

**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-Guerin (BCG) immunotherapy is the gold standard treatment for superficial intermediate/high risk of recurrence or progression bladder tumors. However, approximately 30% of patients fail to respond to treatment. Effective BCG therapy needs precise activation of the Th1 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 bladder cancer patients treated with BCG. Tumors resected prior to 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<sup>+</sup> and CD163<sup>+</sup> 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 accessed through HIF-1 $\alpha$  expression.

**Results:** Patients in which BCG failed had high stroma-predominant CD163<sup>+</sup> macrophage counts (high stroma but low tumor CD163<sup>+</sup> macrophages 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 (HR=2.343; 95%CI: [1.197-4.587];  $p=0.013$ ). Even when adjusted to potential confounders, such as age and therapeutic scheme, multivariate analysis revealed 2.6-fold increased risk of recurrence (HR=2.627; 95%CI: [1.340-5.150];  $p=0.005$ ). High stroma-predominant CD163<sup>+</sup> macrophage counts were also associated with low expression of HIF-1 $\alpha$  in tumor areas, whereas high counts of CD163<sup>+</sup> in the tumor, presented high expression of HIF-1 $\alpha$  in tumor nests.

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

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

## **INTRODUCTION**

Bladder cancer (BC) is the second most common urologic cancer and has the highest recurrence ratio of any malignancy [1]. Approximately 75-85% of all BC are non-muscle invasive (NMIBC), which includes carcinoma in situ (CIS) and papillary tumors confined to the mucosa or submucosa (Ta/T1) [1]. The NMIBC 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, 30–50% 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 two 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, produce IL-2, IL-12 and pro-inflammatory 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]. Also, 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 both 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]. Also, 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 HIF-1 $\alpha$  enhances the expression of VEGF 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 resume, 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<sup>+</sup>) as well as the M2 phenotype, based on CD163 expression, in both stroma and tumor areas. As outlined, we correlated our findings with HIF-1 $\alpha$  expression to disclose the influence of hypoxia in M2-macrophage accumulation and treatment outcome.

## **METHODS**

### Cohort of Patients

In this study were included 99 formalin fixed paraffin embedded (FFPE) tissues from patients treated with transurethral resection of bladder tumor (TURBT) and then submitted to 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 2-3 weeks after surgery (iBCG) and were then submitted to mBCG schedule (the one used in our institute is iBCG + maintenance protocol with 2-weekly instillations every 3 months during 2 years). 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). The male:female ratio was 84:15. The patients were followed 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:10-163months).

Recurrence was defined as the appearance of a tumor after the beginning of the treatment, with at least one tumor-free cystoscopy and cytology in-between. BCG failure, as opposed BCG success, was defined as patients submitted to BCG treatment with 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 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 an experienced pathologist, regarding 2004 WHO grading criteria.

#### Immunohistochemistry

TAMs immunohistochemistry was performed with CD68 antibody (Monoclonal Mouse Anti-Human CD68; Clone PG-M1; DAKO) at a dilution of 1:100 in PBS, after 1h incubation at 37°C. M2 macrophages were accessed 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 (Impact Dab, Vector Labs) was used for color development. Hypoxic sites were evaluated using HIF-1 $\alpha$  antibody (Monoclonal Mouse Anti-Human HIF-1 $\alpha$ ; Clone H1 $\alpha$ 67; Abcam) at a dilution of 1:50 in PBS, after overnight incubation at 37°C.

#### Immunohistochemistry Scoring

CD68<sup>+</sup> and CD163<sup>+</sup> macrophages, infiltrating the stroma and tumor areas were counted by two independent observers (LL, DO) and validated by an experienced pathologist (TA). Each specimen was screened at low magnification (100x) and the five areas with highest number of positively stained cells (hot-spot area) were selected. Photographs were taken, at a 400x magnification, with a real area of 0.035 mm<sup>2</sup>, 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 macrophage characteristic staining pattern; 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. Macrophages counts were classified as low or high according to their distribution in percentiles. The expression of HIF-1 $\alpha$  was determined based on percentage of positive cells and stratified in groups was as follows: Low (negative or 1-10% nuclear or cytoplasmic staining) and High (10-50% or >50% nuclear or cytoplasmic staining).

### Statistical Analysis

Statistical data analysis was performed using the IBM Statistical Package for Social Sciences—SPSS 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.

## **RESULTS**

### Association of Clinical and 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). The median follow-up time of the patients free of recurrence was 97 months (range: 13-163).

Table 1 shows the clinicopathologic parameters and its association with treatment response and RFS. An association was found between patients' age and treatment response, since 69% of the patients presenting BCG failure were over 65 years when compared with 43.9% in the BCG success group ( $p=0.013$ ). Consequently, patients over 65 years presented almost a 3-fold increased risk of recurrence (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, tumour stage, number, grade or size, CIS presence and prior recurrence.

#### Pattern of macrophage infiltration

We started by evaluating the localization of macrophages within tumor specimens. We observed the presence of CD68<sup>+</sup> and CD163<sup>+</sup> 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<sup>+</sup> macrophages was 33 within stroma and 13 within tumor while for CD163<sup>+</sup> macrophages within stroma and tumor was, respectively, 24 and 7. The mean ratio of CD163<sup>+</sup>/CD68<sup>+</sup> macrophages was 51.3% in the tumor and 24.6% in the associated stroma. A moderate to strong CD68<sup>+</sup> macrophage stroma infiltration was observed in 46% of the tumors while only 4% of cases had none CD68<sup>+</sup> macrophage staining in tumor nest. A high CD163<sup>+</sup> macrophages stroma infiltration was observed in 15% tumors while only 8% of tumors presented no CD163 staining in tumor nests.

#### Correlation between clinical characteristics and CD68<sup>+</sup> and CD163<sup>+</sup> macrophage counts

The correlation between clinical variables and the macrophages counts are indicated in Table 2. Our results evidenced that CD68<sup>+</sup> and CD163<sup>+</sup> 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 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.



### CD68<sup>+</sup> and CD163<sup>+</sup> macrophages and BCG treatment outcome

To evaluate the CD68<sup>+</sup> and CD163<sup>+</sup> macrