, in the context of BCG immunotherapy outcome.

- To analyze if a set of functional polymorphisms in genes involved in the several steps of the mechanism of action of BCG immunotherapy is associated with recurrence after treatment.

- To establish a predictive profile of BCG treatment outcome, based the most informative biomarkers evaluated, in order to stratify patients in risk groups of treatment failure allowing to identify patients at higher risk of recurrence after BCG treatment.
To fulfill the outlined in the first objective, the influence of TAMs (CD68⁺) and also the M2-polarized macrophage phenotype (CD163⁺) was evaluated, in the context of BCG treatment outcome. Taking into consideration that the microenvironment plays a determinant role in the modulation of the macrophage lineages the tumor and the stroma were independently evaluated. This work is presented in chapter 4.1.1 of this thesis, entitled “The predominance of M2-polarized macrophages in the stroma of low-hypoxic bladder tumors is associated with BCG immunotherapy failure”.

The work regarding the evaluation of the role tumor-associated carbohydrate antigen sTn in BCG immunotherapy is presented in Chapter 4.1.2, entitled “Response of high-risk of recurrence/progression bladder tumours expressing sialyl-Tn and sialyl-6-T to BCG immunotherapy”.

To address the proposed objective regarding the predictive value of genetic polymorphisms in the BCG treatment response, firstly preliminary studies were conducted, using a dataset of 125 patients. Herein two preliminary studies are presented where genetic polymorphisms in genes involved in immune response, such as TNFA, IL4 (Paper IV, Chapter 4.2.1), FAS and FASL (Paper V, Chapter 4.2.2) were evaluated. Finally, the original dataset was extended to 204 patients and a panel of 42 polymorphisms in 38 genes from key molecules involved in BCG mechanism of action were evaluated. This information was used to establish predictive models of treatment outcome and a risk score of recurrence. This work is presented in the paper VI of Chapter 4.2.3, entitled “The role of functional polymorphism in immune response genes as biomarkers of BCG Immunotherapy outcome in Bladder cancer: Establishment of a predictive profile”.
3. MATERIAL & METHODS, RESULTS and DISCUSSION
3.1. Tumor–associated Markers

3.1.1. Paper II

The predominance of M2–polarized macrophages in the stroma of low-hypoxic bladder tumors is associated with BCG immunotherapy failure


Urologic Oncology (in press)
The predominance of M2-polarized macrophages in the stroma of low-hypoxic bladder tumors is associated with BCG immunotherapy failure

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Abstract

**Objective:** Bacillus Calmette-Guérin (BCG) immunotherapy is the gold standard treatment for superficial bladder tumors with intermediate/high risk of recurrence or progression. However, approximately 30% of patients fail to respond to the treatment. Effective BCG therapy needs precise activation of the type 1 helper cells immune pathway. Tumor-associated macrophages (TAMs) often assume an immunoregulatory M2 phenotype and may directly interfere with the BCG-induced antitumor immune response. Thus, we aim to clarify the influence of TAMs, in particular of the M2 phenotype in stroma and tumor areas, in BCG treatment outcome.

**Patients and methods:** The study included 99 patients with bladder cancer treated with BCG. Tumors resected before treatment were evaluated using immunohistochemistry for CD68 and CD163 antigens, which identify a lineage macrophage marker and a M2-polarized specific cell surface receptor, respectively. CD68\textsuperscript{+} and CD163\textsuperscript{+} macrophages were evaluated within the stroma and tumor areas, and high density of infiltrating cells spots were selected for counting. Hypoxia, an event known to modulate macrophage phenotype, was also assessed through hypoxia induced factor (HIF)-1\textalpha expression.

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Results: Patients in whom BCG failed had high stroma-predominant CD163^+ macrophage counts (high stroma but low tumor CD163^+ macrophage counts) when compared with the ones with a successful treatment (71% vs. 47%, P = 0.017). Furthermore, patients presenting this phenotype showed decreased recurrence-free survival (log rank, P = 0.008) and a clear 2-fold increased risk of BCG treatment failure was observed in univariate analysis (hazard ratio = 2.343; 95% CI: 1.197–4.587; P = 0.013). Even when adjusted for potential confounders, such as age and therapeutic scheme, multivariate analysis revealed 2.6-fold increased risk of recurrence (hazard ratio = 2.627; 95% CI: 1.340–5.150; P = 0.005). High stroma-predominant CD163^+ macrophage counts were also associated with low expression of HIF-1α in tumor areas, whereas high counts of CD163^+ in the tumor presented high expression of HIF-1α in tumor nests.

Conclusions: TAMs evaluation using CD163 is a good indicator of BCG treatment failure. Moreover, elevated infiltration of CD163^+ macrophages, predominantly in stroma areas but not in the tumor, may be a useful indicator of BCG treatment outcome, possibly owing to its immunosuppressive phenotype.

Keywords: Bladder cancer; BCG immunotherapy; Tumor-associated macrophages; CD68; CD163

1. Introduction

Bladder cancer is the second most common urologic cancer and has the highest recurrence rate of any malignancy [1]. Approximately 75% to 85% of all bladder cancers are non–muscle invasive cancers, which includes carcinoma in situ (CIS) and papillary tumors confined to the mucosa or submucosa (Ta/T1) [1]. The non–muscle invasive cancer risk classification divides patients into low-, intermediate-, and high-risk categories for recurrence and progression [1]. The gold standard treatment for intermediate–high-risk patients is intravesical instillations with Bacillus Calmette-Guérin (BCG) [1]. However, 30% to 50% of patients fail to respond, and 15% show progression to muscle-invasive disease. In these cases, radical cystectomy is the treatment to follow [2]. Intravesical instillations of BCG induce a massive local immune response that is characterized by the expression of cytokines in the bladder, as well as an influx of granulocytes and mononuclear cells (lymphocytes and macrophages) into the tumor areas [3,4].

Tumor biology, tumor progression, and response to therapy are influenced by the tumor microenvironment [5,6]. These include stromal cells, infiltrating leukocytes, and blood vessels (depending on tumor size), all of which contribute to the so-called tumor stroma [6]. Tumor-associated macrophages (TAMs) are a major component of the tumor stroma that contribute to tumor progression in several types of cancer [6,7].

Macrophages are polarized in 2 distinct functional forms, M1 and M2 [6–8]. The classical or M1 macrophages activate type 1 helper cells (Th1) that have the capability to kill pathogens and produce IL-2, IL-12, and proinflammatory cytokines that promote responses like cytotoxic T-cell activation [8]. In contrast, alternatively activated M2 macrophages express low levels of IL-12 and high levels of IL-4 and IL-10, promoting Th2 cytokines that inhibit Th1 responses [7]. However, associated to the tumor, M2-polarized macrophages comprise multiple subtypes that may contribute to immunosuppression, angiogenesis, cell invasion, and metastasis depending on the microenvironment [5,9]. Moreover, cytokines and chemokines released by the tumor may recruit and modulate monocyte differentiation into M2 macrophages lineages that may differ from those in the stroma [6,7]. As such, a detailed evaluation of macrophage phenotypes in the tumor and the stroma as well as their microenvironment is needed to fully understand how M2 macrophages influence tumor behavior and ultimately the response to treatment.

The studies presented so far indicate that higher TAM counts are associated with lower recurrence-free survival and high risk of BCG treatment failure [10–12]. Nevertheless, these conclusions were based exclusively on CD68 expression, a macrophage lineage marker that does not allow the discrimination between M1 and M2 phenotypes therefore adding bias to these observations [13,14]. Moreover, patients that respond to BCG commonly release large amounts of Th1 cytokines [15], whereas high levels of Th2 cytokines (i.e., IL-4 and IL-10) seem to be related with BCG failure [16]. These observations support the idea that effective BCG therapy requires precise activation of the Th1 immune pathway [17,18]. However, TAMs assuming an immunoregulatory M2 phenotype release Th2 cytokines that may directly interfere with the BCG-induced antitumor immune response [7,16,18]. Still, no direct evidences have been presented supporting the association between higher counts of M2-polarized macrophages and the failure of BCG treatment.

Furthermore, macrophages in different localizations may present different phenotypes induced by the microenvironment. For example, oxygen shortage is known to promote an accumulation of angiogenic M2 macrophages in tumor hypoxic areas, where hypoxia induced factor (HIF)-1α enhances the expression of vascular endothelial growth factor and decreases the production of classical Th2 cytokines [19]. Despite these observations, the influence of hypoxia in the modulation of M2-polarized macrophage distribution in bladder tumors and stroma and its association with BCG treatment outcome also remains unevaluated.

In addition, a clarification about the expression pattern of M2 macrophages in intermediate and high risk of recurrence bladder tumors and the influence of hypoxia is needed to disclose their true predictive value in the context of BCG response. In this study, we devoted to this matter by evaluating the overall TAMs (CD68^+) as well as the M2 phenotype, based on CD163 expression, in both stroma and tumor areas. As outlined, we correlated our findings with
HIF-1α expression to disclose the influence of hypoxia in M2 macrophage accumulation and treatment outcome.

2. Methods

2.1. Cohort of patients

This study included 99 formalin-fixed paraffin-embedded tissues from patients who were treated with transurethral resection of bladder tumor and then underwent BCG immunotherapy in the Urology Department of Portuguese Institute of Oncology—Oporto (IPO-Porto), between 1998 and 2006. All patients received induction BCG therapy for 6 consecutive weeks, starting 2 to 3 weeks after surgery (iBCG), and were then maintained on a mBCG schedule (the one used in our institute is iBCG + maintenance protocol with 2-weekly instillations every 3 mo during 2 y). The iBCG group includes patients treated before the European Association of Urology guidelines recommending the mBCG scheme [20] and patients showing significant intolerance to long BCG treatment.

The average age of the patients was 68 years (range: 41–85 y). The male:female ratio was 84:15. The patients were followed up every 3 months during the first year, every 6 months in the second year, and every 12 months thereafter by cystoscopy and urine cytology. The median follow-up time was 68 months (range: 10–163 mo).

Recurrence was defined as the appearance of a tumor after the beginning of the treatment, with at least 1 tumor-free cystoscopy and cytology in-between. BCG failure, as opposed to BCG success, was defined as patients who underwent BCG treatment and presented a tumor recurrence. Finally, recurrence-free survival (RFS) was defined as the period between the beginning of the treatment and either recurrence or the most recent tumor-free cystoscopy and cytology. The study was approved by the Ethics Committee of IPO-Porto, and all procedures were performed after patient's informed consent. All clinicopathological information was obtained from patients' clinical records. All tumor samples were revised by an experienced pathologist, with respect to the 2004 World Health Organization grading criteria.

2.2. Immunohistochemistry

TAMs immunohistochemistry was performed with CD68 antibody (Monoclonal Mouse Anti-Human CD68; Clone PG-M1; DAKO) at a dilution of 1:100 in phosphate-buffered saline (PBS), after 1-hour incubation at 37°C. M2 macrophages were assessed with the CD163 antibody (Monoclonal Mouse Anti-Human CD163; Clone 10D6; Novocastra-Leica) at a dilution of 1:100 in PBS, after overnight incubation at 37°C. Immunohistochemical detections were performed using HRP Detection System Kit according to manufacturer's instructions. Diaminobenzidine (ImmPACT DAB, Vector Labs) was used for color development. Hypoxic sites were evaluated using HIF-1α antibody (Monoclonal Mouse Anti-Human HIF-1α; Clone H1667; Abcam) at a dilution of 1:50 in PBS, after overnight incubation at 37°C.

2.3. Immunohischemic scoring

CD68+ and CD163+ macrophages, infiltrating the stroma and tumor areas, were counted by 2 independent observers (L.L. and D.O.) and validated by an experienced pathologist (T.A.). Each specimen was screened at low magnification (×100), and the 5 areas with highest number of positively stained cells (hot spot area) were selected. Photographs were taken, at a ×400 magnification, with a real area of 0.035 mm², and TAMs number was counted. The criteria used for macrophage-specific counting were as follows: (i) cells must present the shape of a macrophage or exhibit the characteristic staining pattern of macrophage, (ii) must present cell nucleus, and (iii) be birefringent if the size is small. Macrophages were evaluated in the tumor stroma, which included the papillary axis, lymphoid aggregates, and stroma, and in tumor islets. Macrophage counts were classified as low or high according to their distribution in percentiles. The expression of HIF-1α was determined based on the percentage of positive cells and stratified into groups as low (negative or 1%–10% nuclear or cytoplasmic staining) and high (10%–50% or >50% nuclear or cytoplasmic staining).

2.4. Statistical analysis

Statistical data analysis was performed using the IBM Statistical Package for Social Sciences for Windows (version 20.0). Chi-square analysis was used to compare categorical variables. Correlation between macrophage counts and clinical variables was performed using Spearman rho test. Kaplan-Meier survival curves were used to evaluate correlation between TAMs counts and RFS; log rank statistical test was used for curves comparison. Multivariate Cox regression analysis was used to assess the effect of TAMs density on the time to recurrence in BCG-treated patients and to adjust for potential confounders.

3. Results

3.1. Association of clinical characteristics with BCG treatment outcome

Approximately 42.4% of the patients presented recurrences, with the median recurrence time of 38.5 months (range: 10–122 mo). The median follow-up time of the patients free of recurrence was 97 months (range: 13–163 mo).

Table 1 shows the clinicopathological parameters and their association with treatment response and RFS.
An association was found between patients’ age and treatment response, as 69% of the patients presenting BCG failure were older than 65 years of age when compared with 43.9% in the BCG success group ($P = 0.013$). Consequently, patients older than 65 years of age presented almost a 3-fold increased risk of recurrence (hazard ratio [HR] = 2.763; 95% CI: 1.431–5.336; $P = 0.002$). Similarly, patients treated with mBCG presented a 50% risk reduction (HR = 0.500; 95% CI: 0.271–0.919; $P = 0.026$). Approximately 70% of the patients successfully treated were submitted to a mBCG scheme (vs. 45% of the patients presenting treatment failure, $P = 0.021$). Interestingly, no association was found between treatment outcome and other characteristics such as gender, tumor stage, number, grade or size, CIS presence, and prior recurrence.

### 3.2. Pattern of macrophage infiltration

We started by evaluating the localization of macrophages within tumor specimens. We observed the presence of CD68$^+$ and CD163$^+$ macrophages in both tumor stroma and in tumor islets. The tumor stroma included the papillary axis, lymphoid aggregates, and stroma. The mean count of CD68$^+$ macrophages was 33 within stroma and 13 within tumor whereas for CD163$^+$ macrophages within stroma and tumor was 24 and 7, respectively. The mean ratio of CD163$^+$/CD68$^+$ macrophages was 51.3% in the tumor and 24.6% in the associated stroma. A moderate to strong CD68$^+$ macrophage stroma infiltration was observed in 46% of the tumors, whereas only 4% of cases had no CD68$^+$ macrophage staining in tumor nest. A high CD163$^+$ macrophages stroma infiltration was observed in 15% tumors, whereas only 8% of tumors presented no CD163 staining in tumor nests.

### 3.3. Correlation between clinical characteristics and CD68$^+$ and CD163$^+$ macrophage counts

The correlation between clinical variables and the macrophages counts are presented in Table 2. Our results evidenced that CD68$^+$ and CD163$^+$ macrophages counts within stroma and tumor were correlated with higher stage, grade, and tumor size (Table 2, $P < 0.05$). Similarly, higher counts of CD68 and CD163 within tumor were observed in

<table>
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<th>Total, n (%)</th>
<th>Responders, n (%)</th>
<th>Nonresponders, n (%)</th>
<th>HR [95% CI]</th>
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<td>≥65</td>
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<td>25 (43.9)</td>
<td>29 (69.0)</td>
<td>2.763 [1.431–5.336]</td>
<td>0.002</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
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<tr>
<td>Male</td>
<td>84 (84.8)</td>
<td>46 (80.7)</td>
<td>38 (90.5)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>15 (15.2)</td>
<td>11 (19.3)</td>
<td>4 (9.5)</td>
<td>0.526 [0.187–1.478]</td>
<td>0.223</td>
</tr>
<tr>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Ta</td>
<td>40 (40.4)</td>
<td>22 (38.6)</td>
<td>18 (42.9)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>59 (59.6)</td>
<td>35 (61.4)</td>
<td>24 (57.1)</td>
<td>0.961 [0.521–1.773]</td>
<td>0.899</td>
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<tr>
<td>Grade</td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Low</td>
<td>39 (39.4)</td>
<td>24 (42.1)</td>
<td>15 (35.7)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>60 (60.6)</td>
<td>33 (57.9)</td>
<td>27 (64.3)</td>
<td>1.410 [0.749–2.654]</td>
<td>0.287</td>
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<td></td>
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<tr>
<td>&lt;3</td>
<td>64 (65.3)</td>
<td>36 (63.2)</td>
<td>28 (68.3)</td>
<td>1.0</td>
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</tr>
<tr>
<td>≥3</td>
<td>34 (34.7)</td>
<td>21 (36.8)</td>
<td>13 (31.7)</td>
<td>0.760 [0.393–1.470]</td>
<td>0.416</td>
</tr>
<tr>
<td>Tumor number</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unifocal</td>
<td>45 (45.5)</td>
<td>29 (50.9)</td>
<td>16 (38.1)</td>
<td>1.0</td>
<td></td>
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<tr>
<td>Multifocal</td>
<td>54 (54.5)</td>
<td>28 (49.1)</td>
<td>26 (61.9)</td>
<td>1.729 [0.924–3.235]</td>
<td>0.087</td>
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<tr>
<td>No</td>
<td>92 (92.9)</td>
<td>53 (93.0)</td>
<td>39 (92.9)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>7 (7.1)</td>
<td>4 (7.0)</td>
<td>3 (7.1)</td>
<td>0.944 [0.291–3.056]</td>
<td>0.923</td>
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<tr>
<td>Recurrence status</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>51 (51.5)</td>
<td>33 (57.9)</td>
<td>18 (42.9)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Recurrent</td>
<td>48 (48.5)</td>
<td>24 (42.1)</td>
<td>24 (57.1)</td>
<td>1.562 [0.847–2.881]</td>
<td>0.153</td>
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<td>BCG schedule</td>
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<td></td>
<td></td>
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<tr>
<td>iBCG</td>
<td>41 (41.4)</td>
<td>18 (31.6)</td>
<td>23 (54.8)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>mBCG</td>
<td>58 (58.6)</td>
<td>39 (68.4)</td>
<td>19 (45.2)</td>
<td>2.002 [1.088–3.684]</td>
<td>0.026</td>
</tr>
</tbody>
</table>

*Wald test. Bold values indicate $P < 0.05$. 

An association was found between patients’ age and treatment response, as 69% of the patients presenting BCG failure were older than 65 years of age when compared with 43.9% in the BCG success group ($P = 0.013$). Consequently, patients older than 65 years of age presented almost a 3-fold increased risk of recurrence (hazard ratio [HR] = 2.763; 95% CI: 1.431–5.336; $P = 0.002$). Similarly, patients treated with mBCG presented a 50% risk reduction of recurrence (HR = 0.500; 95% CI: 0.271–0.919; $P = 0.026$). Approximately 70% of the patients successfully treated were submitted to a mBCG scheme (vs. 45% of the patients presenting treatment failure, $P = 0.021$). Interestingly, no association was found between treatment outcome and other characteristics such as gender, tumor stage, number, grade or size, CIS presence, and prior recurrence.
Table 2
Correlation between clinical parameters and CD68+ and CD163+ macrophages counts, in tumor stroma and tumor nest

<table>
<thead>
<tr>
<th></th>
<th>CD68+ macrophages counts</th>
<th>CD163+ macrophages counts</th>
<th>CD163+/CD68+ macrophage ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Correlation coefficient</td>
<td>P value</td>
<td>Correlation coefficient</td>
</tr>
<tr>
<td>Tumor stroma</td>
<td></td>
<td></td>
<td>Tumor stroma</td>
</tr>
<tr>
<td>Age</td>
<td>0.026</td>
<td>0.802</td>
<td>−0.030</td>
</tr>
<tr>
<td>Sex</td>
<td>−0.046</td>
<td>0.609</td>
<td>0.129</td>
</tr>
<tr>
<td>Stage</td>
<td>0.371</td>
<td>0.009</td>
<td>0.271</td>
</tr>
<tr>
<td>Grade</td>
<td>0.284</td>
<td>0.004</td>
<td>0.194</td>
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<tr>
<td>Size</td>
<td>0.263</td>
<td>0.009</td>
<td>0.290</td>
</tr>
<tr>
<td>Tumor number</td>
<td>−0.081</td>
<td>0.424</td>
<td>−0.023</td>
</tr>
<tr>
<td>CIS</td>
<td>−0.176</td>
<td>0.082</td>
<td>0.072</td>
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<tr>
<td>Primary/ recurrent</td>
<td>−0.120</td>
<td>0.239</td>
<td>−0.273</td>
</tr>
</tbody>
</table>

P value (Chi-square test). Bold values indicate P < 0.05.

primary tumors (Table 2, P < 0.05). Interestingly, higher CD163/CD68 ratios in tumor nests were associated with the CIS presence (Table 2, P < 0.05). No correlations were found regarding gender, age, and multifocality.

3.4. CD68+ and CD163+ macrophages and BCG treatment outcome

To evaluate the CD68+ and CD163+ macrophages infiltration within stroma and tumor areas in the context of BCG treatment outcome, counts were stratified based on percentiles (25th, 50th, and 75th). The same strategy was applied for the CD163/CD68 ratio.

Regarding CD68 expression, no association was found between the counts and treatment outcome. On the contrary, we observed that only CD163+ stroma counts falling within the 25th percentile (>19 macrophages) presented a trend association with treatment outcome; CD163+ macrophage counts in the stroma were classified as low (LS) or high (HS) accordingly. Namely, a higher frequency of patients with BCG failure presented HS (above the 25th percentile) for CD163+ macrophages (83%) when compared with ones where BCG was successful (74%), yet this association was not statistically significant.

We also observed that the LS phenotype was always associated with low macrophage tumor counts (LT, Fig. 1A). Furthermore, the CD163+ LS phenotype (associated with LT) presented BCG treatment response rates similar to the cases with HS and high tumor CD163+ counts (HT, >75th percentile, Fig. 1B). Based on these observations, we decided to merge these 2 groups (LS/LT and HS/HT) and compare it with the cases presenting HS but LT CD163+ counts. Taking into consideration the low CD163+ counts presented by the tumors included in the LT phenotype (<10 macrophages) in comparison with the high stroma counts, the group was termed high stroma-predominant CD163+ macrophage group (HSP, Fig. 1C).

This comparison highlighted that a higher percentage of patients presenting BCG failure had HSP when compared with the ones where the treatment was successful (69% vs. 46%; P = 0.020; sensitivity: 54.4%; specificity: 69.1%; Fig. 2). No association was found regarding CD163+/CD68+ ratio and BCG treatment outcome.

To estimate the influence of higher CD163+ macrophages counts in terms of RFS after BCG treatment, a Kaplan-Meier analysis was performed (Fig. 3). Differences were found in terms of RFS between patients with LS and HS counts of CD163+ macrophages (mean RFS: 126 vs. 92 mo; log rank, P = 0.052; Fig. 3A). Moreover, patients with HSP CD163+ macrophages counts presented a different behavior in terms of RFS (log rank, P = 0.008; Fig. 3B) and a lower RFS (mean = 85 mo) than all the others (mean = 123 mo).

Univariate Cox Regression analysis revealed that patients with tumors presenting CD163+ macrophages HS counts had an increased risk trend for recurrence after treatment, (HR = 2.115; 95% CI: 0.972–4.603; P = 0.059). Moreover, patients with tumors classified as HSP showed a clear 2-fold increased risk of BCG treatment failure (HR = 2.343; 95% CI: 1.197–4.587; P = 0.013).

To assess the individual effect of CD163+ macrophage infiltration in BCG treatment outcome, multivariate analysis was performed. When adjusted to potential confounders, such as age and therapeutic scheme, patients with HS CD163+ and HSP CD163+ counts had more than a 2-fold increased risk of recurrence (HR = 2.402; 95% CI: 1.211–4.763; P = 0.012 and HR = 2.627; 95% CI: 1.340–5.150; P = 0.005; Table 3).

3.5. CD163+ macrophages and expression of HIF-1α

The association between CD163+ macrophages counts within tumor and hypoxia was evaluated based on HIF-1α expression. The expression of HIF-1α is represented by a
nuclear and cytoplasmic staining at the invasive front of the tumor. It was also observed that tumor areas with high density of CD163\(^+\) macrophages expressed high amounts of HIF-1\(\alpha\) (Fig. 4A and B). On the contrary, tumor areas with low CD163\(^+\) macrophages counts, independent of the counts in the stroma, presented lower expression of HIF-1\(\alpha\) (Fig. 4C and D). In addition, although the HSP and LS (that also present LT counts) phenotypes were associated with tumors showing low degree of hypoxia, samples presenting the HS/HT phenotypes are associated with highly hypoxic tumors (\(P < 0.001\)). However, the expression of HIF-1\(\alpha\) was not associated with BCG treatment outcome.

4. Discussion

Although BCG immunotherapy is the primary treatment option for intermediate/high-risk bladder tumors, the failure rate is more than 30\% [1]. Therefore the identification of biomarkers able to predict treatment failure and to provide an early identification of those patients better served by alternative therapies is crucial for the management of this disease [4]. There are some biomarkers emerging in the literature, but at the moment, none could be set as reliable to translate into clinical practice [21,22].

One of the biomarkers with consistent results was the presence of TAMs in bladder tumors before BCG treatment, although more studies are needed to validate its relevance [10–12,21,22]. On the contrary, the marker used was CD68, a lineage marker found in both M1 and M2 macrophages [7]. Several authors showed that to accurately determine TAMs influence in prognosis and treatment outcome, M2-specific markers, such as CD163, should be used [23–26]. To address this subject, we investigated the influence of TAMs (CD68\(^+\)) and also the M2-polarized macrophage phenotype (CD163\(^+\)), in the context of BCG treatment outcome. Taking into consideration that the microenvironment plays a determinant role in the modulation of the macrophage lineages, we evaluated independently the tumor and the stroma.

We started by seeking associations between the patient’s clinicopathological characteristics and the BCG treatment outcome and found that it was influenced by age and treatment scheme (iBCG and mBCG). Therefore, these
variables were considered potential confounders and were taken into account in multivariate analysis models to assess the influence of TAMs in BCG outcome. We also observed that CD68⁺ and CD163⁺ macrophages counts in both the stroma and the tumor were correlated with higher stage, grade, and tumor size. Similar results were observed by other authors for bladder cancer using CD68 [12,27]. The CD163⁺ macrophages identification has also been associated with poor prognosis in several types of cancer [23,28]; however, this is the first study suggesting that the M2 subtype may be a characteristic of bladder tumors with high risk of recurrence or progression.

Three studies have been presented supporting the idea that a higher density of macrophages in the tumor and its surroundings may be associated with BCG treatment failure [10–12]. However, we observed no associations between CD68⁺ macrophage counts in stroma and in tumor nests and the outcome. Even though contradictory, these results may stem from the fact that 2 of these studies were conducted in a low number of samples (27 and 46) and did not take into consideration the localization of the macrophages. A third study involving a localization-based analysis in CIS described that cases with a low density of tumor CD68⁺ macrophages presented higher recurrence-free rate. However, the reduced number of CIS in our series does not allow an accurate comparison. Nevertheless, whether macrophage density influences treatment outcome in different ways depending on the histology of the tumor warrants a deeper evaluation.

In contrast, we observed that a high density of M2-polarized macrophage counts in the stroma but not in the tumor related with BCG treatment failure. Interestingly, cases presenting a high density of macrophages in the tumor presented a more favorable outcome. Furthermore, these cases behaved similarly to those presenting an overall low density of M2 macrophages (LT/LS). These results suggest that M2 macrophages may be affecting treatment outcome in different ways possibly owing to the influence of differentiated microenvironmental stimuli in the stroma and the tumor.

As TAMs may not only be found in vascularized stroma but also significantly accumulate in hypoxic areas within the tumor [19,29,30], we hypothesized that differences in CD163 expression between tumor areas could be owing to hypoxia. This was confirmed by the association between high tumor CD163⁺ macrophage counts and high expression of the hypoxia marker HIF-1α within tumor areas; conversely, in specimens with HSP CD163⁺ counts (and LT, respectively), HIF-1α expression within tumor areas was low. These observations suggest that hypoxic conditions may dictate the accumulation of CD163⁺ macrophages in bladder tumor areas.

Hypoxia not only seems to dictate the accumulation of macrophages in the tumor but may also modulate the
M2-macrophage phenotype. In particular, hypoxia is known to enhance the expression of angiogenic factors, producing high amounts of vascular endothelial growth factor and other proinflammatory cytokines like tumor necrosis factor (TNF)−α, IL-1β, macrophage migration inhibitory factor (MIF), and COX2 that act as promoters of a Th1-mediated response known to favor BCG action [19]. On the contrary, normoxia may favor the M2 immunosuppressive phenotype and the down-regulation of molecules implicated in immunological activation such as IL-12, IL-18, IL-1β, and tumor necrosis factor-α [5]. This selective pressure also up-regulates the expression of Th2-type cytokines, as well as IL-10, IL-1RA, and transforming growth factor (TGF)−β, some of which have been associated with a lack of response to BCG treatment [16]. Based on these observations, we hypothesize that hypoxic conditions may favor the accumulation of M2-polarized macrophages in the tumor and also promote their angiogenic phenotype, ultimately leading to a better treatment outcome. Conversely, nonhypoxic or low-hypoxic conditions (low HIF-1α) decrease the density of macrophages in the tumor area, maintaining them in the stroma area. We may hypothesize that these macrophages present the immunosuppressive phenotype, which in part may explain the higher treatment failure.

Although our results point out that HSP CD163+ macrophage counts is a good predictor of recurrence after BCG treatment, some limitations need to be overcome to use this biomarker in clinical practice. Namely, efforts should be taken to make the macrophage counts reproducible. It would be important to evaluate different counting methodologies, especially involving image acquisition and automatic counting software to create a standard technique and cutoff values. Moreover, this a preliminary study with 99 patients that requires validation in larger series and different cohorts. A careful evaluation of the influence of hypoxia and other microenvironment factors in the modulation of macrophage phenotypes is also needed in this context.

Altogether, our results indicate that discrimination of M2 macrophages (CD163+) is a better indicator of treatment failure than the overall macrophage counting given by CD68. Moreover, our observations suggest that only M2 macrophages under normoxic conditions may exert an inhibitory effect on BCG immunotherapy, possibly owing to its immunosuppressive phenotype.

References


3.1. Tumor–associated Markers

3.1.1. Paper III

Response of high-risk of recurrence/progression bladder tumours expressing sialyl–Tn and sialyl–6–T to BCG immunotherapy


British Journal of Cancer 2013
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Response of high-risk of recurrence/progression bladder tumours expressing sialyl-Tn and sialyl-6-T to BCG immunotherapy

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Background: High risk of recurrence/progression bladder tumours is treated with Bacillus Calmette-Guérin (BCG) immunotherapy after complete resection of the tumour. Approximately 75% of these tumours express the uncommon carbohydrate antigen sialyl-Tn (Tn), a surrogate biomarker of tumour aggressiveness. Such changes in the glycosylation of cell-surface proteins influence tumour microenvironment and immune responses that may modulate treatment outcome and the course of disease. The aim of this work is to determine the efficiency of BCG immunotherapy against tumours expressing sTn and sTn-related antigen sialyl-6-T (s6T).

Methods: In a retrospective design, 94 tumours from patients treated with BCG were screened for sTn and s6T expression. In vitro studies were conducted to determine the interaction of BCG with high-grade bladder cancer cell line overexpressing sTn.

Results: From the 94 cases evaluated, 36 had recurrence after BCG treatment (38.3%). Treatment outcome was influenced by age over 65 years (HR = 2.668; (1.344–5.254); P = 0.005), maintenance schedule (HR = 0.480; (0.246–0.936); P = 0.031) and multifocality (HR = 2.065; (1.033–4.126); P = 0.040). sTn or s6T expression was associated with BCG response (P = 0.024; P < 0.0001) and with increased recurrence-free survival (P = 0.001). Multivariate analyses showed that sTn and/or s6T were independent predictive markers of recurrence after BCG immunotherapy (HR = 0.296; (0.148–0.594); P = 0.001). In vitro studies demonstrated higher adhesion and internalisation of the bacillus to cells expressing sTn, promoting cell death.

Conclusion: s6T is described for the first time in bladder tumours. Our data strongly suggest that BCG immunotherapy is efficient against sTn- and s6T-positive tumours. Furthermore, sTn and s6T expression are independent predictive markers of BCG treatment response and may be useful in the identification of patients who could benefit more from this immunotherapy.

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Bladder cancer is the fifth most common cancer in Western society (van Rhijn et al., 2009), with the highest recurrence rate among solid tumours and poor prognosis when the tumour invades the muscularis propria (Babjuk et al., 2011). To reduce the risk of recurrence and progression to muscle invasion, non-muscle invasive high-grade tumours, multifocal and recurrent lesions are submitted to intravesical instillations with live attenuated Bacillus Calmette-Guérin (BCG) after complete transurethral resection of bladder tumour (TURBT; Babjuk et al., 2011). Although the management of the disease has significantly improved with this therapeutics, 30–40% of the patients relapse and approximately 15% progress to muscle invasive tumours (Palou Redorta, 2006). Predicting patients who could be best served by an alternative treatment or early cystectomy, would avoid progression, reduce disease charge and decrease health expenses. It is consensual that the integration of multiple biomarkers may hold predictive value; still such biomarker panel remains to be established (Lima et al., 2012; Zuiverloon et al., 2012).

The exact mechanism by which BCG mediates anti-bladder cancer immunity remains elusive (Alexandroff et al., 2010). However, the capability of the bacillus to recognise and efficiently bind to tumour cells has a determinant role in the therapeutics outcome (Alexandroff et al., 2010). The bacillus is then internalised triggering tumour cell apoptosis or host adaptive immune responses (Becich et al., 1991; Ratliff, 1992). The bacterial adhesion, fibronectin attachment protein (FAP), was recognised as the main factor mediating BCG attachment and internalisation by bladder tumour cells (Sinn et al., 2008; Alexandroff et al., 2010). FAP binds to \( \alpha5\beta1 \) integrins expressed by tumour cells via a fibronectin bridge and to be responsible for the uptake of BCG–fibronectin–integrin complexes (Sinn et al., 2008; Alexandroff et al., 2010). Malignant transformations may be accompanied by a premature stop in the O-glycosylation of proteins by sialylation, originating the sialyl-Tn (sTn, Neu5Acα2-6GalNAcα-O-Ser/Thr) and sialyl-6-T antigens (s6T, Galβ1-3( Neu5Acα2-6)GalNAcα-O-Ser/Thr; Dall’Olio et al., 2012). We recently reported that approximately 75% of high-grade bladder tumours, presenting elevated proliferation indexes and high risk of recurrence/progression expressed sTn (Ferreira et al., 2013). sTn expression enhanced the invasive capability of bladder cancer cells and was considered a surrogate biomarker of tumour aggressiveness (Ferreira et al., 2013). Hence, efficient therapies to manage these tumours are needed to avoid disease progression and poor outcomes.

sTn expression is known to interfere with cell–cell adhesion, cell–matrix interaction, including integrin-fibronectin binding, module cell morphology (Clement et al., 2004; Julien et al., 2006; Pinho et al., 2007) and immune responses (Gilewski et al., 2007; Julien et al., 2009; Takamiya et al., 2013). Thus, we hypothesise it may modulate BCG attachment to tumour cells and/or immune response and consequently influence BCG immunotherapy outcome. sTn is also a biomarker of concomitant molecular alterations that may further determine the tumour behaviour (Ohno et al., 2006). As such, this work is devoted to evaluating the response of sTn-positive bladder tumours to BCG immunotherapy. The sTn structurally related antigen was also evaluated for the first time in the context of bladder cancer.

**MATERIALS AND METHODS**

**Patient cohort.** This study was performed in a retrospective series of 94 cases with high-risk non-muscle invasive bladder cancer. Patients were treated with TURBT and then submitted to BCG immunotherapy in the Portuguese Oncology Institute of Porto, between 1998 and 2006. No second-look TURBT was performed, although the majority of the samples had muscularis propria tumour free. All received intravesical instillation of BCG for 6 consecutive weeks (induction BCG schedule, iBCG) and 56.4% were submitted to maintenance BCG schedule (iBCG + maintenance protocol with two weekly instillations every 3 months during 2 years, mBCG). The iBCG group includes patients treated before the European Association of Urology guidelines recommending the mBCG (Oosterlinck et al., 2006) scheme and patients showing significant intolerance to long BCG treatment.

The male/female sex ratio was of 78:16. The patients were followed every 3 months for the first year, every 6 months for the second year and every 12 months thereafter by cystoscopy and urine cytology. Recurrence was defined as the appearance of a tumour once the treatment has begun, with at least one tumour-free cystoscopy and cytology in-between. These recurrences were also available for study. The non-responders were defined as patients submitted to BCG treatment with tumour recurrence. Finally, recurrence-free survival (RFS) was defined as the period of time between the beginning of treatment and recurrence or the most recent tumour-free cystoscopy and cytology. All procedures were performed after patient’s informed consent and approved by the Ethics Committee of IPO-Porto. All clinicopathological information was obtained from patients’ clinical records. All tumour samples were revised by a pathologist, regarding 2004 WHO grading criteria.

**Expression of sTn in bladder tumours.** Formalin-fixed paraffin-embedded tissue sections were screened for sTn by immunohistochemistry using the avidin/biotin peroxidase method, as described by Ferreira et al. (2013). sTn expression was evaluated with anti-sTn mouse monoclonal antibody clone TKH2 (Ferreira et al., 2013). The s6T antigen was evaluated in sTn-negative tumours using the same antibody, after treatment with a recombinant \( \beta1-1,3 \)-galactosidase from *Xanthomonas campestris* (R&D systems, Minneapolis, MN, USA) for 1 h at 37°C. This enzyme removes the O-3-linked Gal residues exposing the sTn antigen (Figure 1A).

Both antigens were assessed double-blindly by three independent observers. Upon disagreement, the slides were reviewed, until a consensus was reached. Tumours were classified as positive when immunoreactivity of anti-sTn TKH2 antibody was observed.

**Structural assignments were validated by a combination of enzymatic treatments.** For sTn, positive tissues were first treated with a \( \alpha1,2 \)-neuraminidase from *Clostridium perfringens* (Sigma-Aldrich, St Louis, MO, USA) for 2 h at 37°C to remove the sialic acid and then screened for sTn expression. For s6T, positive tissues were primarily incubated with the \( \beta1-1,3 \)-galactosidase, followed by incubation with the neuraminidase. The absence or decrease in immunoreactivity of TKH2 monoclonal antibody confirmed the presence of these structures.

**Adhesion and internalisation of BCG to bladder cancer cell line**

**Cell lines culture.** The human bladder cancer cell line MCR and the transduced variants of MCR (MCRnc and MCRsTn \( ^+ \)) were grown as described by Videira et al. (2009). The MCRsTn \( ^+ \) cell line results from the stable transduction of MCR cells with a lentivirus expressing the coding region of the human ST6GalNAc1 gene, the enzyme responsible by the biosynthesis of sTn (Ferreira et al., 2013). The MCRnc cell line, not expressing sTn, was used as control (Ferreira et al., 2013).

**Bacterial strain and labelling.** BCG strain RIVM (Medac, Hamburg, Germany) was used in this study. Briefly, 10\(^8\) viable BCG cells were labelled with 10 \( \mu \)g fluorescein isothiocyanate (FITC; Invitrogen, Carlsbad, CA, USA) in 1 ml of 50 m\( \mu \)l sodium carbonate buffer (pH 9.2) for 30 min at 20°C. The labelled bacteria (BCG-FITC) were washed three times with PBS containing 0.05% of Tween-80 (Sigma-Aldrich) and centrifuged for 10 min at 13.000 g to remove excess FITC.
MCR cells were plated into 24-well plates at 0.2×10^6 cell per well. At confluence, cells were washed and BCG-FTIC was added to the cultures at a ratio of 10:1 (BCG/cells). After 2, 6 or 24 h incubation time, the cells were gently washed to remove the excess BCG, harvested and centrifuged at 350 g for 5 min. In order to differentiate between internalised and surface-bound BCG, trypan blue was added to quench surface-attached fluorescence bacteria. Flow cytometry was performed on a FACSCalibur (BD Biosciences, San Jose, CA, USA) and data were analysed using the Flowing v2.4 software (Turku Center for Biotechnology, Turku, Finland).

**BCG adhesion and internalisation assay.** MCR cells were plated into 24-well plates at 0.2×10^6 cell per well. At confluence, cells were washed and BCG-FTIC was added to the cultures at a ratio of 10:1 (BCG/cells). After 2, 6 or 24 h incubation time, the cells were gently washed to remove the excess BCG, harvested and centrifuged at 350 g for 5 min. In order to differentiate between internalised and surface-bound BCG, trypan blue was added to quench surface-attached fluorescence bacteria. Flow cytometry was performed on a FACSCalibur (BD Biosciences, San Jose, CA, USA) and data were analysed using the Flowing v2.4 software (Turku Center for Biotechnology, Turku, Finland).

Estimation of cell viability after exposure to BCG. The influence of BCG treatment on MCR cell viability after the 24 h of exposure was assessed through the visualisation of morphologic changes by flow cytometry. Dot plots of forward-angle light scatter (FSc) vs side-angle light scatter (SSc) of MCR cells before and after exposure to BCG were analysed using the Flowing v2.4 software (Turku Center for Biotechnology).

**RESULTS**

Clinicopathological features and BCG treatment outcome. From the 94 cases evaluated, 36 had recurrence after BCG...
treatment (38.3%). The median follow-up time of the patients free of recurrence was 68.5 months (range: 6.0–135.0) and the median time of recurrence was 38.5 months (range: 10.0–122.0). The median follow-up time considering all the cases under analysis was 61.0 months (range: 6.0–135.0). Table 1 summarises patients and tumour clinicopathological features and its association with BCG response and RFS after treatment. We found that 61.1% of the non-responders were over 65 years old at the time of tumour resection, whereas only 36.2% of responders were over 65 years old ($P = 0.018$).

Furthermore, patient over 65 years of age have approximately three-fold increased risk of recurrence (HR $= 2.668$; (1.344–5.254); $P = 0.005$). Moreover, it was observed a higher percentage of patients treated only with iBCG in the non-responder group when compared with the responder group (58.3% vs 34.5%; $P = 0.018$). Therefore, patients treated with mBCG scheme showed a 52% reduced risk of recurrence (HR $= 0.480$; (0.246–0.936); $P = 0.031$). It was also found that patients with multifocal tumours had an increased risk of recurrence after BCG treatment (HR $= 2.065$; (1.033–4.126); $P = 0.040$). No association was found for other characteristics, such as gender, tumour stage, grade or size, CIS presence and prior recurrence.

### Expression of sTn and s6T and association with clinicopathological features.

Approximately 66% of the studied bladder tumours were sTn positive (Figure 1A and B) and in all cases the antigen was observed in more than 5% of the tumour area. Additionally, 10 out of 32 sTn negative cases were positive for s6T (Figure 1C), which is structurally related to sTn. However, s6T assumed a more diffuse expression that did not exceed 5% of the tumour area in all cases.

The expression of sTn alone or in combination with s6T (sTn/s6T) was associated with high-grade tumours ($P = 0.007$; $P = 0.037$) and also with primary tumours ($P = 0.001$; $P = 0.039$). No association was found for other characteristics, such as gender, tumour stage, grade or size, CIS presence and prior recurrence.

### Table 1. Relation between patients clinical and tumour characteristics and response to BCG treatment and time to recurrence

<table>
<thead>
<tr>
<th>Variables</th>
<th>Total n (%)</th>
<th>Responders n (%)</th>
<th>Non-responders n (%)</th>
<th>$P^a$</th>
<th>HR (95% CI)</th>
<th>$P^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
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<td>51 (54.3)</td>
<td>37 (63.8)</td>
<td>14 (38.9)</td>
<td>0.018</td>
<td>1.0</td>
<td>2.668 (1.355–5.254)</td>
</tr>
<tr>
<td>≥65</td>
<td>43 (45.7)</td>
<td>21 (36.2)</td>
<td>22 (61.1)</td>
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</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Male</td>
<td>78 (83.0)</td>
<td>47 (81.0)</td>
<td>31 (86.1)</td>
<td>0.524</td>
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<td>0.883 (0.342–2.283)</td>
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<tr>
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<td>11 (19.0)</td>
<td>5 (13.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ta</td>
<td>40 (42.6)</td>
<td>23 (39.7)</td>
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<td>0.838 (0.435–1.613)</td>
</tr>
<tr>
<td>T1</td>
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<td>35 (60.3)</td>
<td>19 (52.8)</td>
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</tr>
<tr>
<td>Low</td>
<td>38 (40.4)</td>
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<td>1.295 (0.661–2.537)</td>
</tr>
<tr>
<td>High</td>
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<td>34 (58.6)</td>
<td>22 (61.1)</td>
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<tr>
<td><strong>Size (cm)</strong></td>
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<td></td>
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<td>&lt;3</td>
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<td>0.787 (0.384–1.613)</td>
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<td>≥3</td>
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<td>20 (34.5)</td>
<td>11 (31.4)</td>
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<tr>
<td><strong>Tumour number</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unifocal</td>
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<td>13 (36.1)</td>
<td>0.140</td>
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<td>2.065 (1.033–4.126)</td>
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<td>Multifocal</td>
<td>43 (45.7)</td>
<td>28 (48.3)</td>
<td>23 (63.9)</td>
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</tr>
<tr>
<td><strong>CIS</strong></td>
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</tr>
<tr>
<td>No</td>
<td>88 (93.6)</td>
<td>54 (93.1)</td>
<td>34 (94.4)</td>
<td>1.000</td>
<td>1.0</td>
<td>0.783 (0.188–3.267)</td>
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<td>Yes</td>
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<td>4 (6.9)</td>
<td>2 (5.6)</td>
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<td></td>
</tr>
<tr>
<td><strong>Recurrence status</strong></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Primary</td>
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<td>31 (53.4)</td>
<td>17 (47.2)</td>
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<td>1.0</td>
<td>1.327 (0.686–2.564)</td>
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<tr>
<td>Recurrent</td>
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<td>27 (46.6)</td>
<td>19 (52.8)</td>
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<td><strong>BCG schedule</strong></td>
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<td></td>
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</tr>
<tr>
<td>iBCG</td>
<td>41 (43.6)</td>
<td>20 (34.5)</td>
<td>21 (58.3)</td>
<td>0.023</td>
<td>1.0</td>
<td>0.480 (0.246–0.936)</td>
</tr>
<tr>
<td>mBCG</td>
<td>53 (56.4)</td>
<td>38 (65.5)</td>
<td>15 (41.7)</td>
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</tbody>
</table>

Abbreviations: BCG = Bacillus Calmette-Guérin; CI = confidence interval; CIS = Carcinoma in situ; HR = hazard ratio; iBCG = induction BCG; mBCG = maintenance BCG.

Bold values indicate $P < 0.05$.

$^a$Chi-square test.

$^b$Wald test.
negative tumours prior treatment had sTn-negative recurrences. From the 15 non-responder patients who had sTn-positive tumours, 40% presented sTn-negative recurrences.

Kaplan-Meier analysis was used to evaluate if sTn with or without s6T influenced the RFS after BCG treatment. No differences were found regarding sTn expression alone ($P = 0.603$; Figure 2A). However, when sTn and s6T were considered together, significant differences were found ($P = 0.001$; Figure 2B). Patients with sTn/s6T-positive tumours had higher RFS than negative tumours (100.1 vs 63.2 months).

A Cox regression analysis, adjusted to age, tumour number and treatment scheme, was performed to assess the individual effect of these antigens in recurrence after BCG. Patients with sTn-positive tumours presented a trend to a lower-risk recurrence after BCG ($HR = 0.544; 95\% CI: (0.275–1.076); P = 0.080; Table 4$). Likewise, cases positive for sTn/S6T showed a significant lower risk of recurrence ($HR = 0.296; 95\% CI: (0.148–0.594); P = 0.001$).

**Table 2. Association between sTn and s6T antigens and clinicopathological characteristics**

<table>
<thead>
<tr>
<th>Variables</th>
<th>sTn</th>
<th>sTn + s6T</th>
<th>$P^*$</th>
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<tr>
<td>Age (years)</td>
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</tr>
<tr>
<td>&lt;65</td>
<td>21 (65.6)</td>
<td>13 (59.1)</td>
<td>0.112</td>
</tr>
<tr>
<td>≥65</td>
<td>11 (34.4)</td>
<td>9 (40.9)</td>
<td>0.603</td>
</tr>
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</tr>
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<td>Male</td>
<td>27 (84.4)</td>
<td>17 (77.3)</td>
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</tr>
<tr>
<td>Female</td>
<td>5 (15.6)</td>
<td>5 (22.7)</td>
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<tr>
<td>Grade</td>
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<tr>
<td>Low</td>
<td>19 (59.4)</td>
<td>13 (59.1)</td>
<td>0.007</td>
</tr>
<tr>
<td>High</td>
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<td>9 (40.9)</td>
<td>0.042</td>
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<td>Size (cm)</td>
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<td>&lt;3</td>
<td>23 (74.2)</td>
<td>16 (76.2)</td>
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<tr>
<td>≥3</td>
<td>8 (25.8)</td>
<td>5 (23.8)</td>
<td>0.431</td>
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<td>Tumour number</td>
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<td>7 (31.8)</td>
<td>0.249</td>
</tr>
<tr>
<td>Multifocal</td>
<td>20 (62.5)</td>
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<td>0.134</td>
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<td>CIS</td>
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<td></td>
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<tr>
<td>No</td>
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<td>19 (86.4)</td>
<td>0.406</td>
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<td>3 (9.4)</td>
<td>3 (13.6)</td>
<td>0.112</td>
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<td>Recurrence status</td>
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<td>Primary</td>
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<td>7 (31.8)</td>
<td>0.001</td>
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<tr>
<td>Recurrent</td>
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<td>15 (68.2)</td>
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<tr>
<td>BCG schedule</td>
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<tr>
<td>iBCG</td>
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<tr>
<td>mBCG</td>
<td>18 (56.2)</td>
<td>11 (50.0)</td>
<td>0.490</td>
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</table>

**Table 3. sTn and sTn/s6T frequencies and risk of recurrence after BCG therapy**

<table>
<thead>
<tr>
<th>sTn and/or s6T</th>
<th>Responders n (%)</th>
<th>Non-responders n (%)</th>
<th>$P$ value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>15 (25.9)</td>
<td>17 (47.2)</td>
<td>0.034</td>
</tr>
<tr>
<td>Positive</td>
<td>43 (74.1)</td>
<td>19 (52.8)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3. sTn and sTn/s6T frequencies and risk of recurrence after BCG therapy**

<table>
<thead>
<tr>
<th>sTn and/or s6T</th>
<th>Responders n (%)</th>
<th>Non-responders n (%)</th>
<th>$P$ value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>6 (10.5)</td>
<td>16 (43.2)</td>
<td>0.00001</td>
</tr>
<tr>
<td>Positive</td>
<td>51 (89.5)</td>
<td>21 (56.8)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: BCG = Bacillus Calmette-Guérin; iBCG = induction BCG; CIS = Carcinoma in situ; mBCG = maintenance BCG.

*Chi-square test.

Kaplan-Meier analysis was used to evaluate if sTn with or without s6T influenced the RFS after BCG treatment. No differences were found regarding sTn expression alone (Figure 2A). However, when sTn and s6T were considered together, significant differences were found ($P = 0.001$; Figure 2B). Patients with sTn/s6T-positive tumours had higher RFS than negative tumours (100.1 vs 63.2 months).

A Cox regression analysis, adjusted to age, tumour number and treatment scheme, was performed to assess the individual effect of these antigens in recurrence after BCG. Patients with sTn-positive tumours presented a trend to a lower-risk recurrence after BCG ($HR = 0.544; 95\% CI: (0.275–1.076); P = 0.080; Table 4$). Likewise, cases positive for sTn/s6T showed a significant lower risk of recurrence ($HR = 0.296; 95\% CI: (0.148–0.594); P = 0.001$).

**BCG interaction with MCRsTn$^+$ cell line.** To evaluate the affinity of BCG for cells expressing sTn, we set up in vitro assays with MCRnc and MCRsTn$^+$ cell lines, two genetically modified variants of the original MCR bladder cancer cell line. MCRnc cells
were transduced with an empty vector and MCRsTn⁺ with the cDNA coding for the ST6GalNAc.I sialyltransferase. The phenotype of these transduced variants was previously described by Ferreira et al (2013). MCRsTn⁻ presents a continuous and stable expression of the antigen sTn, whereas the MCRnc does not as shown in Supplementary Figure 1 (in the supplementary section).

MCRsTn⁻ and its negative control MCRnc where then treated with BCG. Fluorescent-labelled BCG was internalised significantly over time, with a significant higher internalisation after 6 h incubation, when comparing with 2 h incubation (Figure 3A). Interestingly, after 6 h, MCRsTn⁺ cells showed a tendency to internalise higher amounts of BCG than MCRnc cells (Figure 4A and B). Small amounts of BCG are internalised, resulting in small shifts of FITC fluorescence of the cells after internalisation (Figure 4B).

We then evaluated apoptosis status in MCR cells after 6 h of BCG challenging, using Annexin V an earlier labelling marker for cells undergoing apoptosis. It was observed a consistent tendency for a higher cell death in MCRsTn⁻ after BCG challenging (higher Annexin V labelling—MFI_MCRsTn⁻ = 2560) compared with MCRnc cells (MFI_MCRnc = 2640). In addition, a population of cells presenting stronger Annexin V labelling was also observed after 6 h BCG, which was higher (7%) in MCRsTn⁺ cells than MCRnc (4%; Figure 4A). After a longer period of BCG challenging (24 h), MCRsTn⁺ cells significantly decreased their size and granularity (80% of FSC<sub>low</sub>SSC<sub>low</sub>), which is usually typical of a rupture of plasma membrane and leakage of the cell’s contents (Figure 4B).

Conversely, MCRnc cells underwent little physical changes, presenting only 10% of FSC<sub>low</sub>SSC<sub>low</sub> (Figure 4B). Furthermore, the internalisation of BCG by viable MCRsTn⁻ cells at 24 h was markedly increased in relation to the controls (MCRnc; Supplementary Figure 2, supplementary section), therefore, in accordance with the observations made for 6h.

These results present evidence that both BCG internalisation and loss of cell viability are correlated and both features are enhanced in cells expressing sTn antigen. Altogether, these findings suggest that the bacillus acts more efficiently in cells expressing sTn probably due to its higher internalisation.

**DISCUSSION**

A significant percentage of high risk of recurrence/progression bladder tumours, conservatively treated with BCG immunotherapy after surgery, express cell-proteins yielding the sTn antigen (Ferreira et al, 2013). Despite the malignant potential of these tumours (Ferreira et al, 2013) and evidences that sTn expression may modulate the cell–BCG interaction (Clement et al, 2004; Julien et al, 2006; Pinho et al, 2007) as well as immune responses (Gilewski et al, 2007; Julien et al, 2009; Takamiya et al, 2013), nothing is known about the way patients exhibiting sTn-positive tumours respond to treatment.

We first observed that treatment outcome was influenced by age, treatment scheme and tumour multifocality, as showed in other reports (Bohle and Bock, 2004; Joudi et al, 2006; Fernandez-Gomez et al, 2008; Malmstrom et al, 2009; Kohimoto et al, 2010; Ajili et al, 2012). To overcome the samples heterogeneity, these variables were taken into account in multivariate analysis models to assess the influence of tumour-associated glycans in BCG response.

We also found that sTn expression was associated with high-grade tumours, which is in agreement with our previous observations (Ferreira et al, 2013). STn expression was also associated with primary tumours. However, this may result from fact that the percentage of high-grade tumours was much higher among primary cases (90%) than in recurrences (67%). Altogether, these data reinforce the notion that sTn is a surrogate marker of high-risk bladder cancer.

Furthermore, we report for the first time that bladder tumours express the sTn-related carbohydrate antigen s6T. From the structural point of view, s6T may be considered a form of the sTn antigen masked by a Gal residue O-3 linked to the GalNAc moiety. To our knowledge, s6T has only been observed in human cancer cell lines (Marcos et al, 2004; Julien et al, 2006; Pinho et al, 2007). We also describe that the incubation of tissue

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**Table 4. Multivariate analysis and risk estimation of sTn and s6T influence on BCG therapy outcome**

<table>
<thead>
<tr>
<th></th>
<th>HR*</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>1.0</td>
<td>Referent</td>
<td>0.080</td>
</tr>
<tr>
<td>Positive</td>
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<td>sTn + s6T</td>
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</tr>
<tr>
<td>Negative</td>
<td>1.0</td>
<td>Referent</td>
<td>0.001</td>
</tr>
<tr>
<td>Positive</td>
<td>0296</td>
<td>0148-0.594</td>
<td>0.001</td>
</tr>
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</table>

Abbreviations: BCG = Bacillus Calmette-Guerin; CI = confidence interval; HR = hazard ratio.

*Adjusted for age, tumour number and BCG schedule.

---

**Figure 2. Effect of sTn and s6T expression in recurrence-free survival (RFS).** Kaplan–Meier analysis to evaluate the association between RFS in the studied patients and: (A) sTn expression; (B) STn plus s6T presence (sTn/s6T). Comparison performed by log-rank test (A: P = 0.157; B: P = 0.001); ± censored sTn or sTn/s6T-negative tumours; ◆ censored sTn or sTn/s6T-negative tumours.
sections with a β-(1-3)-galactosidase removed the O-3-linked Gal residue exposing the sTn antigen, allowing its detection by immunohistochemistry with the same antibody used for sTn without significant time consumption. This approach may now be applied to estimate s6T expression in other solid tumours. Studies in vitro studies have shown that s6T expression influences tumour microenvironment similarly to sTn (Pinho et al., 2007). Therefore, both antigens were evaluated in the context of BCG immune response.

sTn expression alone or in combination with that of s6T was associated with lower recurrence rates after BCG. Furthermore, patients expressing sTn and/or s6T presented longer RFS and these antigens were found to be independent predictive markers of reduced recurrence after BCG immunotherapy. Moreover, recurrences after treatment displayed a reduced expression of sTn antigens suggesting that BCG may be more effective against cells expressing these glycans. Thus, sTn-like O-glycans should be considered in a biomarker panel directed to predict BCG treatment outcome.

sTn-expressing cells presented enhanced capacity for BCG adhesion and internalisation and higher BCG-mediated cell death in vitro. This strongly suggests that sTn expression favours BCG-mediated elimination of tumour cells, which may, in part, explain the high correlation between these glycans and treatment response. The exact mechanism underlying these observations remains unknown. However, BCG is known to bind fibronectin–α5β1 integrin complexes promoting a rearrangement of cytoskeletal actin in host cells, which results in the phagocytosis of the pathogen (Chen et al., 2003; Alexandroff et al., 2010). Clement et al. (2004) described that integrin β1 chains express sTn and that the antigen enhanced integrin-fibronectin adhesion. Thus, sTn may contribute to a more efficient binding of the bacillus to tumour cells and consequently a better response to BCG. The bacillus may also directly target cells in a fibronectin-independent manner (Schneider et al., 1994), namely by binding sTn or specific carbohydrates residues such as α2,6 sialic acids. On the other hand, sTn is a product of incomplete O-glycosylation of proteins (Dall'Olio et al., 2012), a reduction in the structural complexity of
O-glycan may allow the bacillus to bind more efficiently to tumour cells. The expression of sTn also induces profound morphological changes in tumour cells (Clement et al, 2004; Pinho et al, 2007) that may further contribute to the bacillus attachment. A deeper understanding of these phenomena may provide new insights on the mechanism of action of BCG and ways to improve the therapeutics.

The efficiency of BCG therapy among sTn-positive tumours may also be related with the immunogenic properties of the antigen (Gilewski et al, 2007; Julien et al, 2009; Takamiya et al, 2013). sTn-based vaccines elicit strong immune responses against breast, ovarian and colorectal cancers in animal models (Gilewski et al, 2007; Julien et al, 2009). Still, using BCG as an immunologic adjuvant was considered essential for the development of both humoral and cellular immune responses against sTn (Miles et al, 1996; O’Boyle et al, 2006). Similarly, instillations with BCG may be enhancing immune responses against these tumour-associated glycans. On the other hand, we observed a higher internalisation of BCG by sTn-expressing cells. After BCG internalisation, malignant cells often act as antigen-presenting cells contributing to the immunologic cascade that leads to tumour clearance (Ratliff, 1992; Alexandroff et al, 2010). Therefore, one may also hypothesise that the expression of this particular glycan may increase the probability of generating BCG antigen-presenting cells in the tumour niche. Understanding these events may allow developing alternative carbohydrate-based immunotherapies for bladder cancer and should be addressed in future studies.

Although this is a retrospective study involving a limited number of cases, our results strongly suggest that BCG immunotherapy is efficient against sTn-positive tumours. Even though we have not determined the exact mechanisms underlying this event, we demonstrated that BCG adhesion and internalisation is higher for sTn-positive cells in vitro, further reinforcing tumour findings.

In conclusion, it has been demonstrated that sTn and s6T antigens correlated with a better response to this treatment. These glycans, in association with other BCG response-associated molecules, may allow the establishment of a predictive panel that can guide therapeutic decision.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES


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Supplementary Information accompanies this paper on British Journal of Cancer website (http://www.nature.com/bjc)
Figure S1. Flow cytometry analyses of transduced MCR cells. Both mock transduced MCRnc (light grey histogram) and \(ST6GalNAc.I\)-transduced MCRsTn\(^+\) (dark grey histogram) cell lines were stained with the anti-sTn antibody. Over 95\% of the MCRsTn\(^+\) cells expressed the sTn antigen, while MCRnc present negative staining.
Figure S2. Percentage of BCG cells adhered and internalized by MCRnc and MCRSTn+ after 24 hour exposure. Internalization of fluorescent-labelled BCG by mock transduced MCRnc cells (light grey bars) and by ST6GalNAc.I transduced MCRSTn+ cells (dark grey bars), after 24 hours of exposure to BCG. A significantly higher internalisation was observed for MCRSTn+ cells in comparison to MCRnc. Data are the average of 3 independent experiments (mean fluorescence intensity, MFI). ***(p<0.001 (Student t-test).**
3.2. Genetic Polymorphisms

3.2.1. Paper IV

IL-4 and TNF-α polymorphisms are associated with risk of multiple superficial tumors or carcinoma in situ development


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IL-4 and TNF-α Polymorphisms Are Associated with Risk of Multiple Superficial Tumors or Carcinoma in situ Development

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Keywords
Bacillus Calmette-Guérin · Bladder cancer · Multifocality · Genetic polymorphisms

Abstract
Introduction: This study evaluates the influence of clinico-pathological characteristics, bacillus Calmette-Guérin (BCG) therapeutic schedule [maintenance (mBCG) or induction (iBCG)], and TNF-α and IL-4 polymorphisms on the outcome of non-muscle-invasive bladder cancer patients treated with BCG. Material and Methods: DNA was extracted from 125 bladder cancer patients treated with BCG; TNF-308G/A and IL4-590C/T polymorphisms were genotyped. Results: The TNF-308A allele carriers had an increased risk of developing multiple tumors (OR: 2.80, p = 0.031). However, IL4-590T carriers also had an increased risk of developing multiple and carcinoma in situ tumors (OR: 2.52, p = 0.033). For these polymorphisms, no association was found with BCG treatment outcome. When treated with iBCG, patients with multiple tumors had shorter recurrence-free survival (RFS) compared with those with a single tumor (p = 0.004); nevertheless, patients with multifocal tumors have improved RFS when treated with mBCG. Conclusions: Overall, the results suggest that multiple tumors and/or carcinoma in situ development are associated with the IL4-590C/T and TNF-308G/A polymorphisms, and emphasize the effectiveness of the mBCG schedule.

Introduction
Bladder cancer is the second most common urologic malignancy worldwide, and non-muscle-invasive bladder cancer (NMIBC) is a frequent (75–85%) and heterogeneous disease with variable biological behavior and outcomes. Current guidelines and established risk factors are widely used for predicting recurrence and/or progression in the clinical setting [1, 2]. Bladder bacillus
Calmette-Guérin (BCG) instillation with a maintenance schedule (mBCG) is indicated in patients with a high risk for recurrence or progression [2]. Nevertheless, predicting the outcome of BCG therapy has been a long-standing task.

In a multivariate randomized NMICB series, it was observed that tumor multiplicity and carcinoma in situ (CIS) presence were associated with recurrence after BCG immunotherapy [3]. For multifocal NMIBC and CIS, the adequate therapy usually includes the radical removal of genetic unstable urothelium (cystectomy) and mBCG [4].

Several molecular markers have been evaluated in tumor biopsies prior to treatment attempting to predict BCG outcome. Interesting results were achieved with cell proliferative factors p53 and Ki-67 overexpression [5–9], but other studies seem to indicate that these molecules are not suitable for predicting BCG treatment response [10–15]. Markers such as heat shock protein (HSP)-90 and anti-HSP-65 antibodies have also shown a certain relative correlation with BCG outcome [16–19].

In response to mycobacterial stimulation, urothelial and infiltrating immune cells enroll an immunological cascade of events, which involves antigen presentation, chemokines [20], and proinflammatory cytokines, such as TNF-α, promoting a cytotoxic response against the tumor cells.

Based on the molecular mechanism involved in BCG therapy, several studies were conducted to identify more reliable predictors of BCG outcome. Urinary levels of immunological mediators during treatment indicated that cytokines such as IL-8 and IL-2 could be predictors of BCG outcome [21–26].

Some groups have studied the possibility of using genetic polymorphisms as markers to predict BCG response, and several genes were evaluated. Polymorphisms in genes such as XPA, XPC, MMP3, ERCC2, XRCC1, COX2, and NFKB were associated with an increased risk of treatment failure [27–33]. On the other hand, polymorphisms in other genes seem to be associated with a good treatment response [31, 34, 35]. However, all of these polymorphisms need to be evaluated in larger populations and better correlated with clinicopathological characteristics in NMIBC recurrence after BCG treatment.

Although several molecular mechanisms are well-established, the inflammatory pathways involved in cancer are still the subject of discussion [36]. In this study, we hypothesized that functional genetic variants [single nucleotide polymorphisms (SNPs)] in molecules of these pathways might modulate inflammation and immunological surveillance. Therefore, in this study we analyzed two SNPs in molecules involved in Th1/Th2 balance, based on their putative functional effect in these molecules [37, 38].

IL-4 is an anti-inflammatory mediator, with a key role in the Th1/Th2 immunological switch [39, 40]. Higher IL-4 expression was found in bladder cancer patients and notably increased in those with recurrence [41]. It was hypothesized that IL-4 may favor the expansion of Th2 polarity, thereby suppressing the cytotoxic immunological mechanism. A previously described IL-4 genetic polymorphism at locus -590 C/T is associated with IL-4 circulating levels [37, 39]. Recently, it was proposed that this SNP, in which the T allele carriers have increased IL-4, may induce suppression of cellular immunity through Th1 inhibition and consequently promote carcinogenesis development [39].

TNF-α is a strong proinflammatory cytokine with different functions depending on its concentration; however, it is known that higher concentrations may deregulate the immune response, thereby acting as a cancer promoter [42]. A functional genetic polymorphism has been described in the promoter region of TNFA (locus -308), which seems to play a role in the TNF-α circulating levels balance [38, 42]. Bladder cancer patients carrying the A allele seem to have increased TNF-α in serum and tumor tissue [43]. Although the TNF-308G/A polymorphism was described as being associated with grade, much debate still exists concerning its association with stage and other clinicopathological characteristics [43, 44].

The aim of the present study was to evaluate the significance of clinicopathological characteristics in NMIBC recurrence after BCG treatment and to analyze the association of these two features with TNF-308G/A and IL4-590C/T functional polymorphisms.

Subjects and Methods

Population

In this retrospective case-control study, all patients who underwent transurethral resection for intermediate and high-risk NMIBC treated with BCG therapy between 1998 and 2006 at the Portuguese Institute of Oncology, Porto (before the adoption of the European Association of Urology recommendations for mBCG schedule for all high-risk patients by the Urology Department) were eligible to be included in this study. From a total of 200 patients, 14 had already died. The remaining 186 were invited to participate in the study and 71% accepted to be enrolled (7 were excluded). All patients had received intravesical instillation of
BCG for 6 consecutive weeks starting 2–3 weeks after surgery and 56% underwent mBCG schedule.

The mean diagnostic age of the patients was 61.54 ± 10.63, years (36–97), with a male:female sex ratio of 105:20. In 52% of the patients, BCG therapy was performed after transurethral resection of primary tumors, while 48% had prior recurrence (one or more) before the BCG treatment. The patients were followed by cystoscopy and urinary cytological examination every 3 months for the first year, every 6 months for the second year, and every 12 months thereafter. The median follow-up time was 60 months (6–135). Tumor recurrence was defined as a newly found bladder tumor after the treatment, with at least one tumor-free cystoscopy between.

The endpoint of the study was recurrence-free survival (RFS), defined as the period between the beginning of BCG treatment and the date of the most recent cystoscopy or recurrence date. Nonresponders, as opposed to responders, were defined as patients submitted to BCG treatment with tumor recurrence.

Based on the concept that multifocal tumor (≥2 lesions) and/or presence of CIS could be an indicator of field carcinogenesis [45], we combined tumors with these characteristics (multifocal, n = 61; CIS, n = 8; multifocal and CIS, n = 5) when necessary for analysis.

All clinicopathological information was obtained from patients' clinical records. Informed and written consent from each patient was obtained. The institutional ethics committee approved the study.

**DNA Extraction and Genotyping**

Peripheral blood samples were collected following a standard venipuncture technique in EDTA-containing tubes, and the DNA was extracted from the white blood cell fraction using a salting-out protocol [46]. The polymorphism analysis was carried out using methodologies published by other authors [47, 48].

**TaqMan 308GA polymorphism analysis** was carried out using TaqMan SNP Genotyping Assay C_7544879_10 (Applied Biosystems, Foster City, Calif., USA). Reactions were performed on an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems) as per the manufacturer's instructions.

**IL4-590C/T polymorphism genotyping** was performed with the PCR-RFLP method as previously described [46, 48], and it was successfully amplified in 89.4% of the samples. A second PCR-RFLP analysis was carried out in 10% of all samples for genotype confirmation. For analysis, we combined both the homozygous and the heterozygous genotype naming 'allele carriers' (TNF-308G/A 'A allele carrier' comprises both AA and GA genotypes; IL4-590C/T 'T allele carriers' comprises both TT and CT genotypes).

**Statistical Analysis**

Statistical data analysis was carried out using the computer software Statistical Package for Social Sciences – SPSS for Windows (version 15.0). χ² analysis was used to compare categorical variables. The OR and its 95% CI were calculated as a measurement of the association between genotypes and recurrence and multiple tumors development risk. Kaplan-Meier survival curves were used to evaluate correlation of BCG schedule and multifocality presence with RFS, and were compared by a log-rank or Breslow (when curves cross over) statistical test.
are a well-known characteristic of NMIBC. Presently, two theories are being discussed, the ‘field effect’ and the ‘single progenitor cell’ hypotheses, which assert that the multifocal development is caused, respectively, by the seeding or intraepithelial spread of transformed cells [45, 49]. Concordantly, our group reported recently that CIS is a surrogate marker of genetic instability and urothelial mucosa field carcinogenesis [4]. These transformed cells may lead to subsequent tumor relapse [49].

This study points out that multifocality is related with a short disease-free interval after iBCG, as observed by others [28]. In accordance with our results, several authors reported multiplicity as an important variable for prediction of recurrence with or without BCG treatment [28]. It is already included in a nomogram to predict recurrence in patients with a history of NMIBC [50]. A ‘field effect treatment’ approach, such as mBCG protocol, is associated with the best outcome, underlying that mBCG is indicated in multiple tumors [51, 52].

Fig. 1. Kaplan-Meier curves representing RFS. a In all patients treated with BCG, patients with multiple tumors have shorter RFS (mean: 84 months) than patients with single ones (mean: 99 months). b According to treatment schedule, patients treated with iBCG have shorter RFS (mean: 79 months) than those treated with mBCG (mean: 100 months). c According to multifocality status in patients treated with iBCG, patients with multifocal tumors have shorter RFS (mean: 53 months) than patients with a single tumor (mean: 95 months). d In those treated with mBCG, no significant differences were found in RFS between patients with multiple and single tumors. p is according to Breslow test; + = censored mBCG or single tumors; ◆ = censored iBCG or multiple tumors.
To investigate the significance of correlating these clinicopathological features and other parameters in predicting BCG outcome, we addressed known functional polymorphisms in the IL-4 and TNF-α cytokines. We observed that IL4-590 T allele carrier patients had an increased risk for multiple tumors and/or CIS development. Agarwal et al. [41] hypothesized previously that IL-4 favors the expansion of Th2 cells in bladder cancer patients and leads to the suppression of Th1 response. This mechanism is required for suppressing the generation of effector cytotoxic T lymphocytes that may lyse tumor cells and subsequently decrease immune surveillance [41, 53]. Since patients carrying the IL4-590 T allele seem to have a higher production of IL-4 [37], a state of immunosuppression is likely to occur (Th1 inhibition). This environment may induce urothelial bladder carcinogenesis as translated in multiple papillary tumors or CIS lesions [39]. Therefore, properties of IL-4 might help tumor cells escape lymphocyte killing and could facilitate the development of multiple tumors in the bladder urothelium.

We also observed an increased risk associated with multifocality in TNF-308 A allele carriers. A previous study established that the A allele is associated with increased TNF-α serum levels and mRNA levels in bladder tumors [43]. Other authors suggest that the TNF-308G/A polymorphism is associated with tumor grade [44]; in this work we found that patients carrying the A allele have a higher risk of developing multiple superficial tumors.

However, the polymorphisms analyzed in this study did not show any association with BCG response and RFS after treatment. Other groups also observed that TNF-308 and IL4-590 are not associated with BCG therapy response [54, 55].

Our results showed that while IL-4 and TNFα genetic polymorphisms alone are not predictors of BCG response, they are associated with the development of multiple and/or CIS tumors, and thus associated with higher recurrence and progression rates. Therefore, these genetic variants could help to identify a subset of patients that are at risk of developing these tumors and consequently identify those patients with reduced RFS after treatment.

To the best of our knowledge, this is the first study to investigate the influence of IL4-590C/T and TNF-308G/A polymorphisms in a multiple tumor and/or CIS setting.

### Conclusions

The genetic polymorphisms studied cannot be used alone as predictive markers of BCG treatment response. However, this study shows that both IL4-590 T and TNF-308 A allele genotyping could help to identify individuals with an increased risk of multiple NMIBC and/or CIS development. This information could be crucial to personalize treatment and improve outcome.

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| Table 1. Polymorphism genotype distribution and risk of multifocality or CIS development |
|------------------------------------------|-----------------|----------|---------|---------|---------|
|                                         | Single n (%)    | Multiple n (%)  | OR       | 95% CI   | p       |
| TNF-308G/A                              |                 |                   |          |         |         |
| GG                                      | 48 (82.8)       | 40 (61.5)         | 1.0      | reference |         |
| A carrier                                | 10 (17.2)       | 25 (38.5)         | 3.00     | 1.29–6.98 | 0.009   |
| IL4-590C/T                              |                 |                   |          |         |         |
| CC                                      | 46 (82.1)       | 39 (64.8)         | 1.0      | reference |         |
| T carrier                               | 10 (17.9)       | 18 (35.2)         | 2.12     | 0.88–5.13 | 0.091   |
| IL4-590C/T                              |                 |                   |          |         |         |
| CC                                      | 45 (85.4)       | 44 (67.7)         | 1.0      | reference |         |
| T carrier                               | 7 (14.6)        | 21 (32.3)         | 2.80     | 1.08–7.27 | 0.031   |

IL-4/TNF-α Polymorphisms and Multifocality in BCG-Treated Patients


462 Urol Int 2011; 87:457–463

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3.2. Genetic Polymorphisms

3.2.2. Paper V

FASL polymorphism is associated with response to bacillus Calmette–Guerin immunotherapy in bladder cancer

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FASL polymorphism is associated with response to bacillus Calmette-Guérin immunotherapy in bladder cancer

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Abstract

**Objective:** Deregulation of FAS/FASL system may lead to immune escape and influence bacillus Calmette-Guérin (BCG) immunotherapy outcome, which is currently the gold standard adjuvant treatment for high-risk non–muscle invasive bladder tumors. Among other events, functional promoter polymorphisms of FAS and FASL genes may alter their transcriptional activity. Therefore, we aim to evaluate the role of FAS and FASL polymorphisms in the context of BCG therapy, envisaging the validation of these biomarkers to predict response.

**Patients and methods:** DNA extracted from peripheral blood from 125 patients with bladder cancer treated with BCG therapy was analyzed by Polymerase Chain Reaction—Restriction Fragment Length Polymorphism for FAS-670 A/G and FASL-844 T/C polymorphisms. FASL mRNA expression was analyzed by real-time Polymerase Chain Reaction.

**Results:** Carriers of FASL-844 CC genotype present a decreased recurrence-free survival after BCG treatment when compared with FASL-844 T allele carriers (mean 71.5 vs. 97.8 months, \( P = 0.030 \)) and have an increased risk of BCG treatment failure (Hazard Ratio = 1.922; 95% Confidence Interval: [1.064–3.471]; \( P = 0.030 \)). Multivariate analysis shows that FASL-844 T/C and therapeutic scheme are independent predictive markers of recurrence after treatment. The evaluation of FASL gene mRNA levels demonstrated that patients carrying FASL-844 CC genotype had higher FASL expression in bladder tumors (\( P = 0.0027 \)). Higher FASL levels were also associated with an increased risk of recurrence after BCG treatment (Hazard Ratio = 2.833; 95% Confidence Interval: [1.012–7.929]; \( P = 0.047 \)). FAS-670 A/G polymorphism analysis did not reveal any association with BCG therapy outcome.

**Conclusions:** Our results suggest that analysis of FASL-844 T/C, but not FAS-670 A/G polymorphisms, may be used as a predictive marker of response to BCG immunotherapy.

**Keywords:** Bladder cancer; BCG immunotherapy; Polymorphisms; Fas/Fasl; Predictive markers

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1. Introduction

The Fas/Fas ligand (FasL) system is one of the major pathways of apoptosis and an important regulator of cell
proliferation and immune system regulation. During carcinogenesis, tumor cells may alter this mechanism to subvert the immune system and suppress the antitumor immune response [1].

Fas/FasL further controls T-cell apoptosis after immune reaction by the process of activation-induced cell death (AICD) [2]. This mechanism involves the overexpression of both Fas and FasL upon activation by antigen or other stimuli, subsequently promoting cell “suicide” or “fratricide” [3]. Decreased Fas and elevated FasL expression has been found in many types of cancers including bladder cancer [4]. It has been shown that tumor cells may counterattack Fas-sensitive tumor-infiltrating lymphocytes (TILs) by using heightened expression of FasL. This mechanism is thought to lead to tumor cell immune escape, thus contributing to cancer formation and progression [5].

Functional polymorphisms in the promoter region of FAS and FASL genes have been identified, and they alter the transcriptional activity of these genes, which may have implications in the Fas/FasL pathways [6,7]. Moreover, they have been associated with a higher risk in various cancer models [8,9]. In patients with bladder cancer, FASL-844 CC genotype was associated with a significantly increased risk of bladder cancer [10]. However, the role of Fas/FasL and its functional polymorphisms have not been addressed in the context of bladder cancer treatment.

At the moment, the most effective treatment for high-risk non–muscle invasive bladder cancer (NMIBC) consists of an immunotherapy with bacillus Calmette-Guérin (BCG) [11]. Generally it is performed as adjuvant to transurethral resection of bladder tumor in intermediate- and high-risk patients [11]. However, several studies demonstrated that 30% of the patients failed to respond to this therapeutics and in some cases, the tumor may become more aggressive [12]. Thus, early identification of patients who were better served by alternative or more aggressive approaches, such as cystectomy, or both is a critical aspect in the management of high-risk NMIBC [13,14]. However, at the moment, there are no established biomarkers to determine the outcome of BCG immunotherapy. Recently, several authors suggest that immunological predictive markers may yield promising clinical value in the context of predicting BCG immunotherapy outcome [14–16].

One of the main pathways of BCG antitumoral response is mediated by BCG and lymphokine-activated killer cells [17]. Although it has been demonstrated that Fas/FasL pathway is not directly involved in BCG antitumoral effect [17,18], BCG effector cells are sensitive to FasL-dependent AICD regulation [2] and possibly to FasL counterattack by tumor cells. Hypothesizing that a deregulation of Fas/FasL pathway may lead to immune escape and influence the efficacy of BCG immunotherapy, in this study we aimed to evaluate the role of FAS and FASL polymorphisms in the context of BCG therapy response. To the best of our knowledge, this is the first study addressing this subject and may provide important insights about the role of these molecules as predictive markers of BCG treatment outcome.

2. Material and methods

2.1. Population

In this retrospective case-control study, all patients with intermediate and high risk of NMIBC who underwent transurethral resection followed by BCG therapy between 1998 and 2006 at the Portuguese Institute of Oncology, Porto were eligible for this study. From a total of 193 patients, 14 had already died. The remaining 179 patients were invited to participate in the study, 70% of which accepted [19]. Blood samples were collected from the patients during 2006 and 2008 on follow-up consultation. All patients received intravesical instillation of BCG for 6 consecutive weeks starting 2 to 3 weeks after surgery, i.e. induction scheme (iBCG), and 56% underwent further instillations every 3 months for 2 years, i.e. maintenance scheme (mBCG). The iBCG group includes patients treated before the European Association of Urology guidelines recommending the mBCG scheme and patients showing significant intolerance to long-term BCG treatment. The mean diagnostic age of the patients was 61.54 ± 10.63 (min: 36; max: 97) years, with a male to female sex ratio of 105:20. The patients were followed by cystoscopy and urinary cytology examination every 3 months for the first year, every 6 months for the second year, and every 12 months thereafter. The median follow-up time was 60 months (from 6 to 135 months). Tumor recurrence was defined as a newly found bladder tumor after the treatment, with at least 1 tumor-free cystoscopy in between. The end point of the study was recurrence-free survival (RFS), defined as the period between the beginning of BCG treatment until the date of the most recent cystoscopy or recurrence date. Nonresponders, as opposed to responders, were defined as patients submitted to BCG treatment who showed tumor recurrence. All clinicopathological information was obtained from patients' clinical records. Informed and written consent from each patient was obtained. The institutional ethics committee approved the study.

2.2. DNA extraction and genotyping

Peripheral blood samples were collected following standard venipuncture technique in Ethylenediaminetetraacetic acid (EDTA)-containing tubes, and the DNA was extracted from the white blood cell fraction using a salting-out protocol [20] and stored at −20°C.

The FAS and FASL genetic polymorphisms were genotyped using Restriction Fragment Length Polymorphism-Polymerase Chain Reaction (RFLP-PCR) technique. The primers and enzymes used in FAS-670 A/G and FASL-844 T/C polymorphisms genotyping were the ones described by other authors [6,7]. The PCR-RFLP conditions were the same as used by aforementioned authors [20]. Genotyping data was read blind to the study end point. For quality control, 10% of the samples were randomly selected to a
second PCR-RFLP analysis and 100% of concordance was observed.

2.3. RNA extraction and FASL mRNA expression analysis

A total of 34 tumor samples were randomly selected from the initial population according to the patient genotype for FASL-844 T/C polymorphism (17 TT/TC and 17 CC). RNA was isolated from formalin-fixed paraffin-embedded (FFPE) tissue samples using “Absolutely RNA FFPE Kit” (Stratagene, La Jolla, CA). Up to 2 μg of total RNA was reverse transcribed with random primers, using the “High Capacity cDNA Reverse Transcription Kit” (Applied Biosystems, Foster City, CA). Real-time PCR amplification of cDNA samples was performed in a StepOne Real-Time PCR System (Applied Biosystems) using TaqMan Gene Expression Master Mix, primers, and probes provided by Applied Biosystems. Expression of FASL was measured with TaqMan expression assay (ID: Hs00181225_m1) from Applied Biosystems.

The raw $-\Delta Ct$ was used to analyze the FASL expression in FASL-844 CC or TT/TC genotypes carriers and therefore used as an estimate of the mRNA relative levels. $\Delta Ct$ stands for the difference between the cycle threshold (Ct) of the amplification curve of the target gene and that of the GAPDH. The efficiency of the amplification reaction for each primer-probe is more than 95% (as determined by the manufacturer).

2.4. Assessment of FasL expression by immunohistochemistry (IHC)

The same 34 FFPE tissue sections were screened for FasL by IHC with the streptavidin/biotin peroxidase method using anti-FasL mouse monoclonal antibody clone 5D1 (Leica Biosystems, Wetzlar, Germany). Tissues were incubated with primary antibody overnight in a 1:35 dilution. A semiquantitative approach was established to score the immunohistochemical labeling based on the percentage of cells that stained positively. The entire section was screened and the cases were classified as Low FasL IHC expression when the percentage of positive cells was more than median value of the overall expression (<25%). When the overall expression was more than 25%, it was classified as High FasL IHC expression.

2.5. Statistical analysis

Statistical data analysis was carried out using the computer software Statistical Package for Social Sciences—SPSS for Windows (version 15.0). Chi-square analysis was used to compare categorical variables. The odds ratio (OR) and its 95% confidence interval (CI) were calculated as a measurement of the association between genotypes and the risk of recurrence. Kaplan-Meier survival curves were used to evaluate correlation between genotypes and RFS and were compared by log-rank statistical test. Further, multiple Cox regression analysis was used to assess the effect of individual polymorphisms on the time to recurrence in BCG-treated patients and to adjust for potential confounders. Nonparametric Mann-Whitney test was used to compare the differences in the mRNA expression between the study groups.

3. Results

3.1. FAS and FASL polymorphisms as predictors of BCG immunotherapy outcome

Regarding the evaluation of FAS-607 A/G and FASL-844 T/C polymorphisms, Table 1 presents genotype frequencies and the risk of recurrence after BCG treatment. It was observed that FASL-844 CC genotype frequency is higher in patients with recurrence when compared with responders group (37.5% vs. 24.7%). An increased risk was noticed (OR = 1.8), although not statistically significant. Kaplan-Meier function plots and probabilities analysis showed that FASL-844 CC genotype carriers have a shorter recurrence-free survival after BCG treatment (mean 71.5 months) when compared with T allele carriers (mean 97.8 months, $P = 0.030$, Fig. 1). To estimate the risk associated with this difference, Cox regression was performed and it was found that FASL-844 CC genotype carriers have an increase risk of BCG treatment failure (Hazard Ratio (HR) = 1.897; 95% CI: [1.051–3.424]; $P = 0.034$). Regarding FAS-670 A/G polymorphism, no association was found with recurrence risk nor RFS.

The relation between clinicopathological characteristics and response to BCG treatment (i.e. responders vs. non-responders) is presented in Table 2. It was observed that the treatment scheme is associated with recurrence after
treatment (35.1% vs. 58.3%, \(P = 0.011\)). Our previous report, with the same sample data set, showed that iBCG-treated patients or patients with multiple tumors have a shorter RFS [19]. In the present study, we performed univariate Cox Regression and found that the patients treated only with iBCG scheme have 2-fold risk of recurrence after BCG immunotherapy (HR = 2.096; 95% CI: [1.177–3.731]; \(P = 0.012\)). However, the presence of multiple tumors revealed a trend toward an increase risk of recurrence (HR = 1.760; 95% CI: [0.981–3.159]; \(P = 0.058\)). There are no association between the evaluated polymorphism and the clinicopathological characteristics.

Therefore, multivariate Cox regression analysis adjusted to treatment scheme and multifocality was performed to assess the individual effect of FASL-844 T/C on the risk of recurrence in BCG-treated patients. The results shown in Table 3 reveal that independently of the treatment scheme adopted, patients carrying FASL-844 CC genotype have approximately a 2-fold risk of early recurrence and that independently of the patient’s FASL-844 genotype, patients treated only with iBCG scheme have approximately 2.5-fold risk of early recurrence. Thus, FASL-844 T/C, BCG scheme, and multifocality are independent predictive factors of BCG immunotherapy outcome.

### 3.2. FASL mRNA expression and its relation with FASL-844 T/C and treatment response

Of the 125 patients, 34 tumor tissues were available for evaluation of FASL mRNA expression levels. As shown in Fig. 2, significantly higher FASL mRNA levels were found in tumors from FASL-844 CC genotype patients than from TT or TC genotype patients (median mRNA relative levels, −7.80 vs. −6.14 ± 0.46, \(P = 0.0027\)). In our samples, the normalizing gene (GAPDH) presents stable Ct values (mean ± standard deviation: 29.95 ± 2.26), demonstrating that RNA recovered from FFPE is suitable for gene expression analysis.

Individuals were categorized as high expressers (High FASL) when the normalized transcripts levels (mRNA relative levels) were more than the geometric mean of all cases (\(>−7.04\)), whereas individuals with mRNA relative levels less than −7.04 were categorized as low FASL expressers (Low FASL). From the High FASL group, 78.6% individuals were FASL-844 CC genotype carriers, whereas among Low FASL cases, only 30% were CC genotype (\(P = 0.005\)). Kaplan-Meier analysis (Fig. 3) showed that High FASL individuals have a reduced RFS (mean 51.4 months) when compared with Low FASL individuals (mean 96.2 months, \(P = 0.030\)). Univariate Cox Regression analysis demonstrated that High FASL individuals have approximately 3-fold risk of recurrence after BCG treatment (HR = 2.833; 95% CI: [1.012–7.929]; \(P = 0.047\)). IHC was used to confirm FasL localization. It was observed that FasL was present both in tumors and the immune infiltrates, independently of the FASL-844 T/C genotype. The FasL overall expression was in accordance with the levels of gene expression (\(P < 0.001\)—Fig. 4). No association was found between the FasL expression in the tumor and BCG response as well as other clinicopathological characteristics.

### Table 2
Relation between patients, clinical and tumor characteristics, and BCG treatment outcome

<table>
<thead>
<tr>
<th>Variables</th>
<th>Total n (%)</th>
<th>Responders n (%)</th>
<th>Nonresponders n (%)</th>
<th>(P) valuea</th>
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</thead>
<tbody>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>74 (59.2)</td>
<td>45 (58.4)</td>
<td>29 (60.4)</td>
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<tr>
<td>Grade</td>
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</tr>
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<tr>
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<td>60 (77.9)</td>
<td>39 (81.2)</td>
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</tr>
<tr>
<td>Size (cm)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>≤3</td>
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<td>47 (69.1)</td>
<td>30 (69.8)</td>
<td>0.942</td>
</tr>
<tr>
<td>&gt;3</td>
<td>34 (30.6)</td>
<td>21 (30.9)</td>
<td>13 (30.2)</td>
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</tr>
<tr>
<td>Tumor number</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>59 (47.2)</td>
<td>40 (51.9)</td>
<td>19 (39.6)</td>
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<tr>
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<td>29 (60.4)</td>
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</tr>
<tr>
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<td>67 (87.0)</td>
<td>45 (93.8)</td>
<td>0.230</td>
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<tr>
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<td>10 (13.0)</td>
<td>3 (6.2)</td>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>Primary</td>
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<td>41 (53.2)</td>
<td>24 (50.0)</td>
<td>0.724</td>
</tr>
<tr>
<td>Recurrent</td>
<td>60 (48.0)</td>
<td>36 (46.8)</td>
<td>24 (50.0)</td>
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</tr>
<tr>
<td>Treatment scheme</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>iBCG</td>
<td>55 (44.0)</td>
<td>26 (35.1)</td>
<td>41 (58.3)</td>
<td>0.011</td>
</tr>
<tr>
<td>mBCG</td>
<td>70 (56.0)</td>
<td>60 (64.9)</td>
<td>5 (41.7)</td>
<td></td>
</tr>
</tbody>
</table>

aChi-square test.

b1.6% of them are pure CIS and 8.8% are concomitant CIS. CIS = carcinoma in situ.
4. Discussion

The management of high-risk NMIBC relies mostly on an adjuvant immunotherapy consisting of intravesical instillations with BCG, performed after transurothelial resection of the tumor [11]. BCG promotes a strong local immune response that results in tumor elimination [13,21,22]. In our most recent systematic review on this subject, we point out several markers such as CD68, ezrin, HSP90, CD83, and IL2 urinary levels related with BCG response [14]. Several functional polymorphisms in genes such as NRAMP1, hGPX1, XPA, ERCC2, and ERCC6 as well as in genes involved in inflammatory pathways (IL8, TNFA, IL6, TGFB1, COX2, and IFNG) have been appointed as putative predictive markers of BCG treatment response [14]. All these genetic markers have been identified based on a candidate gene approach using well-established polymorphisms. Still there are no reliable biomarkers to determine the outcome of this therapeutics, thereby permitting the early identification of patients better served by alternative therapeutics or cystectomy [14,16]. It has been long thought that such biomarkers may be encountered among molecules/cells involved in the modulation of immune responses at the tumor site [14–16]. Among these putative immune-related biomarkers is the Fas/FasL pathway. Still, its involvement in BCG-mediated tumor cells elimination remains controversial, because some authors demonstrated that BCG antitumoral effect is not mediated by Fas/FasL [17,18], whereas others found higher levels of these molecules in T lymphocytes after treatment [23]. Nevertheless, this pathway is responsible for the regulation of cell apoptosis, namely T-cell depletion by increasing AICD rates [2], therefore a deregulation of this pathway may compromise the immune response mediated by BCG immunotherapy and consequently contribute to modulate the therapeutics outcome. Tumor cells are known to modulate and evade Fas-mediated apoptosis, by simultaneously down-regulating their own Fas expression and promoting the expression of FasL on their surface [5]. This protects tumor cells against FasL-induced apoptosis and, at the same time, promotes the apoptosis of activated T-cell expressing FasL, in what has been called as the FasL “counterattack” [5]. Functional polymorphisms in FAS and FASL genes may unbalance the expression of Fas/FasL by tumor cells and contribute to the establishment of an immune-suppressive environment that favors tumor proliferation [5]. The unbalanced expression of Fas/FasL was observed in several tumors, including bladder cancer [4]. Moreover, the presence of the FAS and FASL
polymorphism was correlated with an increased risk of developing bladder tumors [10] and with an enhanced T-cell apoptosis after immune reaction [24].

Given the putative involvement of Fas/FasL pathway in the immune response, one may hypothesize that functional polymorphisms affecting expression levels of these molecules may deregulate this pathway, contributing to the failure of BCG therapeutics [15]. Therefore, it may be used as a predictive biomarker of BCG immunotherapy outcome.

As the FASL-844 T/C polymorphism was never evaluated in the context of BCG therapy, in this study we have evaluated these polymorphism in a data set of Portuguese patients and found that those carrying FASL-844 CC genotype presented an increased risk of recurrence after treatment. To corroborate if FASL-844 CC genotype was associated with higher basal expression levels of FasL, we have also evaluated mRNA levels of FASL gene in tumor samples. This showed that patients carrying FASL-844 CC genotype had higher FASL expression in bladder tumors, as previously reported for others models [7,24]. We have also demonstrated that higher FASL levels were associated with an increased risk of recurrence after BCG treatment. This suggests that patients carrying FASL-844 CC genotype have higher FASL expression in T-cell membrane or in tumors that may indeed compromise the efficiency of BCG treatment by balancing the immunological boost promoted by the bacillus. Our results suggest that this may not occur by an overexpression of FasL in tumors. More studies are needed to determine whether it may result from enhanced AICD in T cells.

Herein, we opted to extract RNA from FFPE tissues. Although mRNA extracted from FFPE is partially degraded, it is possible to use this in real-time PCR to obtain an accurate and specific gene expression using amplicons under 100 bp [25]. Based on these observations, we choose amplicons lower than 100 bp. We also observed that GADPH expression was stable among our samples and that the mean Ct was around 30. According to several authors, this is an indicator of mRNA quality and amplification efficiency [25,26]. Based on these concepts, we can say that our RNA samples were suitable for expression analysis.

Regarding FAS-670 A/G analysis, we could confirm that this polymorphism is not associated with BCG treatment outcome in the Portuguese population, as described for the Indian population [27], suggesting that FAS-670 A/G may not influence the molecular mechanisms underlying this therapeutics.

Clinicopathological characteristics as predictors of BCG immunotherapy outcome have been long studied, although the findings are controversial [28]. Herein, we evaluated the predictive value of all clinicopathological characteristics and found that only patients treated with iBCG or with multiple tumors present a higher risk of failure, which is in accordance with previous findings [19,29,30]. However, multivariate analysis showed that this did not influence the biomarker value of FASL-844 T/C in the context of response to BCG immunotherapy. Still, the main limitation of this study was the low number of mBCG cases, thus the analysis of a larger patient set only treated with mBCG scheme would be required to evaluate more accurately the value of this polymorphism in the context of the currently adopted therapeutic scheme.

This is the first report addressing the potential role of FASL-844 T/C polymorphism as a predictive marker of

### Table: FasL expression

<table>
<thead>
<tr>
<th>Low FasL IHC expression</th>
<th>High FasL IHC expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low FASL mRNA expression</td>
<td>17 (85%)</td>
</tr>
<tr>
<td>High FASL mRNA expression</td>
<td>3 (21.4%)</td>
</tr>
<tr>
<td>Low FasL IHC expression</td>
<td>3 (15%)</td>
</tr>
<tr>
<td>High FasL IHC expression</td>
<td>11 (78.6%)</td>
</tr>
</tbody>
</table>

Fig. 4. IHC using anti-FasL antibody. Representative image of tumor (dark arrow) and immune infiltrate (white arrow) expressing FasL in specimens with low and high expression of FASL mRNA (400×). In cases with low FASL mRNA, the FasL protein expression was lower and less intense (A). In cases with high FASL mRNA expression, FasL was markedly and diffusely expressed in both the tumor and the immune infiltrate (B). Expression of FASL mRNA is associated with FasL expression evaluated by IHC, \( P < 0.001 \) (Chi-square test) (C). (Color version of the figure is available online.)
BCG immunotherapy response. Despite the limited number of samples, a significant association with the outcome of the therapeutics was observed. This highlights that FASL-844 T/C polymorphism could be included in a biomarker panel to guide the management of high-risk bladder cancers.

Acknowledgments

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References

3.2. Genetic Polymorphisms

3.2.3. Paper VI

The role of functional polymorphism in immune response genes as biomarkers of BCG Immunotherapy outcome in Bladder cancer: Establishment of a predictive profile


The Pharmacogenomics Journal (Submitted)

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ABSTRACT

The management of high-risk non-muscle invasive bladder cancer relies on first instance in intravesical instillations with Bacillus Calmette-Guérin, after tumor removal. However, approximately 30% of the patients present recurrence after this therapeutic and ultimately progression of the disease. At the moment there are no reliable biomarkers to determine the outcome of this therapeutics, thereby permitting the early identification of patients better served by alternative therapeutics. In a dataset of 204 patients treated with BCG, we evaluate 42 genetic polymorphism in 38 genes involved in the BCG mechanism of action, using Sequenom MassARRAY technology. Using stepwise multivariate Cox Regression analysis we propose the first predictive profile of BCG immunotherapy outcome and a risk score that permits to categorize patients into risk groups. According to our model, patients within the Low Risk groups have a 90% chance of successful treatment, whereas patients in the High Risk groups present 75% chance of recurrence after BCG treatment.

KEYWORDS: BCG immunotherapy; bladder cancer; genetic polymorphisms; predictive profile; risk score of recurrence.
INTRODUCTION

Bladder Cancer (BC) is the most common malignancy of the urinary tract [1] and has the highest lifetime treatment costs per patient of all cancers [2]. In Europe, BC is the fourth most common cancer in men and the eighth most common cause of cancer-specific mortality [1]. At the time of diagnosis, 75-80% of cancers are non-muscle invasive BC (NMIBC) tumors of stages pTa, pT1 or Carcinoma in situ (CIS). After transurethral resection (TUR), these tumors present high recurrence and progression rates at five years [3]. TUR followed by a schedule of intravesical administration of bacille Calmette-Guerin (BCG) as allowed delaying the time to recurrence and is currently considered the most effective treatment for intermediate and high risk of recurrence/progression NMIBC tumors [3]. However, several studies have demonstrated that 30% to 50% of the patients fail to respond to this therapeutic, in such cases the tumor may become more aggressive [4, 5].

Since the response to BCG is unpredictable, it is important to find biomarkers able to accurately determine treatment outcome and/or a profile that could identify patients at elevated risk of treatment failure.

The exact mechanism of BCG immunotherapy antitumor activity is not well known [6]. The initial event comprehends the attachment of BCG to matrix fibronectin at sites of urothelial disruption [7, 8]. Subsequently the bacillus is internalised and bacterial antigens are presented by either tumor cells or APC (antigen presenting cells), such as macrophages, B-lymphocytes and dendritic cells [8, 9]. These events induce the production of various cytokines and chemokines including interferon-γ (IFN-γ), interleukin-1 (IL-1), IL-2, IL-5, IL-6, IL-8, IL-10, IL-12, IL-18 and tumor necrosis factor-α (TNF-α) that mediate the initiation and maintenance of the inflammatory process that contributes to eliminate residual tumor cells [6, 10]. Qualitative analyses of BCG treatment-associated immune responses indicates that the effective establishment of a Th1-cytokine profile is crucial for an effective antitumor activity [6, 11]. The importance of the Th1/Th2-dichotomy is further supported by observations that high
expression levels of immunoregulatory or Th2-cytokines, like IL-10, arrest Th1-cytokine responses and abrogate the therapeutic effect of BCG [12]. Experimental evidences further support that tumor cells may be eliminated directly by the bacillus upon its internalization by the production of iNOS or by the effector cells such as cytotoxic T-cells, via perforin or TRAIL [13-15]. These observations support the notion that alterations in these pathways may influence treatment outcome.

Several markers that could predict BCG immunotherapy response have been appointed, and some authors suggest an important role for functional polymorphisms (SNPs) in key genes of immune system and inflammatory response [6, 16, 17]. Supporting these observations, we have recently presented evidences that a polymorphism in FASL may influence treatment outcome [18]. Therefore, a deeper investigation of functional SNPs in genes involved in different steps of BCG mechanism of action, such as antigen presentation, cytokines and chemokines production and tumor elimination, is regarded crucial to define a therapy failure profile.

Our aim is to evaluate genetic polymorphisms as potential predictive biomarkers and, in combination with clinicopathological characteristics information, establish a predictive profile of BCG immunotherapy outcome.

MATERIAL AND METHODS

Population

In this retrospective case-control study, all intermediate and high-risk NMIBC patients who underwent transurethral resection followed by BCG therapy between 1998 and 2009 at the Portuguese Institute of Oncology – Porto were eligible for this study. A total of 204 blood samples were collected during 2006 and 2012 on patient’s follow-ups. All patients received intravesical instillation of BCG for 6 consecutive weeks starting 2-3 weeks after surgery, i.e., induction scheme (iBCG) and the majority underwent further instillations every 3 months for two years, i.e.,
maintenance scheme (mBCG). The treatment age of the patients was 64.19±9.99 (min:37; max:84) years, with a male:female sex ratio of 175:29. The patients were followed by cystoscopy and urinary cytology every 3 months for the first year, every 6 month for the second year and every 12 months thereafter. The median follow-up time was 57 months (from 8 till 163 months). Tumor recurrence was defined as a newly found bladder tumor after the treatment, with at least one tumor-free cystoscopy in-between. The end point of the study was recurrence-free survival (RFS), defined as the period between the beginning of BCG treatment until the date of the most recent cystoscopy or recurrence. All clinicopathological information was obtained from patients’ clinical records. Informed and written consent from each patient was obtained. The institutional ethics committee approved the study.

**DNA extraction and genotyping**

Peripheral blood samples were collected following standard venipuncture technique in EDTA-containing tubes, and the DNA was extracted from the white blood cell fraction using a salting out protocol [19] and stored at -20ºC.

A total of 42 SNPs in 38 genes involved in the various steps of BCG immunotherapy mechanism of action were selected based in the following criteria: i) have putative or published functional implication in molecule expression, ii) have a minor allele frequency of 15% (Table S1).

Gene polymorphisms were determined using a Sequenom MassARRAY system (San Diego, CA, USA). The genotyping was undertaken using the Sequenom-iPLEX platform, according to the manufacturer’s instructions (Sequenom, San Diego, CA, USA). The detection of SNP was carried out by analyzing the primer extension products generated from previously amplified genomic DNA using a Sequenom chip-based matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry platform. Sequenom Assay Design 3.1 software was used to design the primers for polymerase chain reaction (PCR) amplification and
single base extension assays (Sequenom, San Diego, CA, USA) according to the manufacturer’s instructions.

Genotyping data was read blindly until the study endpoint. For quality control, 10% of the samples were randomly selected for a second analysis and 100% of concordance was observed.

**Statistical analysis**

Statistical data analysis was carried out using the computer software IBM Statistical Package for Social Sciences—SPSS for Windows (version 22.0; IBM, Armonk, NY, USA). Chi-square analysis was used to compare categorical variables. Kaplan-Meier survival curves were used to evaluate correlation between genotypes and RFS and were compared by log-rank statistical test. Further, multivariate Cox regression analysis was used to assess the effect of individual polymorphisms on the time to recurrence in BCG-treated patients and to adjust for potential confounders.

Stepwise multivariate Cox regression with backward elimination (P-value for retention =0.10) was conducted in all clinical pathological characteristics. A similar approach was applied for genetic variants, including all polymorphisms with aHR <0.6 or aHR >1.4 (40% decrease or increase in odds of the outcome; Table S2). The variables that remained in the two previous models were inserted in another stepwise multivariate Cox regression with backward elimination to create a combined (clinical and genetic variables) model. The concordance (c) index was used to compare the predictive ability of the three Cox regression models, with c>0.5 being considered with a good prediction ability [20].

We constructed an inclusive multi-locus genetic risk score for each participant by summing the coefficients for each of the resulting variables after stepwise regression analyses. For each SNP, the risk genotypes were coded as 1 and the non-risk alleles as 0. The model was determined by multiplying the β coefficients (Hazard Ratios) by the SNPs and the clinico-pathological variables included in the model. Parsimonious risk scores
were calculated based on the models that included separately the clinicopathological and the genetic variables.

We assessed the clinical value of the above three scores in correctly predict disease status by receiver operating characteristic (ROC) curve analysis. The comparison of the areas under the ROC curves (AUC) constructed with the scores (with and without genetic information) was performed. Risk groups were created based in the best sensitivity/specificity ratio, as well as, the best negative and positive predictive values (NPV and PPV) possible of the best predictive model.

RESULTS

Clinicopathological features and BCG treatment outcome

The 204 evaluated cases presented a recurrence after BCG treatment of 34.3%. The median time to recurrence was 29.5 months (range: 8.0-122.0), whereas patients without recurrence the median follow-up time was 68.5 months (range: 12.0-163.0). Table 1 presents patients and tumour clinicopathological characteristics and its association with treatment response and RFS after BCG treatment.

Our results show that patients over 65 years have approximately 2-fold increased risk of recurrence (HR=1.973, [1.212-3.212], p=0.006). Moreover, it was observed that men have a 2.5-fold increased risk of recurrence (HR=2.533, [1.018-6.303], p=0.046). Furthermore, patients treated only with iBCG showed a 2-fold risk of recurrence (HR=2.034, [1.270-3.256], p=0.003). We performed a stepwise Cox regression analysis using backward elimination and evaluated all the clinicopathological features (gender, age, tumour stage, grade, multifocality, size, CIS presence and prior recurrence and treatment scheme) in terms of recurrence after BCG treatment. Only gender, age, tumor multifocality and treatment scheme remained independently associated to BCG outcome. The variables retained by this analysis were termed as Model 1 in the
predictive model establishment phase. These variables were considered as possible confounders and were used as covariates in the subsequent analysis.

<table>
<thead>
<tr>
<th>Variables</th>
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<th>p'</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
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</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>&lt;65 years</td>
<td>97 (47.5)</td>
<td>71 (53.0)</td>
<td>26 (37.1)</td>
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<td>63 (47.0)</td>
<td>44 (62.9)</td>
<td>1.973 [1.212-3.212]</td>
<td>0.006</td>
</tr>
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<td>24 (17.9)</td>
<td>5 (7.1)</td>
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</tr>
<tr>
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<td>110 (82.1)</td>
<td>65 (92.9)</td>
<td>2.533 [1.018-6.303]</td>
<td>0.046</td>
</tr>
<tr>
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<td></td>
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<tr>
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<td>80 (59.7)</td>
<td>42 (60.0)</td>
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<tr>
<td>High</td>
<td>149 (73.0)</td>
<td>98 (73.1)</td>
<td>51 (72.9)</td>
<td>1.188 [0.700-2.017]</td>
<td>0.524</td>
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<tr>
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<td>&lt;3</td>
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<tr>
<td>≥3</td>
<td>61 (32.1)</td>
<td>42 (33.6)</td>
<td>19 (29.2)</td>
<td>0.828 [0.485-1.414]</td>
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<td>68 (50.7)</td>
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<tr>
<td>Multifocal</td>
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<td>66 (49.3)</td>
<td>42 (60.0)</td>
<td>1.554 [0.963-2.510]</td>
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<tr>
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<td>124 (92.5)</td>
<td>62 (88.6)</td>
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<tr>
<td>Yes</td>
<td>18 (8.8)</td>
<td>10 (7.5)</td>
<td>8 (11.4)</td>
<td>1.238 [0.592-2.588]</td>
<td>0.570</td>
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<tr>
<td>Recurrence Status</td>
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<td></td>
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<tr>
<td>Primary</td>
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<td>72 (53.7)</td>
<td>32 (45.7)</td>
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<tr>
<td>Recurrent</td>
<td>100 (49.0)</td>
<td>62 (46.3)</td>
<td>38 (54.3)</td>
<td>1.304 [0.814-2.089]</td>
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<tr>
<td>mBCG</td>
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<td>33 (24.6)</td>
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<tr>
<td>iBCG</td>
<td>138 (67.6)</td>
<td>101 (75.4)</td>
<td>37 (52.9)</td>
<td>2.034 [1.270-3.256]</td>
<td>0.003</td>
</tr>
</tbody>
</table>

HR: Hazard Ratio; CI: Confidence Interval; *: Wald test; Bold values indicate p<0.05
Genetic polymorphism and BCG treatment outcome

Antigen presentation

In this study we evaluated five polymorphisms in genes involved in antigen presentation. From the studies SNPs, only patients carrying GG genotype for ICAM1 rs5498 presented a 2-fold risk of recurrence after BCG treatment (aHR=1.759, [1.050-2.949], p=0.032; Table 2). In terms of RFS, using Kaplan-Meier analysis, a trend was observed for this SNP. Patients carrying GG genotype had a mean RFS of 80 months, whereas patients carrying AA and GA genotype presented a mean RFS of 116 months (log rank, p=0.07; Fig. 1A).

Cytokines and chemokines

We evaluated 33 polymorphisms in 29 cytokine/chemokine genes and its receptors (Table S1 e S2). Our results demonstrate that among the studied polymorphisms IL2RA rs2104286 C/T, IL17A rs2275913 (-197G/A), TNFA rs1799964 (-1031T/C) and CCR2 rs391835 G/A (V64I) are associated with BCG treatment outcome. As showed in table 2, an increased risk of recurrence was found in patients carrying the variants of the mentioned polymorphisms (IL2RA rs2104286 CC vs.CT: aHR=2.007, [1.207-3.335], p= 0.007; IL17A rs2275913 GG+GA vs. AA: aHR=2.097, [1.118-3.993], p= 0.021; TNFA rs1799964 TT+TC vs.CC: aHR=2.427, [1.144-5.149], p= 0.021; CCR2 rs391835 GG vs. GA+AA: aHR=2.197, [1.120-4.312], p=0.022). Individuals carrying each of these genotypes presented lower recurrence free survival (log rank p<0.05; Fig. 1B-E)

Effectors molecules

We also evaluated iNOS gene, due to its involvement in the direct killing of tumor cells upon BCG internalization and TRAIL and its receptor TRAILR1, that are important mediators of cell death promoted by neutrophils and cytotoxic T-cell. Our analysis demonstrated that only patients carrying G allele (GG or TG genotypes) of TRAILR1 had a 3-fold risk of recurrence (TT vs. TG+GG, aHR=3.195, [1.373-7.433], p= 0.007;
Table 2). In terms of RFS, we found differences when we compared patients carrying the GG or TG genotypes with the ones with TT genotype (log-rank, \( p=0.018 \); Fig 1F).

<table>
<thead>
<tr>
<th>Table 2 – Multivariate analysis and risk estimation of the most significant polymorphisms</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ICAM1 rs5498 A/G (K469E)</strong></td>
</tr>
<tr>
<td>AA</td>
</tr>
<tr>
<td>AG</td>
</tr>
<tr>
<td>GG</td>
</tr>
<tr>
<td>AA+AG</td>
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<table>
<thead>
<tr>
<th><strong>IL2RA rs2104286 C/T</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>TT</td>
</tr>
<tr>
<td>CT</td>
</tr>
<tr>
<td>CC</td>
</tr>
<tr>
<td>CT+CC</td>
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<table>
<thead>
<tr>
<th><strong>IL17A rs2275913 (-197G/A)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
</tr>
<tr>
<td>GA</td>
</tr>
<tr>
<td>AA</td>
</tr>
<tr>
<td>GG+GA</td>
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<table>
<thead>
<tr>
<th><strong>TNFA rs1799964 (-1031T/C)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>TT</td>
</tr>
<tr>
<td>TC</td>
</tr>
<tr>
<td>CC</td>
</tr>
<tr>
<td>TT+TC</td>
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<td>CC</td>
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<table>
<thead>
<tr>
<th><strong>CCR2 rs391835 G/A (V64I)</strong></th>
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</thead>
<tbody>
<tr>
<td>GG</td>
</tr>
<tr>
<td>GA</td>
</tr>
<tr>
<td>AA</td>
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<tr>
<td>GA+AA</td>
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</table>

<table>
<thead>
<tr>
<th><strong>TRAILR1 rs13278062(-397T/G)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>TT</td>
</tr>
<tr>
<td>TG</td>
</tr>
<tr>
<td>GG</td>
</tr>
<tr>
<td>TG+GG</td>
</tr>
</tbody>
</table>

HR: Hazard ratio; 95%CI: 95% Confidence Interval; \( ^{a} \): adjusted to BCG scheme, age, gender and tumor multifocality; Bold values indicate \( p<0.05 \).
Figure 1 - Effect of the most significant polymorphisms in recurrence-free survival (RFS). Kaplan-Meier analysis to evaluate the association between RFS in the studied patients and the genotypes of: A- ICAM1 rs5498 A/G (K469E); B- IL2RA rs2104286 C/T; C- IL17A rs2275913 (-197G/A); D- TNFA rs1799964 (-1031T/C); E- CCR2 rs391835 G/A (V64I); F- TRAILR1 rs13278062 (-397T/G). Comparison performed by log-rank test (A: p=0.070; B: p=0.013; C: p=0.005; D: p=0.039; E: p=0.032; F: p=0.018); + and ◆ censored cases of each group analyzed.
Establishment of predictive models of BCG immunotherapy outcome

To determine the best predictive model of BCG outcome, we compared a clinical, a genetic and a combined model using stepwise Cox regression analysis (Table 3).

The concordance (c) index was used to compare the predictive ability of each model; the predictive value was assessed with Harrell’s concordance indexes, where a c-index of 1 indicates perfect concordance [20]. The predictive model of clinicopathological characteristics mentioned earlier presented a c-index of 0.698 (Model 1, Table 3).

To test if genetic variability in functional SNPs in molecules involved in BCG treatment mechanism could contribute to a combined effect for treatment outcome, we estimated the overall mutually-adjusted effects by stepwise multivariate Cox regression (Model 2). The SNPs in TNFA-1031T/C (rs1799964), IL4-33T/C (rs2070874), IL2RA rs2104286 T/C, IL17A-197G/A (rs2275913), IL17RA-809A/G (rs4819554), IL18R1 rs3771171 T/C, IL6R Asp358Ala (rs8192284), ICAM1 K469E (rs5498), FASL-844T/C (rs763110) and TRAILR1-397T/G (rs79037040) remained independently associated with risk of recurrence after BCG immunotherapy and this model presented a c-index of 0.735 (Table 3).

To evaluate the combined effect of clinicopathological features and genetic variants in BCG immunotherapy outcome we performed stepwise multivariate Cox regression with the variables obtained in the two previous models. Age and the IL6R polymorphism were removed from the combined model (Model 3) and the c-index of this model was 0.821 (Table 3).
### Table 3 – Predictive models of recurrence after BCG treatment obtained by Multivariate Stepwise Cox Regression

<table>
<thead>
<tr>
<th></th>
<th>HR</th>
<th>95%CI</th>
<th>P</th>
<th>c-index</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Model 1</strong></td>
<td>0.698</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Age (&lt;65 vs. ≥65)</td>
<td>1.78</td>
<td>[1.06-3.01]</td>
<td>0.031</td>
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<tr>
<td>Gender (female vs. male)</td>
<td>2.72</td>
<td>[1.09-6.81]</td>
<td>0.033</td>
<td></td>
</tr>
<tr>
<td>Tumor Number (unifocal vs. multifocal)</td>
<td>1.64</td>
<td>[0.97-2.76]</td>
<td>0.063</td>
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</tr>
<tr>
<td>Treatment Scheme (mBCG vs. iBCG)</td>
<td>2.01</td>
<td>[1.22-3.32]</td>
<td>0.006</td>
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</tr>
<tr>
<td><strong>Model 2</strong></td>
<td>0.735</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TNFA-1031T/C (rs1799964)</strong> (TT+TC vs. CC)</td>
<td>2.45</td>
<td>[1.31-5.33]</td>
<td>0.023</td>
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</tr>
<tr>
<td><strong>IL4-33T/C (rs2070874)</strong> (CT vs. TT)</td>
<td>1.72</td>
<td>[0.86-3.38]</td>
<td>0.116</td>
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<tr>
<td><strong>IL2RA rs2104286 T/C (TT vs. TC+CC)</strong></td>
<td>1.80</td>
<td>[1.08-2.99]</td>
<td>0.023</td>
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<tr>
<td><strong>IL17A-197G/A (rs2275913)</strong> (GG+GA vs. AA)</td>
<td>2.27</td>
<td>[1.16-4.45]</td>
<td>0.017</td>
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<tr>
<td><strong>IL17RA-809A/G (rs4819554)</strong> (AA+AC vs. CC)</td>
<td>2.19</td>
<td>[1.19-4.01]</td>
<td>0.011</td>
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<tr>
<td><strong>IL18R1 rs37711171 T/C (CC vs. TC+TT)</strong></td>
<td>2.88</td>
<td>[0.88-9.47]</td>
<td>0.081</td>
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<tr>
<td><strong>IL6R Asp358Ala (rs8192284)</strong> (CC vs. CA+AA)</td>
<td>2.00</td>
<td>[0.85-4.67]</td>
<td>0.110</td>
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<tr>
<td><strong>ICAM1 K469E (rs5498)</strong> (AA+AG vs. GG)</td>
<td>1.87</td>
<td>[1.08-3.26]</td>
<td>0.027</td>
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<td><strong>FASL-844T/C (rs763110)</strong> (TT+TC vs. CC)</td>
<td>1.71</td>
<td>[1.02-2.86]</td>
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<tr>
<td><strong>TRAILR1-397T/G (rs79037040)</strong> (TT vs. TG+GG)</td>
<td>2.59</td>
<td>[1.11-6.06]</td>
<td>0.028</td>
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<td><strong>Model 3</strong></td>
<td>0.820</td>
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<tr>
<td>Gender (female vs. male)</td>
<td>4.45</td>
<td>[1.71-11.6]</td>
<td>0.002</td>
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<td>Tumor Number (unifocal vs. multifocal)</td>
<td>2.29</td>
<td>[1.37-3.84]</td>
<td>0.002</td>
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<td>Treatment Scheme (mBCG vs. iBCG)</td>
<td>3.57</td>
<td>[2.03-6.28]</td>
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<td><strong>TNFA-1031T/C (rs1799964)</strong> (TT+TC vs. CC)</td>
<td>3.49</td>
<td>[1.58-7.70]</td>
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<td><strong>IL4-33T/C (rs2070874)</strong> (CT vs. TT)</td>
<td>1.93</td>
<td>[0.96-3.86]</td>
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<td><strong>IL2RA rs2104286 T/C (TT vs. TC+CC)</strong></td>
<td>2.79</td>
<td>[1.63-4.78]</td>
<td>&lt;0.001</td>
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<tr>
<td><strong>IL17A-197G/A (rs2275913)</strong> (GG+GA vs. AA)</td>
<td>2.65</td>
<td>[1.32-5.32]</td>
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<tr>
<td><strong>IL17RA-809A/G (rs4819554)</strong> (AA+AC vs. CC)</td>
<td>2.89</td>
<td>[1.49-5.63]</td>
<td>0.002</td>
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<tr>
<td><strong>IL18R1 rs37711171 T/C (CC vs. TC+TT)</strong></td>
<td>2.75</td>
<td>[0.82-9.18]</td>
<td>0.101</td>
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<tr>
<td><strong>ICAM1 K469E (rs5498)</strong> (AA+AG vs. GG)</td>
<td>2.47</td>
<td>[1.42-4.29]</td>
<td>0.001</td>
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<tr>
<td><strong>FASL-844T/C (rs763110)</strong> (TT+TC vs. CC)</td>
<td>1.70</td>
<td>[1.02-2.84]</td>
<td>0.042</td>
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<tr>
<td><strong>TRAILR1-397T/G (rs79037040)</strong> (TT vs. TG+GG)</td>
<td>5.19</td>
<td>[2.05-13.1]</td>
<td>0.001</td>
<td></td>
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</tbody>
</table>

HR: Hazard ratio; 95%CI: 95% Confidence Interval
The linear risk scores were computed based on the above Cox regression models and tested as overall risk predictors. Figure 2 shows the ROC curves for the risk scores of each model. The AUC estimates for the risk score of Model 3 was higher than the two other risk scores (Model 1, AUC: 0.684; Model 2, AUC: 0.734; Model 3, AUC: 0.820).

Figure 2 - ROC curves and AUC for the risk scores constructed based on the three different models. Solid line corresponds to the risk score of Model 3 (combination of clinical-pathological and genetic variables), dashed line represents the risk score of Model 2 (genetic variables alone) and dotted line refers to the risk score of Model 1 (Clinical-pathological variables alone). Model 1, AUC: 0.684; Model 2, AUC: 0.734; Model 3, AUC: 0.820.

Risk groups stratification was performed based on the best cut-off values to obtain the highest sensitivity and specificity. Three risk groups have been created based on the best predictive model (Model 3), as shown in Table 5. Patients within Low Risk group representing approximately 44% of all patients presented a negative predictive value of 92.2%, meaning that patients with a risk score below 17.2 had approximately 92% chance to
have a successful BCG treatment. On the other hand, the High Risk group had a positive predictive value of 75.6% and represented 22% of all patients treated with BCG immunotherapy. This encompasses patients with a risk score above 21.1, which have approximately 76% chance of BCG treatment failure. Moreover patients with a score among the two mentioned cut-off values were placed in the Intermediate Risk group, which have a 42% chance of treatment failure.

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>BCG success</th>
<th>BCG failure</th>
<th>HR</th>
<th>95% CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Risk</td>
<td>90 (44.1)</td>
<td>83 (61.9)</td>
<td>7 (10.0)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
</tr>
<tr>
<td>Intermediate</td>
<td>69 (33.8)</td>
<td>40 (29.9)</td>
<td>29 (41.4)</td>
<td>6.58</td>
<td>[2.88-15.1]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>High Risk</td>
<td>45 (22.1)</td>
<td>11 (8.2)</td>
<td>34 (48.6)</td>
<td>18.7</td>
<td>[8.24-42.6]</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

HR: Hazard Ratio; CI: Confidence Interval; *: Wald test

Kaplan-Meier plots showed significant differences in terms of RFS, for patients belonging to the different risk groups (Log-rank, p=0.0001, Fig. 3). Patients in the High risk group presented recurrence at the median time of 32 months, whereas patients in the Intermediate Risk group recurred at 93 months. Moreover, patients in the Low risk groups presented a mean time to recurrence of 140 months. Cox regression analysis showed that patients in the Intermediate and High risk groups have a substantial increase in recurrence risk (Intermediate Risk: HR=6.58, [2.88-15.05], p<0.001; High Risk: HR=18.7, [8.24-42.6], p=<0.001; Table 5).
Figure 3 - Recurrence-free survival (RFS) of the patients of the different risk groups based on the predictive Model 3. Kaplan-Meier analysis to evaluate the RFS of the studied patients within each risk group. Comparison performed by log-rank test (p<0.001); + Low Risk patients; • censored Intermediate Risk patients; ◆ censored High Risk patients.

DISCUSSION

Intravesical immunotherapy with BCG is the gold standard therapeutics for NMIBC at intermediate/high of recurrence or progression [3]. Nevertheless, approximately 30% of the patients recur after the treatment and 15% progress to muscle-invasive disease associated with poor prognosis [5]. Several clinicopathological characteristics influence the course of treatment, however there is no consensus regarding its predictive value [21]. Furthermore, there are no biomarkers to assess the outcome, making impossible the early identification of patients that could be better served by alternative treatments [16, 21].
Our previous reports using a smaller dataset have demonstrated that age, tumor multiplicity and the treatment scheme are associated with treatment failure [18, 22-24]. Herein, with a larger sample we confirmed that patients with age over 65 years or with multiple tumors or treated only with induction scheme are at risk of treatment failure. We also observed that male patients presented an increased risk of recurrence after treatment. Our findings reinforce previous associations of these clinical variables with BCG immunotherapy response [18, 21-24] and highlight the need to include them in predictive models for this therapeutics.

Genetic polymorphisms in molecules involved in the BCG mechanism of action have also been appointed as good candidates to predict treatment outcome and few polymorphisms have already been studied individually, in small sample sets, mainly using non-integrative approaches [16, 25-27]. In an attempt to establish of a predictive model of treatment response we evaluated 45 functional SNPs in 41 genes of molecules involved in the several steps of BCG immunotherapy mechanism of action.

Among molecules implicated in antigen presentation, only ICAM1 K469E polymorphism was associated with BCG outcome. This is the first study evaluating the role of ICAM-1 SNPs in BCG immunotherapy context. ICAM-1 (Intercellular Adhesion Molecule-1) is a transmembrane protein involved in adhesion of antigen-presenting cells to T lymphocytes, ICAM-1 binding produces proinflammatory effects such as inflammatory leukocyte recruitment [28]. Interestingly, circulating forms of ICAM-1 (sICAM1) were found to inhibit the interaction between T cells and tumors [29], and block NK cell-mediated toxicity [30]. The ICAM1 K469E polymorphism has been recognized to affect sICAM-1 levels and individuals carrying the GG genotype of this SNP have been found to present higher levels of sICAM-1 and consequently a decreased immune response [31]. Our results now show that patients carrying this genotype present a higher risk of BCG failure. Therefore, we postulate that higher levels of sICAM-1 may inhibit BCG antigen presentation and T-cell interaction with bladder tumor cells, reducing the cytotoxic effect of BCG immunotherapy.
We also evaluated several SNPs in cytokines and chemokines genes and its receptors and found that patients carrying the AA genotype of *IL17-197G/A* polymorphism presented an increased risk of recurrence after BCG treatment. It was recently described that IL-17 (also known as IL-17A), a T-cell-derived proinflammatory cytokine, is produced by γδ T-cells and plays a key role in the BCG-induced recruitment of neutrophils to the bladder, which is essential for the antitumor activity against bladder cancer [32]. The presence of AA genotype of *IL17-197G/A* polymorphism has been associated to reduced the levels of this molecule [33], therefore explaining its association with decreased response to treatment.

At the moment, IL-2 urinary levels are recognized as the most promising predictive biomarker of BCG treatment response. However, this molecule can only be measured during the treatment period and therefore cannot be used previously in the treatment definition [16]. Therefore, we evaluate genetic polymorphism that may exert some effect in IL-2 production and its receptor. Among the SNPs evaluated only *IL2RA* SNP was associated with BCG immunotherapy outcome. Studies revealed that patients carrying C allele of *IL2RA* rs2104286 presented lower production of soluble IL-2Rα and reduced T-cell activation [34]. Since we found that patients carrying this allele presented higher risk of recurrence, we may hypothesize that T-cell activation through IL-2 may be diminished in these patients, compromising a key role feature of BCG mechanism of action.

Due to the important role of TNF-α in the establishment of a Th1 immune response, we evaluated functional polymorphisms in *TNFA* gene. We found that *TNFA-1031T/C* was associated with recurrence after BCG treatment. This is the only SNP that we found to be associated to treatment outcome that already has been evaluated by other authors [25], however we obtained contradicting results. We found that patients carrying CC genotype presented an increased risk of recurrence, whereas the results published so far show a protective effect of this genotype. This may be explained by the different ethnicities background of the population evaluated in both studies; it also may result from a bias of the sample number, once the number of patients that we evaluated was significantly...
higher (204 vs 70); or a consequence from the higher percentage of patients treated with mBCG in the population that we studied (treatment scheme was not considered in the referenced study, once they only had 7 patients treated with mBCG).

We and others have reported that macrophages plays a key role in BCG mechanism of action [6, 22, 35]. Therefore, we elected to evaluate polymorphisms in genes involved in macrophage attraction and activation, such as, monocyte chemoattractant protein-1 (MCP-1) that acts as a chemoattractant for monocytes, macrophages and other inflammatory cells to sites of inflammation [36] and its receptor (CCR2). From the SNPs studied only the \textit{CCR2}-960T/A polymorphism demonstrated an association with treatment outcome. This SNP is located within the functional promoter [37] and patients carrying A allele present reduced monocyte migration activity [38]. Moreover, our results showed that patients carrying this allele had an increased risk of recurrence possible due to a deficient monocyte migration and consequently a decrease macrophage attraction influencing the immunological activation promoted by BCG therapy.

Another line of evidence indicates a that for neutrophils plays an important part in BCG immunotherapy, especially through the release of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). This molecule is one of the key effector of BCG antitumor action [14]. Therefore we evaluated genetic polymorphism that could influence TRAIL expression as well as its receptor. Our results demonstrated patients carrying the G allele of \textit{TRAILR1}-397T/G polymorphism had an increased risk of recurrence after BCG treatment. Wang \textit{et al.} showed that the presence of this allele increases the transcriptional activity of \textit{TRAILR1} promoter, possibly leading to higher expression of the receptor [39]. Although an increased expression of \textit{TRAILR1} may improve death receptor activation in BC cells promoted by BCG, the higher expression of this receptor in T-cells may enhance T-cell apoptosis, especially in T helper (Th) 1 cell clones which are sensitive to TRAIL-induced apoptosis [40]. Moreover, the activation of T cells with interleukin (IL)-2 resulted in TRAIL susceptibility and TRAIL caused death of antigen-specific memory CD8+ T cells [40].
Taking in account all these facts, we may postulate that an increased expression of this receptor, enhanced by TRAILR1-397T/G polymorphism may lead to higher TRAIL-induced T-cell apoptosis, compromising BCG immunotherapy efficacy.

The effects exerted by these SNPs contribute to impaired T-cell activation, reduced macrophage and neutrophil attraction, and Th1 cell apoptosis, all of them key mediators of tumor cell death. Our data suggests that the presence of several of these deleterious effects may contribute to an inefficient immune response and consequently BCG treatment failure.

The main objective of this work was to establish a predictive profile of BCG immunotherapy outcome. We started by evaluating the influence of clinical variables and genetic variants separately and then in combination. We found that the association of clinicopathological and genetic information provides a good predictive model of recurrence after BCG treatment and the risk score created based in this model may be suitable to stratify patients based on their chances of a successful treatment. This is the first report combining several genetic polymorphisms that could influence BCG mechanism of action and also clinical variables. A predictive model and a risk score of recurrence after BCG treatment is also being proposed for the first time. Noteworthy, we created a model based on information from more than 200 patients, which contrasts with the small populations used in previous studies involving genetic polymorphisms in the context of BCG immunotherapy (less than 70 patients). The established risk groups are expected to help clinical decision; however patients that fall in the Intermediate Risk group present a 40% probability of recurrence that is similar to the recurrence chance without performing this risk stratification. More studies are needed to corroborate our findings in even higher samples from different population. It is also necessary to include other potential markers, such as tumor markers, this may help to a better stratification of the patients.

In conclusion, this work has allowed the identification of novel genetic markers of BCG immunotherapy outcome and the establishment of
a genetic risk score that could stratify patients according to the risk of recurrence after BCG immunotherapy. The established risk groups are able to identify patients with poor prognosis and is this may be a helpful tool improve clinical decision.

**Acknowledgments**
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**REFERENCES**


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Table S2 - Genotype distribution and risk estimates of the different polymorphisms evaluated

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<th>Genotype</th>
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<td>4 (5.7%)</td>
<td>0.488</td>
<td>[0.171-1.396]</td>
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<td>G carrier (TG+GG)</td>
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<td>[0.574-1.506]</td>
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<td>38 (54.3%)</td>
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<td>G carrier (TG+GG)</td>
<td>68 (50.7%)</td>
<td>32 (45.7%)</td>
<td>0.930</td>
<td>[0.574-1.506]</td>
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<td>T carrier (TT+TG)</td>
<td>118 (88.1%)</td>
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<td>GG</td>
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<td>4 (5.7%)</td>
<td>0.488</td>
<td>[0.171-1.396]</td>
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| IL2RA rs2104286 | TT         | 82 (61.2%) | 33 (47.1%) | 1.0 | Referent | -     |
|                | TG         | 41 (30.6%) | 32 (45.7%) | 2.007 | [1.207-3.335] | 0.007 |
|                | CC         | 11 (8.2%)  | 5 (7.1%)    | 1.245 | [0.476-3.257] | 0.655 |
|                | G carrier (TG+GG) | 52 (38.8%) | 37 (52.8%) | 1.603 | [1.001-2.567] | 0.050 |
|                | T carrier (TT+TC) | 123 (91.8%) | 65 (92.8%) | 1.0 | Referent | -     |
|                | CC         | 11 (8.2%)  | 5 (7.1%)    | 0.828 | [0.329-2.078] | 0.687 |

| IL4 rs2243250 | CC         | 104 (77.6%) | 57 (81.4%) | 1.0 | Referent | -     |
|              | T carrier (CT+TT) | 29 (21.6%) | 11 (15.7%) | 0.795 | [0.415-1.522] | 0.488 |

<p>| IL6 rs1800795 | GG         | 67 (50.4%) | 26 (38.2%) | 1.0 | Referent | -     |
|              | GC         | 48 (36.1%) | 31 (45.6%) | 1.189 | [0.736-1.922] | 0.479 |
|              | CC         | 18 (13.5%) | 11 (16.2%) | 0.914 | [0.444-1.880] | 0.807 |
|              | G carrier (GG+GC) | 66 (49.6%) | 42 (61.8%) | 1.177 | [0.713-1.944] | 0.523 |
|              | CC         | 18 (13.5%) | 11 (16.2%) | 0.876 | [0.457-1.677] | 0.689 |</p>
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<th>BCG failure</th>
<th>HR</th>
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<td>C carrier (TC+TT)</td>
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<td>7 (5.2%)</td>
<td>5 (7.1%)</td>
<td>0.960</td>
<td>[0.384-2.398]</td>
<td>0.930</td>
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<td>39 (57.4%)</td>
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<td>Referent</td>
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<tr>
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<td>57 (42.5%)</td>
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<td>0.703</td>
<td>[0.414-1.195]</td>
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<td>11 (8.2%)</td>
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<td>[0.281-1.612]</td>
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<td>39 (57.4%)</td>
<td>1.0</td>
<td>Referent</td>
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<tr>
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<td>68 (50.7%)</td>
<td>29 (42.6%)</td>
<td>0.694</td>
<td>[0.425-1.135]</td>
<td>0.146</td>
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<td>G carrier (GG+GA)</td>
<td>123 (91.8%)</td>
<td>62 (91.2%)</td>
<td>1.0</td>
<td>Referent</td>
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<td></td>
<td>AA</td>
<td>11 (8.2%)</td>
<td>6 (8.8%)</td>
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<td>[0.332-1.802]</td>
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<td>Model</td>
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<td>BCG success N (%)</td>
<td>BCG failure N (%)</td>
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<td>p</td>
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<td>GG</td>
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<td>33 (47.1%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
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<td></td>
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<td>GA</td>
<td>58 (43.3%)</td>
<td>25 (35.7%)</td>
<td>0.856</td>
<td>[0.506-1.449]</td>
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<td>AA</td>
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<td>[0.988-3.836]</td>
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<td>GG</td>
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<td>33 (47.1%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
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<td>37 (52.8%)</td>
<td>1.042</td>
<td>[0.648-1.675]</td>
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<td>Recessive</td>
<td>G carrier (GG+GA)</td>
<td>125 (93.3%)</td>
<td>58 (82.8%)</td>
<td>1.0</td>
<td>Referent</td>
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<td>[1.118-3.933]</td>
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<td><em>IL17RA</em> rs4819554</td>
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<td>44 (32.8%)</td>
<td>21 (30.4%)</td>
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<td>Referent</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TC</td>
<td>69 (51.5%)</td>
<td>33 (47.8%)</td>
<td>1.093</td>
<td>[0.631-1.895]</td>
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<td>21 (15.7%)</td>
<td>15 (21.7%)</td>
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<td>[0.849-3.257]</td>
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<td>TT</td>
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<td>21 (30.4%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
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<td></td>
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<td>C carrier (TC+CC)</td>
<td>90 (67.2%)</td>
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<td>[0.726-2.034]</td>
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<td>Recessive</td>
<td>T carrier (TT+TC)</td>
<td>113 (84.3%)</td>
<td>54 (78.2%)</td>
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<td>Referent</td>
<td>-</td>
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<td></td>
<td></td>
<td>CC</td>
<td>21 (15.7%)</td>
<td>15 (21.7%)</td>
<td>1.606</td>
<td>[0.903-2.858]</td>
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<td>CC</td>
<td>74 (55.2%)</td>
<td>34 (49.3%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
</tr>
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<td></td>
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<td>GC</td>
<td>51 (38.1%)</td>
<td>28 (40.6%)</td>
<td>0.913</td>
<td>[0.552-1.510]</td>
<td>0.722</td>
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<td>GG</td>
<td>9 (6.7%)</td>
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<td>1.250</td>
<td>[0.548-2.851]</td>
<td>0.596</td>
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<td>CC</td>
<td>74 (55.2%)</td>
<td>34 (49.3%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
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<td></td>
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<td>G carrier (GC+GG)</td>
<td>60 (44.8%)</td>
<td>35 (50.7%)</td>
<td>0.959</td>
<td>[0.597-1.541]</td>
<td>0.862</td>
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<td>C carrier (CC+GC)</td>
<td>125 (93.3%)</td>
<td>62 (89.9%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
</tr>
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<td>GG</td>
<td>9 (6.7%)</td>
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<td>1.262</td>
<td>[0.574-2.772]</td>
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<td>14 (20.9%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
</tr>
<tr>
<td></td>
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<td>AC</td>
<td>68 (51.1%)</td>
<td>38 (56.7%)</td>
<td>1.176</td>
<td>[0.617-2.240]</td>
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<td>26 (19.5%)</td>
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<td>1.163</td>
<td>[0.520-2.601]</td>
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<td>14 (20.9%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
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<td>A carrier (AC+AA)</td>
<td>94 (70.6%)</td>
<td>53 (79.1%)</td>
<td>1.080</td>
<td>[0.585-1.994]</td>
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<td>107 (80.4%)</td>
<td>52 (77.6%)</td>
<td>1.0</td>
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<td>AA</td>
<td>26 (19.5%)</td>
<td>15 (22.4%)</td>
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<td>Genotype</td>
<td>BCG success N (%)</td>
<td>BCG failure N (%)</td>
<td>HR*</td>
<td>95% CI</td>
<td>p</td>
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<td>IL18R1 rs3771171</td>
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<tr>
<td>Cytokines</td>
<td></td>
<td></td>
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<td>61 (45.9%)</td>
<td>37 (54.4%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TC</td>
<td>60 (45.1%)</td>
<td>27 (39.7%)</td>
<td>0.827</td>
<td>[0.504-1.356]</td>
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<td>12 (9.0%)</td>
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<td>61 (45.9%)</td>
<td>37 (54.4%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C carrier (TC+CC)</td>
<td>72 (54.1%)</td>
<td>31 (45.6%)</td>
<td>0.754</td>
<td>[0.465-1.221]</td>
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<tr>
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<td>T carrier (TT+TC)</td>
<td>121 (91.0%)</td>
<td>64 (94.1%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
</tr>
<tr>
<td></td>
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<td>CC</td>
<td>12 (9.0%)</td>
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<td>0.422</td>
<td>[0.132-1.347]</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GG</td>
<td>89 (66.9%)</td>
<td>48 (71.6%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>GA</td>
<td>39 (29.3%)</td>
<td>16 (23.9%)</td>
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<td>[0.443-1.207]</td>
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<td>[0.197-1.580]</td>
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<td>89 (66.9%)</td>
<td>48 (71.6%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
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<td>A carrier (GA+AA)</td>
<td>44 (33.1%)</td>
<td>19 (28.4%)</td>
<td>0.697</td>
<td>[0.430-1.128]</td>
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<td>G carrier (GG+GA)</td>
<td>128 (96.2%)</td>
<td>64 (95.5%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
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<td>AA</td>
<td>5 (3.8%)</td>
<td>3 (4.5%)</td>
<td>0.602</td>
<td>[0.219-1.658]</td>
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<td></td>
<td></td>
<td></td>
<td>CC</td>
<td>63 (47.0%)</td>
<td>38 (55.9%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
</tr>
<tr>
<td></td>
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<td>CA</td>
<td>57 (42.5%)</td>
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<td>0.777</td>
<td>[0.461-1.307]</td>
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<td>14 (10.4%)</td>
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<td>[0.468-2.521]</td>
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<td>CC</td>
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<td>38 (55.9%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
</tr>
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<td>A carrier (CA+AA)</td>
<td>71 (52.9%)</td>
<td>30 (44.1%)</td>
<td>0.843</td>
<td>[0.522-1.362]</td>
<td>0.485</td>
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<td>C carrier (CC+CA)</td>
<td>120 (89.5%)</td>
<td>61 (89.7%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
</tr>
<tr>
<td></td>
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<td>AA</td>
<td>14 (10.4%)</td>
<td>7 (10.3%)</td>
<td>1.235</td>
<td>[0.553-2.760]</td>
<td>0.607</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TT</td>
<td>72 (53.7%)</td>
<td>38 (55.9%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TC</td>
<td>56 (41.8%)</td>
<td>22 (32.4%)</td>
<td>0.778</td>
<td>[0.458-1.324]</td>
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<td>CC</td>
<td>6 (4.5%)</td>
<td>8 (11.8%)</td>
<td>2.112</td>
<td>[0.970-4.598]</td>
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<td>Dominant</td>
<td></td>
<td>TT</td>
<td>72 (53.7%)</td>
<td>38 (55.9%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>C carrier (TC+CC)</td>
<td>62 (46.3%)</td>
<td>30 (44.2%)</td>
<td>0.935</td>
<td>[0.576-1.517]</td>
<td>0.785</td>
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<td>T carrier (TT+TC)</td>
<td>128 (95.5%)</td>
<td>60 (88.3%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CC</td>
<td>6 (4.5%)</td>
<td>8 (11.8%)</td>
<td>2.427</td>
<td>[1.144-5.149]</td>
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<td>Model</td>
<td>Genotype</td>
<td>BCG success N (%)</td>
<td>BCG failure N (%)</td>
<td>HR</td>
<td>95%CI</td>
<td>p</td>
</tr>
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</tr>
<tr>
<td><strong>Cytokines</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GG</td>
<td>100 (74.6%)</td>
<td>49 (70.0%)</td>
<td>1.0</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GA</td>
<td>32 (23.9%)</td>
<td>19 (27.1%)</td>
<td>0.964</td>
<td>[0.561-1.655]</td>
<td>0.894</td>
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<td>2 (1.5%)</td>
<td>2 (2.9%)</td>
<td>2.938</td>
<td>[0.658-13.12]</td>
<td>0.158</td>
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<td>GG</td>
<td>100 (74.6%)</td>
<td>49 (70.0%)</td>
<td>1.0</td>
<td>Referent</td>
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<td>A carrier (GA+AA)</td>
<td>34 (25.4%)</td>
<td>21 (30.0%)</td>
<td>1.029</td>
<td>[0.611-1.734]</td>
<td>0.894</td>
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<td>68 (97.1%)</td>
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<td>Referent</td>
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<td>2 (1.5%)</td>
<td>2 (2.9%)</td>
<td>2.131</td>
<td>[0.499-9.099]</td>
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<td>Referent</td>
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<td>AG</td>
<td>71 (53.0%)</td>
<td>38 (55.1%)</td>
<td>1.194</td>
<td>[0.680-2.097]</td>
<td>0.537</td>
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<td>20 (14.9%)</td>
<td>12 (17.4%)</td>
<td>1.404</td>
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<td>19 (27.5%)</td>
<td>1.0</td>
<td>Referent</td>
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<td>[0.673-2.365]</td>
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<td><strong>TGFB1 rs1800469</strong></td>
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<td>Referent</td>
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</tr>
<tr>
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<td></td>
<td>CT</td>
<td>66 (49.3%)</td>
<td>27 (38.6%)</td>
<td>0.659</td>
<td>[0.394-1.103]</td>
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<td>[0.451-2.030]</td>
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<td>1.0</td>
<td>Referent</td>
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<td>T carrier (CT+TT)</td>
<td>79 (59.0%)</td>
<td>36 (51.5%)</td>
<td>0.728</td>
<td>[0.452-1.174]</td>
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<td>C carrier (CC+CT)</td>
<td>121 (90.3%)</td>
<td>61 (87.2%)</td>
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<td>TT</td>
<td>13 (9.7%)</td>
<td>9 (12.9%)</td>
<td>1.154</td>
<td>[0.569-2.340]</td>
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<tr>
<td><strong>TGFBR1 rs334354</strong></td>
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<td>GG</td>
<td>89 (66.9%)</td>
<td>48 (71.6%)</td>
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<tr>
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<td>GA</td>
<td>39 (29.3%)</td>
<td>16 (23.9%)</td>
<td>1.061</td>
<td>[0.589-1.912]</td>
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<td>5 (3.8%)</td>
<td>3 (4.5%)</td>
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<td>[0.575-6.290]</td>
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<td>48 (71.6%)</td>
<td>1.0</td>
<td>Referent</td>
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<td>A carrier (GA+AA)</td>
<td>44 (33.1%)</td>
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<td>1.153</td>
<td>[0.663-2.006]</td>
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<td>G carrier (GG+GA)</td>
<td>128 (96.2%)</td>
<td>64 (95.5%)</td>
<td>1.0</td>
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<td>5 (3.8%)</td>
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<td>[0.589-6.180]</td>
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<td>Polymorphism</td>
<td>Model</td>
<td>Genotype</td>
<td>BCG success N (%)</td>
<td>BCG failure N (%)</td>
<td>HR(a)</td>
<td>95%CI</td>
<td>p</td>
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<td>Chemokines</td>
<td>TGFBR2 rs3087465</td>
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<td>GG</td>
<td>82 (61.2%)</td>
<td>45 (64.3%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
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<td></td>
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<td></td>
<td>GA</td>
<td>50 (37.3%)</td>
<td>21 (30.0%)</td>
<td>0.852</td>
<td>[0.506-1.435]</td>
<td>0.548</td>
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<td>2 (1.5%)</td>
<td>4 (5.7%)</td>
<td>1.283</td>
<td>[0.459-3.590]</td>
<td>0.635</td>
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<td>Dominant</td>
<td>GG</td>
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<td>45 (64.3%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
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<td>A carrier (GA+AA)</td>
<td>52 (38.8%)</td>
<td>25 (35.7%)</td>
<td>0.889</td>
<td>[0.543-1.456]</td>
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<td>G carrier (GG+GA)</td>
<td>132 (98.5%)</td>
<td>66 (94.3%)</td>
<td>1.0</td>
<td>Referent</td>
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<td>2 (1.5%)</td>
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<td>1.296</td>
<td>[0.463-3.628]</td>
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<td>38 (28.4%)</td>
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<td>1.0</td>
<td>Referent</td>
<td>-</td>
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<td>GA</td>
<td>61 (45.5%)</td>
<td>38 (54.3%)</td>
<td>1.990</td>
<td>[0.984-4.024]</td>
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<td>35 (26.1%)</td>
<td>22 (31.4%)</td>
<td>2.439</td>
<td>[1.138-5.226]</td>
<td>0.022</td>
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<td>Dominant</td>
<td>GG</td>
<td>38 (28.4%)</td>
<td>10 (14.3%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
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<td>A carrier (AA+GA)</td>
<td>96 (71.6%)</td>
<td>60 (85.7%)</td>
<td>2.197</td>
<td>[1.120-4.312]</td>
<td>0.022</td>
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<td>Recessive</td>
<td>G carrier (GA+GG)</td>
<td>99 (73.9%)</td>
<td>48 (68.6%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
</tr>
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<td>AA</td>
<td>35 (26.1%)</td>
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<td>1.484</td>
<td>[0.881-2.500]</td>
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<td>19 (27.1%)</td>
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<td>Referent</td>
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<tr>
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<td>GA</td>
<td>103 (76.9%)</td>
<td>51 (72.9%)</td>
<td>0.725</td>
<td>[0.404-1.301]</td>
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<td>GMCSF rs1469149</td>
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<td>41 (31.1%)</td>
<td>27 (39.7%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
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<td>AC</td>
<td>64 (48.5%)</td>
<td>31 (45.6%)</td>
<td>0.947</td>
<td>[0.563-1.591]</td>
<td>0.837</td>
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<td>27 (20.5%)</td>
<td>10 (14.7%)</td>
<td>0.716</td>
<td>[0.344-1.490]</td>
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<td>AA</td>
<td>41 (31.1%)</td>
<td>27 (39.7%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
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<td>C carrier (AC+CC)</td>
<td>91 (69.0%)</td>
<td>41 (50.3%)</td>
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<td>[0.551-1.466]</td>
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<td>105 (79.6%)</td>
<td>58 (85.3%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
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<td>CC</td>
<td>27 (20.5%)</td>
<td>10 (14.7%)</td>
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<td>[0.380-1.463]</td>
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<td>BCG failure N (%)</td>
<td>HR^95%CI</td>
<td>p</td>
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<td>39 (29.3%)</td>
<td>20 (29.9%)</td>
<td>1.0</td>
<td>Referent</td>
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<td></td>
<td>TA</td>
<td>69 (51.9%)</td>
<td>36 (53.7%)</td>
<td>1.050</td>
<td>0.862</td>
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<td>AA</td>
<td>25 (18.8%)</td>
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<td>20 (29.9%)</td>
<td>1.0</td>
<td>Referent</td>
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<td>A carrier (TA+AA)</td>
<td>94 (70.7%)</td>
<td>47 (70.1%)</td>
<td>1.065</td>
<td>0.815</td>
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<td>TA</td>
<td>69 (51.9%)</td>
<td>36 (53.7%)</td>
<td>1.050</td>
<td>0.862</td>
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<td>AA</td>
<td>25 (18.8%)</td>
<td>11 (16.4%)</td>
<td>1.0</td>
<td>Referent</td>
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<td><em>MIF</em> rs755622</td>
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<td>GG</td>
<td>98 (73.1%)</td>
<td>50 (71.4%)</td>
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<tr>
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<td>GC</td>
<td>33 (24.6%)</td>
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<td>1.205</td>
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<td>CC</td>
<td>3 (2.2%)</td>
<td>0 (0.0%)</td>
<td>1.0</td>
<td>Referent</td>
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<tr>
<td></td>
<td></td>
<td>Recessive</td>
<td>GG</td>
<td>98 (73.1%)</td>
<td>50 (71.4%)</td>
<td>1.0</td>
<td>Referent</td>
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<td>C carrier (GC+CC)</td>
<td>36 (26.8%)</td>
<td>20 (28.6%)</td>
<td>1.127</td>
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<td>5 (3.7%)</td>
<td>4 (5.7%)</td>
<td>0.971</td>
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<td><em>RANTES</em> rs2107538</td>
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<td>GG</td>
<td>90 (67.2%)</td>
<td>51 (72.9%)</td>
<td>1.0</td>
<td>Referent</td>
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<td>AG</td>
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<td>15 (21.4%)</td>
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<td>4 (5.7%)</td>
<td>0.971</td>
<td>0.955</td>
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<td>Recessive</td>
<td>GG</td>
<td>90 (67.2%)</td>
<td>51 (72.9%)</td>
<td>1.0</td>
<td>Referent</td>
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<td></td>
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<td>A carrier (AG+AA)</td>
<td>44 (32.8%)</td>
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<td>GG</td>
<td>29 (21.6%)</td>
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<td>1.365</td>
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<td>AA</td>
<td>5 (3.7%)</td>
<td>4 (5.7%)</td>
<td>1.057</td>
<td>0.915</td>
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<td>Apoptosis and Cell Death</td>
<td><em>FAS</em> rs1800682</td>
<td>Dominant</td>
<td>AA</td>
<td>38 (28.4%)</td>
<td>20 (28.6%)</td>
<td>1.0</td>
<td>Referent</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>GA</td>
<td>67 (50.0%)</td>
<td>31 (44.3%)</td>
<td>1.022</td>
<td>0.943</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GG</td>
<td>29 (21.6%)</td>
<td>19 (27.1%)</td>
<td>1.365</td>
<td>0.366</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Recessive</td>
<td>AA</td>
<td>38 (28.4%)</td>
<td>20 (28.6%)</td>
<td>1.0</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>G carrier (GA+GG)</td>
<td>96 (71.6%)</td>
<td>50 (71.4%)</td>
<td>1.110</td>
<td>0.705</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AA</td>
<td>105 (78.4%)</td>
<td>51 (72.9%)</td>
<td>1.0</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GG</td>
<td>29 (21.6%)</td>
<td>19 (27.1%)</td>
<td>1.335</td>
<td>0.293</td>
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<td>Pathway</td>
<td>Polymorphism</td>
<td>Model</td>
<td>Genotype</td>
<td>BCG success</td>
<td>BCG failure</td>
<td>HRa</td>
<td>95%CI</td>
<td>p</td>
</tr>
<tr>
<td>-------------------------------</td>
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<td></td>
<td></td>
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<td></td>
<td>N (%)</td>
<td>N (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apoptosis and Cell Death</td>
<td><em>FASL</em> rs763110</td>
<td></td>
<td>TT</td>
<td>25 (18.7%)</td>
<td>8 (11.6%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TC</td>
<td>69 (51.5%)</td>
<td>35 (50.7%)</td>
<td>1.444</td>
<td>[0.654-3.189]</td>
<td>0.363</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CC</td>
<td>40 (29.9%)</td>
<td>26 (37.7%)</td>
<td>1.640</td>
<td>[0.724-3.715]</td>
<td>0.236</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>TT</td>
<td>25 (18.7%)</td>
<td>8 (11.6%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C carrier (CC+TC)</td>
<td>109 (81.3%)</td>
<td>61 (88.4%)</td>
<td>1.539</td>
<td>[0.726-3.258]</td>
<td>0.260</td>
</tr>
<tr>
<td>Recessive</td>
<td></td>
<td></td>
<td>T carrier (TC+TT)</td>
<td>94 (70.1%)</td>
<td>43 (62.3%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CC</td>
<td>40 (29.9%)</td>
<td>26 (37.7%)</td>
<td>1.440</td>
<td>[0.883-2.350]</td>
<td>0.144</td>
</tr>
<tr>
<td></td>
<td><em>INOS</em> rs2779249</td>
<td></td>
<td>AA</td>
<td>67 (50.0%)</td>
<td>38 (54.3%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AC</td>
<td>56 (41.8%)</td>
<td>29 (41.4%)</td>
<td>0.916</td>
<td>[0.559-1.501]</td>
<td>0.727</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CC</td>
<td>11 (8.2%)</td>
<td>3 (4.3%)</td>
<td>0.699</td>
<td>[0.211-2.314]</td>
<td>0.558</td>
</tr>
<tr>
<td>Dominant</td>
<td></td>
<td></td>
<td>AA</td>
<td>67 (50.0%)</td>
<td>38 (54.3%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C carrier (AC+CC)</td>
<td>67 (50.0%)</td>
<td>32 (45.7%)</td>
<td>0.867</td>
<td>[0.539-1.396]</td>
<td>0.557</td>
</tr>
<tr>
<td>Recessive</td>
<td></td>
<td></td>
<td>A carrier (AA+AC)</td>
<td>123 (91.8%)</td>
<td>67 (95.7%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CC</td>
<td>11 (8.2%)</td>
<td>3 (4.3%)</td>
<td>0.690</td>
<td>[0.215-2.221]</td>
<td>0.534</td>
</tr>
<tr>
<td></td>
<td><em>TRAILR1</em> rs79037040</td>
<td></td>
<td>TT</td>
<td>31 (27.9%)</td>
<td>6 (10.2%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TG</td>
<td>51 (45.9%)</td>
<td>33 (55.9%)</td>
<td>3.546</td>
<td>[1.477-8.513]</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GG</td>
<td>29 (26.1%)</td>
<td>20 (33.9%)</td>
<td>3.078</td>
<td>[1.251-7.573]</td>
<td>0.014</td>
</tr>
<tr>
<td>Dominant</td>
<td></td>
<td></td>
<td>TT</td>
<td>31 (27.9%)</td>
<td>6 (10.2%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>G carrier (GG+TG)</td>
<td>80 (72.0%)</td>
<td>53 (89.8%)</td>
<td>3.195</td>
<td>[1.373-7.433]</td>
<td>0.007</td>
</tr>
<tr>
<td>Recessive</td>
<td></td>
<td></td>
<td>T carrier (TG+TT)</td>
<td>82 (73.8%)</td>
<td>39 (66.1%)</td>
<td>1.0</td>
<td>Referent</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GG</td>
<td>29 (26.1%)</td>
<td>20 (33.9%)</td>
<td>1.310</td>
<td>[0.803-2.137]</td>
<td>0.279</td>
</tr>
</tbody>
</table>
4. CONCLUSIONS
Patients presenting intermediate/high risk of recurrence NMIBC are treated with intravesical instillation with BCG. However, approximately one third of the patients fail to respond to the treatment and ultimately may present progression to muscle invasive disease. An early identification of patients better served by alternative therapeutics is currently considered of pivotal importance for the management of intermediate/high risk NMIBC. Despite several studies on this matter, at the moment there are no validated biomarkers to determine BCG immunotherapy outcome.

In the systematic review presented in this thesis, it was highlighted that macrophage infiltration and genetic polymorphisms in genes of immune system present potential as biomarkers of treatment response. As such, attention was devoted to evaluate these features.

In addition, it was observed that the tumors of patients treated with BCG express cell-surface proteins carrying the cancer-associated carbohydrate antigen sTn. This posttranslational modification of proteins influences both the adhesion to tumor cells and immune responses and may, therefore, directly interfere with BCG action. Based on these considerations we included sTn in the biomarker panel to be addressed in this thesis. The ultimate goal was to create a predictive profile of treatment response.

Each biomarker was extensively discussed in the papers presented in the previous chapters. Herein we summarize the main findings and conclusions of each work.

The idea that macrophages counts are a useful predictive marker of recurrence after BCG treatment was corroborated. It was also described for the first time that discrimination of M2 macrophages (CD163⁺) is a better indicator of treatment failure than the overall macrophage count, given by CD68. Moreover, our observations suggest that only M2 macrophages under normoxic conditions may exert an inhibitory effect on BCG immunotherapy, possibly due to its immunosuppressive phenotype. More studies are needed to corroborate this hypothesis.

It was also describe, for the first time, that tumor-associated carbohydrate sialyl-Tn, and its related antigen sialyl-6-T, may be suitable
predictive biomarkers of treatment recurrence. Our results strongly suggest that BCG immunotherapy is efficient against sTn positive tumors. Although the exact mechanisms underlying this event was not determined, it was demonstrated that BCG adhesion and internalization is higher for sTn positive cells \textit{in vitro}.

As outlined, genetic polymorphisms may affect several key molecules involved in BCG immunotherapy; therefore their identification may help to personalize treatment. This subject was primarily addressed in a dataset of 125 patients by evaluating the \textit{TNF-308G/A} and \textit{IL4-590C/T} functional polymorphisms. Although the results showed that these genetic polymorphisms alone are not predictors of BCG treatment response, they are associated with the development of multiple and/or CIS tumors, which are associated with higher recurrence and progression rates. The influence of \textit{FAS-670A/G} and \textit{FASL-844T/C} polymorphisms was also evaluated and it was observed that patients carrying \textit{FASL-844} CC genotype presented higher expression of FasL and an increased risk of recurrence after treatment.

Then, the number of patients was increased and a panel of 45 functional polymorphisms in genes involved in the several steps of BCG mechanism of action was elected for subsequent studies. To establish a predictive profile of recurrence after BCG immunotherapy, we used stepwise multivariate Cox Regression analysis with backward elimination to select the genetic variants that may help to predict BCG treatment outcome. In this new dataset we repeated the previously evaluated polymorphisms. Results corroborate previews findings, where \textit{TNF-308G/A}, \textit{IL4-590C/T} and \textit{FAS-670A/G} polymorphisms were not associated with BCG treatment outcome. Conversely, \textit{FASL-844T/C} polymorphism was confirmed to be a good predictor of recurrence. This showed that the preliminary dataset was representative of the study population and reinforced the consistency of the first results.

It was further observed that the polymorphisms \textit{TNFA-1031T/C} (rs1799964), \textit{IL2RA} rs2104286 T/C, \textit{IL17A-197G/A} (rs2275913), \textit{IL17RA-809A/G} (rs4819554), \textit{IL18R1} rs3771171 T/C, \textit{IL6R} Asp358Ala (rs8192284),
ICAM1 K469E (rs5498), FASL-844T/C (rs763110) and TRAILR1-397T/G (rs79037040) were independently associated with risk of recurrence after BCG immunotherapy.

The association of this genetic information with clinicopathological variables provided a good predictive model of recurrence after BCG treatment. A risk score was established based in this model and the results presented here showed that this score may be suitable to stratify patients according to their chances to have a successful treatment. Accordingly, patients within the Low Risk groups have a 90% chance of successful treatment, whereas patients in the High Risk groups present 75% chance of recurrence after BCG treatment.

The established risk groups are expected to help clinical decision; however patients that fall in the Intermediate Risk group present a 40% probability of recurrence that is similar to the recurrence chance without performing this risk stratification. More studies are needed to corroborate these findings using a larger sample set representing different populations. It is also essential to include other potential markers, such as tumor markers, which may help to better stratify the patients.

In conclusion, this thesis has allowed the identification of novel tumor-associated markers of BCG immunotherapy outcome, such as M2-macrophage infiltration and tumor-associated carbohydrate sialyl-Tn, and its related antigen sialyl-6-T, and the establishment of a genetic risk score that could stratify patients according to the risk of recurrence after BCG immunotherapy.
5. FUTURE PERSPECTIVES
This study has provided important insights about predictive biomarkers of BCG immunotherapy outcome. However, more studies are needed to truly disclose the role of these biomarkers in the BCG mechanism of action.

Regarding the role of high stroma-predominant CD163<sup>+</sup> macrophage counts, some limitations need to be overcome in order to use this biomarker in clinical practice. Namely, efforts should be conducted to make the macrophage counts reproducible. Therefore, it would be important to evaluate macrophage counts using different counting methodologies using, for instance, image acquisition and automatic counting software. This would help to create a standard technique and cut-off values.

A careful evaluation of the influence of hypoxia and other microenvironment factors in the modulation of macrophage phenotypes is also needed in this context. It would be important to analyze if the high stroma-predominant CD163<sup>+</sup> macrophages have indeed an immunosuppressive phenotype, which could be achieved by evaluating Th2 cytokines expression, such as IL-10. Moreover, it will be necessary to determine if CD163<sup>+</sup> macrophages within the hypoxic tumor areas present an angiogenic phenotype with the analysis of pro-angiogenic factors, such as VEGF.

*In vitro* studies should be conducted using M2-polarized macrophages co-cultured with bladder tumor cells, to determine if the cytotoxic effect of BCG may be impaired with the presence of this macrophage population. The use of antibodies blocking Th2 cytokines produced by tumors with high stroma-predominant CD163<sup>+</sup> macrophages may also help to subvert the inhibitory effect of these entities, restoring the anti-tumor effect of BCG.

Regarding the influence of sTn and/or s6T expression in BCG immunotherapy response, it will be important to disclose the specificity of BCG binding to these glycans. Whether binding to tumor cells is mediated by bacterial adhesins able to recognize the sTn or sTn related antigens (such as s6T) or these events stem from alterations in the glycosylation of
integrins should be addressed in future studies. An approach using glycoarray technology would greatly assist in this matter. The identification of a bacterial adhesin capable of recognizing tumor-associated glycans may have practical implications, as it can be used to guide therapeutics to tumor cells.

Regarding the genetic polymorphism study, it will also be necessary to evaluate how the polymorphisms used to build the predictive model contribute to a differential expression of the target molecules, as performed in the study regarding FasL. Disclosing the role of these effects in the context of BCG anti-tumor activity is also warranted.

The inclusion of sTn/s6T antigen expression and CD163+ macrophages counts to the proposed model may also improve its predictive capability, and provide a better stratification of the patients. In an explorative approach, the introduction of this biomarkers improved the predictive ability of the model. However, since our dataset of tumor samples was small, we could not create a suitable risk score. The risk score is based on the Hazard Ratio provided by the stepwise Cox regression and with only 100 samples the 95% confidence intervals were very wide. This not allow a accurate measurement of the influence of these markers, making difficult to establish a risk score with this model. In order to overcome this, it will be necessary to obtain tumors specimens of all patients enrolled in this study and evaluate the mentioned tumor-associated markers. The resultant risk score may allow to better discriminate patients within the Intermediate Risk (using the proposed risk score), into the Low and the High Risk groups.

The addition of the tumor-associated markers that were described in this thesis to the proposed genetic risk score will allow to identify, more accurately, patients with poor prognosis and is this may be a helpful tool improve clinical decision.
6. REFERENCES
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APPENDIXS

Overexpression of tumour-associated carbohydrate antigen sialyl-Tn in advanced bladder tumours


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Overexpression of tumour-associated carbohydrate antigen sialyl-Tn in advanced bladder tumours

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\textbf{ABSTRACT}

Little is known on the expression of the tumour-associated carbohydrate antigen sialyl-Tn (STn), in bladder cancer. We report here that 75% of the high-grade bladder tumours, presenting elevated proliferation rates and high risk of recurrence/progression expressed STn. However, it was mainly found in non-proliferative areas of the tumour, namely in cells invading the basal and muscle layers. STn was also found in tumour-adjacent mucosa, which suggests its dependence on a field effect of the tumour. Furthermore, it was not expressed by the normal urothelium, demonstrating the cancer-specific nature of this antigen. STn expression correlated with that of sialyltransferase ST6GalNAc.I, its major biosynthetic enzyme. The stable expression of ST6GalNAc.I in the bladder cancer cell line MCR induced STn expression and a concomitant increase of cell motility and invasive capability. Altogether, these results indicate for the first time a link between STn
Tumour-associated glycans
Proliferative bladder cancer
expression and malignancy in bladder cancer. Hence, therapies targeting STn may constitute new treatment approaches for these tumours.

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1. Introduction

Bladder cancer, the fifth most common cancer in Western society, is a growing concern, owing to increased incidence during the past years (Ploeg et al., 2009; van Rhijn et al., 2009). Most of the newly diagnosed bladder cancer cases are superficial, or low-grade non-muscle invasive papillary tumours, being conservatively treated by complete transurethral resection of the tumour (Babjuk et al., 2012). However, approximately half of the patients show a high-percentage of recurrences and an elevated risk of progression to muscle invasive disease, which correlates with poor prognosis (Hussain et al., 2009). The risk of recurrence and/or progression is mostly determined by clinicopathological features (Babjuk et al., 2012). According to the European Organization for Research and Treatment of Cancer (EORTC), this group includes high grade (HG) papillary tumours and carcinoma in situ (CIS) and those with multifocal or recurrent lesions (Babjuk et al., 2012). The evaluation of the nuclear protein Ki-67 (Ki-67 proliferation index), an established marker of cell proliferation, is often used to enhance the prognostic accuracy of risk classification given by clinicopathological features (Margulis et al., 2009; Santos et al., 2003), since it is considered a surrogate biomarker of bladder cancer aggressiveness, disease recurrence and progression (Margulis et al., 2009; Santos et al., 2003).

Tumour resection followed by a schedule of intravesical instillations with live attenuated strains of Mycobacterium bovis (Bacillus Calmette–Guérin, BCG) is the standard adjuvant therapeutic option for high-risk of recurrence/progression bladder tumours (Askeland et al., 2012; Babjuk et al., 2012). Although BCG has improved the management of high-risk patients, 30–40% of cases either show intolerance or relapse after treatment (Yates and Roupret, 2011). Consequently, these patients require life-long follow-up and repeated courses of treatment making bladder cancer the costliest to treat among solid tumours (Askeland et al., 2012; Dovedi and Davies, 2009; Sievert et al., 2009). Upon therapeutic failure and/or muscle invasion, cystectomy is advocated for oncological control (Askeland et al., 2012; Dovedi and Davies, 2009; Sievert et al., 2009). Furthermore, at the moment there is a lack of specific biomarkers to target aggressive cell phenotypes and direct molecular-based therapy, which may be used to avoid preventive cystectomy (Dovedi and Davies, 2009).

Vaccines using tumour-associated glycans, in association with immunological boosters, are emerging as potential therapeutic strategies against cancer (Hakomori, 2001; Lakshminarayanan et al., 2012; Ryan et al., 2010; Sorensen et al., 2006). In the forefront of these antigens is sialyl-Tn (STn; Neu5Ac2-6GalNAcZ-O-Ser/Thr) (Gilewski et al., 2007; Julien et al., 2009; Miles et al., 2011). STn has been mostly observed in tumour-associated mucins due to their high number of potential O-glycosylation sites (Clement et al., 2004; Conze et al., 2010; Julien et al., 2006; Marcos et al., 2011; Pinto et al., 2012). However, integrins (Clement et al., 2004) and CD44 (Julien et al., 2006), among other proteins, may also carry this posttranslational modification. Overexpression of STn antigen has been detected in breast (Leivonen et al., 2001), oesophagus (Ikeda et al., 1993), colon (Itzkowitz et al., 1989), pancreas (Kim et al., 2002), stomach (David et al., 1996; Marcos et al., 2011), endometrium (Inoue et al., 1991), and ovary (Numa et al., 1995) carcinomas, whereas low or no expression was observed in the respective normal tissues. STn overexpression was also reported in several cancer precursor lesions, such as esophageal dysplastic squamous epithelia (Itoh et al., 1996), gastric intestinal metaplasia (Baldus et al., 1998; Ferreira et al., 2006) and colonic moderate dysplasia (Cao et al., 1997).

STn is known to influence cell recognition by the immune system (Angata et al., 2007), affect processes as cell cycle, apoptosis, and actin cytoskeleton dynamics, decrease cell–cell aggregation and increase extra-cellular adhesion, migration, invasion (David et al., 1996; Julien et al., 2006, 2005; Pinho et al., 2007) and metastatization (Ozaki et al., 2012). In line with these observations, STn positive tumors have been frequently observed at the invasion front of tumours and in peritoneal and pleural effusions in ovarian cancer patients; yet they are less common in metastatic lesions than in primary tumours (Davidson et al., 2000). In gastric carcinomas, STn was correlated with the depth of invasion and metastatization (Ikeda et al., 1993), and thus poor prognosis (Terashima et al., 1998). Conversely, STn was not correlated with the depth of invasion in studies concerning colorectal (Itzkowitz et al., 1989; Ogata et al., 1998) and breast cancers (Schmitt et al., 1995). However, some contradicting results have been presented regarding its association with metastasis and decreased survival in these cancers (Julien et al., 2012). Hence, a recent review suggests that the biological role of STn in tumour development may be dependent on each cancer type or sub-type (Julien et al., 2012).

Despite these observations, there is little information regarding STn in the context of bladder cancer. Given its clinical relevance and the fact that there are available therapies based on this antigen, we addressed the presence of STn in bladder tumours and the mechanisms underlying its expression.

2. Materials and methods

2.1. Patient and sampling

Formalin-fixed, paraffin embedded (FFPE) tissues were prospectively collected from 69 patients, mean age of 69 years (age range 45–89), who underwent transurethral resection (TUR) of the bladder tumour in the Portuguese Institute for Oncology of Porto (IPO-Porto, Portugal), between July 2011
and May 2012. Based on urothelial carcinoma grading and staging criteria of the World Health Organization (WHO), three different groups were considered (Table 1), low-grade (LG, \( n = 24 \)) and high-grade HG non muscle-invasive (NMIBC, \( n = 26 \)) and muscle-invasive (MIBC, \( n = 19 \)) bladder cancers. Of HG NMIBC, 21 were papillary tumours and 5 were carcinoma in situ (CIS). None of these patients had received prior adjuvant therapy. Six normal urothelium tissues of necropsied male individuals without bladder cancer history, within the same mean of age range, were also included.

Additionally, FFPE tissues from 16 radical cystectomy cases including the main lesion in each specimen, responsible for therapeutic decision, the adjacent mucosa, which may or may not include a concomitant tumour, and the ureter representing a distant mucosa, were also studied. Mucosa without visible histopathological alterations was defined as “histologically normal” mucosa.

All procedures were performed under the approval of the Ethics Committee of IPO-Porto, after patient’s informed consent. All clinicopathological information was obtained from patients’ clinical records.

2.2. Tissue expression of STn and Ki-67

FFPE tissue sections were screened for STn and Ki-67 by immunohistochemistry using the avidin/biotin peroxidase method. Briefly, 3 \( \mu m \) sections were deparaffinised with xylene, rehydrated with graded ethanol series, microwaved for 15 min in boiling citrate buffer (10 mM Citric Acid, 0.05% Tween 20, pH 6.0), and exposed to 3% hydrogen peroxide in methanol for 20 min. The expression of STn was then evaluated using anti-STn mouse monoclonal antibody, clone TKH2 (Kjeldsen et al., 1988), that identifies both single and clustered STn residues (Ogata et al., 1998), whereas Ki-67 was evaluated using monoclonal mouse anti-human Ki-67 antibody, clone MIB-1 (Dako). After blockage with BSA (5% in PBS), the antigens were identified with Vectastain Elite ABC peroxidase kit (Vector Lab) followed by incubation with 3,3-diaminobenzidine tetrahydrochloride (DAB, Dako). Finally, the slides were counterstained with haematoxylin.

A semi-quantitative approach was established to score the immunohistochemical labelling based on the intensity of staining and the percentage of cells that stained positively. The STn and Ki-67 expression were assessed double-blindly by two independent observers and validated by an experienced pathologist. Whenever there was a disagreement, the slides were reviewed, and consensus was reached. Tumours were classified as proliferative whenever Ki-67 expression was higher than 18%, as described by Santos et al. (Santos et al., 2003).

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>STn expression</th>
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<tbody>
<tr>
<td>Normal urothelium</td>
<td>6</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
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<tr>
<td></td>
<td></td>
<td>++</td>
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<tr>
<td></td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>Total STn+</td>
<td>0 (0%)</td>
<td>–</td>
</tr>
<tr>
<td>Low-grade papillary tumours</td>
<td>24</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
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<tr>
<td></td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>Total STn+</td>
<td>5 (21%)</td>
<td>–</td>
</tr>
<tr>
<td>High-grade (CIS + papillary tumours)</td>
<td>26</td>
<td>–</td>
</tr>
<tr>
<td>Carcinoma in situ (CIS)</td>
<td>5</td>
<td>–</td>
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<tr>
<td></td>
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<td>++</td>
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<tr>
<td></td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>Total STn+</td>
<td>1 (20%)</td>
<td>–</td>
</tr>
<tr>
<td>High-grade papillary tumours</td>
<td>21</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
</tr>
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<td>+++</td>
</tr>
<tr>
<td>Total STn+</td>
<td>19</td>
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<tr>
<td>MIBC</td>
<td>16 (76%)</td>
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<tr>
<td></td>
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<td>+</td>
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<td></td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>Total STn+</td>
<td>14 (74%)</td>
<td>–</td>
</tr>
</tbody>
</table>

Table 1 – STn expression in the healthy urothelium and in non-muscle invasive (NMIBC) and muscle invasive (MIBC) bladder cancers of different clinicopathological natures.

2.3. Cell lines culture

The human bladder cancer cell line MCR and the transduced variants of MCR (MCRnc and MCRSTn+), were grown as described by Videira et al. (2009b).

2.4. Generation of STn+ bladder cancer cells

MCR cells were transduced with a retroviral vector generated with the ViraPower™ Lentiviral Expression System (Invitrogen), according to manufacturer’s instructions. The whole coding region of human ST6GalNAc.I was PCR amplified and cloned in the pLenti6/V5 Directional TOPO cloning vector which drives the expression of inserted genes through the CMV promoter. A negative control retroviral vector was prepared with an empty plasmid. After transduction with negative control- or ST6GalNAc.I-expressing vectors, MCR cells were selected with 4 \( \mu g \) ml\(^{-1} \) blasticidin. An additional immunomagnetic enrichment of the STn+ cells was performed by using mouse anti-STn (HB-STn1 clone from Dako), followed by the secondary antibody anti-mouse IgG associated to paramagnetic microbeads (Miltenyi Biotec). The stable transduction of the enzyme was confirmed by evaluation of ST6GalNAc.I expression and activity. STn expression was
determined by analysis of the mean fluorescence intensity (MFI) ± SE through flow cytometry analysis using monoclonal antibody TKH2.

2.5. **Evaluation of STn expression in cell lines**

For phenotypic characterization, cells were stained with 1:50 diluted anti-STn TKH2 monoclonal antibody for 16 h at 4 °C, and 1:100 diluted goat fluorescein isothiocyanate (FITC)-labelled anti-mouse IgG (Dako) for 15 min at 4 °C in the dark and then acquired in a FacsCalibur Flow cytometer (Becton Dickinson). Data were analysed using the WinMDI v2.9 software (The Scripps Research Institute, San Diego, CA, USA).

2.6. **Analysis of ST6GalNAc.I expression**

RNA extraction from FFPE sections was performed after deparaffinization of the tissue using Absolutely RNA FFPE kit (Agilent technologies) while for cell lines it was used the GenElute Mammalian Total RNA Purification kit and DNase treatment (Sigma), according to the manufacturer’s instructions. The purity of RNA extracts was determined based on the A260/A280 ratio. Only ratios between 1.9 and 2.1 were considered further.

Approximately 250–500 ng of total RNA (1 μg for cell lines) was converted by reverse transcription into cDNA, using the random-primers-based High Capacity cDNA Archive Kit (Applied Biosystems). The expression levels of ST6GalNAc.I were determined by TaqMan assay (Applied Biosystems), the reference sequences detected by each primer/probe set and the Assay ID provided by the manufacturer were the following: ST6GalNAc.I (NM018414.2/Hs00300842_m1). Real time PCR was performed in a 7500 Fast Real-Time PCR System using the TaqMan Universal PCR Master Mix Fast from the manufacturer’s instructions. Subsequently, the CFSE-labelled cells were seeded into 24-well microplates, incubated in a 5% CO2 incubator at 37 °C and harvested at 24, 48, 72 and 96 h post-culture. Flow cytometry using a FACSCalibur (Becton–Dickinson) was performed and the data collected were analysed with ModFit LT 3.2 software (Verity Software House, Topsham, ME), allowing to assess the cell proliferation index (PI). The PI represents the average number of cells that were originated from a single cell of the parental generation. The parental generation was set based on the analysis of data obtained from the cells corresponding to the 24 h of culture.


MCR cell pellets were homogenized in H2O and the protein concentration was determined using the RC-DC protein quantification kit (BioRad) according to the manufacturer’s instructions. Sialyltransferase activity was assayed in whole cell homogenates as previously described by Dall’Olio et al. (1997) with some modifications. Briefly, the reaction mixture contained 80 mM sodium cacodylate buffer pH 6.5, 0.5% Triton X-100, 6 μg ml⁻¹ of asialo bovine submaxillary mucin (ABS M, prepared by acid desialylation of BSM) as acceptor substrate, 30 μM (1280 Bq) of CMP-[14C]Sia (Amersham) and 2 μg ml⁻¹ of homogalactose proteins. Endogenous controls were prepared in the absence of acceptor substrate. The enzyme reactions were incubated at 37 °C for 2 h and the acid insoluble radioactivity was measured as previously described by Dall’Olio et al. (1997). The incorporation on endogenous substrates was subtracted.

2.8. **Cell proliferation measurement**

To study their proliferative capacity, cells were labelled with CellTrace CFSE Cell Proliferation Kit (Invitrogen). The MCR cells were resuspended into medium at final concentration of 1 × 10⁶ cells ml⁻¹ and incubated with 10 μM CFSE, following the manufacturer’s instructions. Subsequently, the CFSE-labelled cells were seeded into 24-well microplates, incubated in a 5% CO2 incubator at 37 °C and harvested at 24, 48, 72 and 96 h post-culture. Flow cytometry using a FACSCalibur (Becton–Dickinson) was performed and the data collected were analysed with ModFit LT 3.2 software (Verity Software House, Topsham, ME), allowing to assess the cell proliferation index (PI). The PI represents the average number of cells that were originated from a single cell of the parental generation. The parental generation was set based on the analysis of data obtained from the cells corresponding to the 24 h of culture.

2.9. **Analysis of cell motility using a wound-healing assay**

Cell motility was tested in a wound-healing migration assay. MCR cells were seeded into 12-well microplates and grown to confluence. A scratch was made in the monolayer with a sterile 200 μl pipette tip. After wounding, the suspended cells and debris were washed away and fresh medium was added. At 0 and 24 h after wounding, scratched regions were photographed with an inverted microscope equipped with a digital camera.

2.10. **Invasion assay**

Invasion assays were performed using BD Biocoat Matrigel invasion chambers, comprised by an 8-μm diameter pore size filter coated with a thin layer of matrigel, and placed in a two-compartment system in a 24-well plate. Prior to each experiment, filters were re-hydrated in serum-free DMEM medium for 2 h at 37 °C. After detachment of subconfluent cells with trypsin/EDTA, cells were suspended in culture medium supplemented with 5% inactivated FBS, counted and seeded on the upper side of the matrigel-coated filter at a density of 5 × 10⁴ cells/ well. After 24 h at 37 °C, filters were fixed in 4% paraformaldehyde and non-invading cells, present on the upper side, were completely removed, to facilitate analysis. Cells that had invaded the underside of the filters were mounted in Vectashield®+4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, CA, USA), and visualized through a Zeiss Axiolov 200M fluorescence microscope (Carl Zeiss, Germany). Invasive cells were scored in at least 12 microscopic fields (20 × objective) when DAPI-counterstained nuclei passed through the filter pores. Results are presented as means ± SD for each sample. Invasion levels are expressed as a ratio of the results obtained with the mock-transfected control cell line.

2.11. **Statistical analysis**

Statistical analysis was performed using the Student’s T-test for unpaired samples. Differences were considered to be significant when p < 0.05. A chi-square test was used to analyse correlations between clinicopathological features and STn and Ki-67 expressions.
3. Results

3.1. Expression of STn in bladder tumours

STn expression in bladder tumours was evaluated by immunohistochemistry using mouse monoclonal antibody clone TKH2. As shown in Table 1, STn is not expressed in the healthy urothelium; conversely 46% of the bladder tumours presented cells with STn membrane and cytoplasmic staining (32/69) (Figure 1), demonstrating the tumour-specific nature of this antigen. The removal of sialic acids from the tissue sections with a α-neuraminidase impaired the recognition by TKH2 and confirmed STn expression.

STn expression was lower in low-grade (LG) NMIBC (21% STn+ tumours; Figure 1A–B) compared to high-grade lesions (HG; 67%), which include papillary tumours (76% STn+ tumours; Figure 1C–E), CIS (20% of STn+ tumours; Figure 1F), and MIBC (74% STn+ tumours; Figure 1G–H). Noteworthy, STn was absent from the majority of CIS (4/5; 80%) and showed an expression comparable to LG tumours. Altogether, these results highlight an association between the STn antigen and high grade NMIBC (p < 0.002; Figure 2) as well as with muscle invasive tumours (p < 0.03; Figure 2).

The O-acetylation of sialic acid residues prevents TKH2 from recognizing STn antigens in certain tissues (Ogata et al., 1998). To exclude this possibility in bladder cancer, the slides were chemically de-O-acetylated prior to immunohistochemistry. This procedure did not alter STn expression patterns demonstrating that STn antigens were not encrypted by O-acetylation.

3.1.1. Pattern and extension of STn expression in bladder tumours

The STn antigen presented a focal expression that for the majority of the STn positive cases (26/36) did not exceeded 15% of the tumour section (Table 1). Furthermore, in 25% of the STn positive cases (9/36) the antigen was detected in less than 5% of the tissue (data omitted from Table 1). Higher expression patterns were restricted to HG papillary NMIBC, where 27% of the cases (7/26) presented STn levels between 15% and 45% of the tumour section (Table 1) and locally diffuse staining (Figure 1C, D, G). STn was mainly observed in basal layer cells (75% of STn+ cases; Figure 1A, C–E), but it could be also detected throughout the papillae (Figure 1C–E) and cells of the luminal surface (Figure 1F) in cases presenting locally diffuse staining. STn was further observed in cells invading the basal (50% of STn+ of HG NMIBC; Figure 1C–E, G) and muscle layers (57% of STn+ MIBC; Figure 1G, H), suggesting a role in invasion.

3.1.2. STn antigen expression in advanced tumours and in the surrounding areas

The STn antigen was also evaluated in a series of radical cystectomy specimens which included the tumour used for therapeutic decision (termed “main tumour” in Figure 3) and the tumour-adjacent mucosa. The ureters were included as distant mucosa (Figure 3). In agreement with the observations from Table 1, STn was detected in 69% (11/16) of all main tumours as well as in their adjacent mucosa (Figure 3), independently of their histological classification. Noteworthy, STn was absent from 90% of the distant mucosas of STn positive cases; the only exceptions being a ureter with pre-neoplastic and another with a neoplastic lesions (Figure 3). These results point out that the STn+ tumour-adjacent mucosa may display molecular changes similar to those of the main lesions. Thus, this antigen may be useful as a marker of field carcinogenesis in the bladder.

3.2. Expression of ST6GalNAc.I in bladder tumours

The presence of STn has been strongly associated with the overexpression of ST6GalNAc.I in several human malignancies. To assess this event in bladder tumours, mRNA levels of ST6GalNAc.I gene were analysed and normalized in relation to β-actin, which proved to be a stable expressed gene in previous studies concerning bladder tumours (Videira et al., 2007). As shown by Figure 4, low gene expression levels were detected in tumours that did not express STn. In addition, the levels of ST6GalNAc.I increased with the expression of STn, and were significantly higher in the tumours with STn expression superior to 15%. Figure 4 also shows that this behaviour was similar in LG and HG tumours. However, as a result of higher STn expression, the average ST6GalNAc.I mRNA levels were more elevated in HG (53%) tumours than LG (9%). These observations suggest that overexpression of ST6GalNAc.I gene is one of the main events leading to STn expression in bladder tumours.

3.3. STn expression and tumour proliferation

As shown above, the expression pattern of STn correlates with HG tumours, known to present elevated proliferation rates (Margulis et al., 2009; Santos et al., 2003). To assess a possible association between STn and proliferation, 24 cases from the initial series of 69 bladder tumours, comprehending 12 LG and 12 HG tumours (7 NMIBC, none of them CIS, and 5 MIBC), were screened for STn and Ki-67 expression. Tumours presenting Ki-67 expression superior to 18% were classified as proliferative. As highlighted by the graphical matrix in Figure 5A, 8% (1/12) LG and 75% (9/12) HG cases showed elevated Ki-67, confirming the higher proliferation of HG tumours (p < 0.0012). Similarly, Figure 5A also shows an association between proliferative phenotypes and STn expression (p < 0.001). However, in all STn positive cases, the examination of sequential sections revealed that STn antigen expression was mainly seen in areas that did not express Ki-67 (Figure 5A), although some overlap was present in 25% of the cases (3/12, Figure 5B). This indicates that the STn antigen is mostly expressed in non-proliferative areas of the tumour. Nevertheless, the majority of the non-proliferative tumours also did not express STn (12/14), demonstrating an interdependence between both phenomena.

3.4. In vitro assessment of the biological significance of STn expression

3.4.1. Development of a high-grade bladder cancer cell line overexpressing STn

To further corroborate the role of ST6GalNAc.I in the expression of STn antigen by bladder cancer cells, we induced the overexpression of ST6GalNAc.I in a bladder cancer cell line. The MCR bladder cell line, that showed negligible expression...
of ST6GalNAc.I and no STn (data not shown), was transduced with a lentivirus expressing the coding region of the human ST6GalNAc.I gene. The obtained cell line variant, herein named MCRSTn⁺, showed markedly increased expression of ST6GalNAc.I mRNA levels (Figure 6A). It also showed significantly higher sialyltransferase activity towards the ABSTM, a substrate for the ST6GalNAc.I enzyme, when compared with the negative control cell line (MCRnc) transduced with void lentivirus (Figure 6A). The overexpression of STn antigen by MCRSTn⁺ cell line variant was confirmed by flow cytometric analysis (Figure 6B).

3.4.2. STn influence on cell proliferation, migration and invasion
STn expression was correlated with tumours with higher proliferative indexes (Figure 5). To assess the influence of STn in proliferation, migration and invasion, we evaluated the effect of STn on cell proliferation, migration and invasion. We observed that STn expression was associated with an increase in cell proliferation, migration and invasion.

Figure 1 — Expression of STn in FFPE bladder tumours. A) Low-grade papillary tumour showing a predominance of STn⁺ cells in the basal layer; B) Magnification which shows tumour cells with membrane and cytoplasmic STn⁺ staining; C) High-grade papillary tumour evidencing the focal nature of STn expression. Positive cells were found both in the basal layer and throughout the papillae; D) High-grade papillary tumour showing locally extensive STn positivity; E) High-grade papillary tumour evidencing STn⁺ in the basal layer; F) CIS showing STn⁺ in the cells facing the lumen of the bladder; G) MIBC showing locally extensive STn expression including at the muscle invasive front; H) MIBC highlighting STn⁺ cells invading the muscle layer.
proliferation, MCR cells (MCRnc and MCRSTn\(^+\)) were cultured for 48, 72 and 96 h and then evaluated in relation to their proliferation index. The comparison between the two cell line variants showed that the proliferation index of MCRSTn\(^+\) cells was generally higher than the index of MCRnc cells, although only statistically different at 72 h of culture (\(p < 0.05\); Figure 7). However, this effect was no longer significant at 96 h of culture (Figure 7).

STn positive cells were observed invading the basal and muscle layers (Figures 1 and 2) and in the adjacent mucosa of advanced stage bladder tumours (Figure 5), suggesting a correlation of STn with invasion and migration. Thus, the influence of STn expression in MCR cell invasion was assessed using the Matrigel invasion assay. Our results evidence that MCR cells transduced with ST6GalNAc.I (MCRSTn\(^+\)) are approximately four folds more invasive than bladder cells transduced with the negative control (MCRnc; Figure 8A). The effect of STn expression on cell migration was estimated by a wound-healing assay. Therefore, uniform scratches were made in confluent monolayers of MCRnc and MCRSTn\(^+\) cell lines and the capability of the cells to migrate and fill the scratches was monitored. As observed in Figure 8A, by 24 h after wounding, the MCRSTn\(^+\) cells had almost completely covered the empty space. Conversely, the negative control, MCRnc cells, displayed a large “gap”, thus demonstrating their lower capability to closure the wound. Our results evidence that MCR cells expressing STn present increased invasion and wound repair capacities.

4. Discussion

The STn antigen is highly expressed by several human carcinomas and preneoplastic lesions (Julien et al., 2012) and is explored as a tumour marker in serological assays (CA72-4) (Reis et al., 2010).

Despite the clinical relevance of STn in human malignancies, scarce information is available about its role in bladder tumours. Over twenty years ago, Langkilde et al. (1992) addressed this antigen on series of transitional cell carcinomas (currently classified as high-grade urothelial cell carcinomas according to current WHO guidelines (Babjuk et al., 2012)). Normal mucosal biopsy specimens from patients with non-malignant bladder urologic diseases were included as controls. According to the authors, STn was not expressed by the control group, showed a very restricted pattern of expression in bladder tumours and no association with recurrence and progression. Subsequent in vitro studies found that MCR cells transduced with ST6GalNAc.I (MCRSTn\(^+\)) are approximately four folds more invasive than bladder cells transduced with the negative control (MCRnc; Figure 8A). The effect of STn expression on cell migration was estimated by a wound-healing assay. Therefore, uniform scratches were made in confluent monolayers of MCRnc and MCRSTn\(^+\) cell lines and the capability of the cells to migrate and fill the scratches was monitored. As observed in Figure 8A, by 24 h after wounding, the MCRSTn\(^+\) cells had almost completely covered the empty space. Conversely, the negative control, MCRnc cells, displayed a large “gap”, thus demonstrating their lower capability to closure the wound. Our results evidence that MCR cells expressing STn present increased invasion and wound repair capacities.

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mucins MUC1, MUC2 and MAUB (mucin antigen of the urinary bladder) isolated from bladder cancer cell lines carried STn (Bergeron et al., 1996, 1997). However, no evidence of such an expression was found in tumours. Herein, we readdressed this matter and found that the STn antigen was associated with advanced stage bladder tumours. More important, STn was absent in the healthy urothelium, which demonstrates its tumour-associated nature. Since this study was performed on a recent prospective series it is not possible, at this point, to determine correlations with disease outcome. Nevertheless, STn was mainly expressed by HG papillary NMIBC, known for their elevated risk of recurrence and progression to muscle invasive disease and MIBC that encompass an elevated risk of metastization and present decreased overall survival (Babjuk et al., 2012). STn expression was further associated with elevated Ki-67, a proliferation-related molecule and a surrogate biomarker of increased risk to recurrence and progression in bladder tumours (Margulis et al., 2009; Santos et al., 2003). In addition, the majority of non-proliferative tumours did not express STn, which demonstrates that the expression of the antigen is indeed a characteristic of proliferative tumours. Still, STn was mainly detected in non-proliferative areas of the tumours. However, the STn antigen was frequently observed in areas of invasion of the basal and muscle layers, suggesting it may be associated with the process of cell migration and invasion. This reinforces the notion that STn is part of a malignant bladder cancer phenotype, as previously observed for other carcinomas (Clement et al., 2004; Julien et al., 2006; Ohno et al., 2006; Ozaki et al., 2012; Pinho et al., 2007). We also found the STn antigen in tumour-adjacent mucosa, which may be explained by the migration of STn+ cells to the tumour surroundings. On the other hand, this may be a consequence of field carcinogenesis previously observed in bladder cancers (Jones et al., 2005; Palmeira et al., 2011). Nevertheless, the STn antigen holds potential as a biomarker of bladder disseminated disease.

STn is a product of an incomplete O-glycosylation process due to the premature O-6 sialylation of the glycoside GalNAcα1-O-Ser/Thr (Tn antigen) by ST6GalNAc.I (Marcos et al., 2004). In several epithelial tumours STn results from an increased ST6GalNAc.I expression and/or activity (Marcos et al., 2011; Sewell et al., 2006; Vazquez-Martin et al., 2004). Previous studies have reported ST6GalNAc.I expression by the urothelium at the mRNA level (Yamamoto et al., 2003); however we and others (Langkilde et al., 1992) have not detected STn expression in the histologically healthy tissues. These observations suggest either the absence of the antigen or the insufficient sensitivity of the method. ST6GalNAc.I localization in the Golgi apparatus and the competitive action of other glycosyltransferases for the Tn antigen may also favour the extension of
in vitro ability to express this antigen in cell models, demonstrating that tumour cells may lose the (Pinho et al., 2007). These findings associated with the absence of the antigen from most bladder tumours non-proliferative areas strongly suggests that STn expression does not play a direct role in tumour proliferation.

On the other hand, STn expression significantly enhanced the migration and invasive capacity of MCR cells, demonstrating that this antigen plays an important role in bladder cancer cell invasion, as suggested by the observation of bladder tumours. Enhanced migration capabilities of STn+ cells on components of the extracellular matrix, such as fibronectin and collagen, have been described for other cancer cell lines (Julien et al., 2005, 2006; Pinho et al., 2007), and result, among several factors, from impaired integrin binding (Clement et al., 2004). In addition, STn expression has been shown to increase the invasion potential of tumour cells (Clement et al., 2004; Julien et al., 2006; Ohno et al., 2006; Ozaki et al., 2012; Pinho et al., 2007), supporting a similar role in bladder tumours. Further experiments are however required to clarify the molecular mechanisms underlying promotion of cancer cell invasion and migration. These findings reinforce however that alterations in the glycosylation patterns of cell-surface proteins may strongly interfere with events like cell–cell adhesion, cell–matrix interaction, tumour growth, motility and invasion (Dall’Olio et al., 2012).

In resume, our work comprehensively describes the expression of the STn antigen in bladder cancer. Namely, it demonstrates the tumour-specific nature of this type of glycosylation and its association with advanced, highly proliferative tumours, invasion and organ disseminated disease. Thus, the evaluation of STn antigen may add valuable information about the aggressiveness of proliferative tumours, complementing the information given by Ki-67. Studies are ongoing in broader retrospective series to determine the association of STn with disease outcome and corroborate these findings. We are also devoted to the identification of the glycoproteins yielding STn, which is expected to bring insights...
about the role of this type of glycosylation in bladder carcinogenesis and provide novel therapeutic vectors. The antigen STn may also be monitored noninvasively in urine or serum using as is the case for other human carcinomas using the CA72-4 test (Reis et al., 2010). This could allow decreasing the number of cystectomies in post-surgery follow-ups of patients with high-grade tumours, a particularly critical matter for the elderly that constitute the majority of the cases.

Furthermore, the STn antigen is associated to high-grade NMIBC which currently constitutes one of the main therapeutic concerns due to their elevated risk of recurrence/

progression (Babjuk et al., 2012). Adjuvant immunotherapy with BCG has allowed to delay recurrence and decrease the risk of progression into muscle invasive disease (Babjuk et al., 2012); still more than half of the patients either recur within two-years after TUR of the tumour or show intolerance to the treatment (Askeland et al., 2012; Yates and Roupret, 2011). Due to the lack of efficient therapies, upon therapeutic failure and/or muscle invasion, the patient is faced with cystectomy (Babjuk et al., 2012).

Carbohydrate antigens associated with advanced-stage tumours and malignant phenotypes such as STn, are expressed at the cell surface and, therefore, available for antibody or lectin-mediated recognition (Neutsch et al., 2012). Thus, these antigens may present an opportunity for the introduction of novel therapeutics, such as selective drug-delivery approaches (Neutsch et al., 2012) or carbohydrate-based immunotherapy (Heimburg-Molinaro et al., 2011). An anti-cancer vaccine named Theratope, comprehending a synthetic STn coupled to the immunogenic carrier keyhole haemocyanin has already been developed (Julien et al., 2009; Miles et al., 2011; Sandmaier et al., 1999). Tests in animal models and humans for breast, ovarian, and colorectal cancers have showed that the antigen is safe and produces a strong immune response against these tumours (Julien et al., 2009, 2012; Miles et al., 2011). Even though Theratope failed to improve overall survival of metastatic breast cancer patients in a phase III clinical study, the design of the study disregarded the heterogeneous STn expression between patients (Miles et al., 2011), compromising the outcome (Julien et al., 2012; Zeichner, 2012). Thus, Theratope or other STn-based vaccine designs may constitute valuable therapeutic options for STn positive advanced bladder tumours. However, given the low association of STn with more proliferative areas of the tumour, one is led to speculate that advanced stage bladder cancer patients may better benefit from the combination of anti-STn immunotherapy and anti-proliferative drugs. Furthermore, these approaches may allow targeting disseminated disease in the adjacent and distant mucosa from the main tumour.

Figure 7 — Comparison between the proliferation capacity of MCRnc and MCRSTn⁺ cells. The transduced MCR cells were labelled with CFSE and cultured for various periods of time (48, 72 and 96 h). The cells were harvested and analysed by flow cytometry with Modfit software, allowing the calculation of the proliferation index, which represents the average number of cells that was originated by a single cell of the parent generation. At the various periods of culture, MCRSTn⁺ cells show a higher proliferation index than the negative control, but this difference was only statistically significant at 72 h of culture. The data are presented as a mean ± standard deviation of 3 independent studies. *** p < 0.05 (Student’s T-test).

Figure 8 — STn expression promotes MCR cells wound healing closure and invasion. A) Wound healing closure assay. Uniform scratches were made using a 200 μL pipette tip in confluent monolayers of MCRSTn⁺ and MCRnc cells. Cells were allowed to heal and the extent of closure was monitored by microscopic analysis. After 24 h culture, the MCRSTn⁺ cells had almost completely covered the wound, in clear contrast to negative control, MCRnc, where unoccupied space was still observed. B) Invasion assay. MCRSTn⁺ and MCRnc cells were incubated for 24 h, in the upper compartment of Matrigel invasion chambers, in complete DMEM medium and in the absence of other chemoattractants. Invasive cells were determined as described in Materials and methods. The data are presented as a mean ± standard deviation of 4 independent studies. *** p < 0.001 (Student’s T-test).
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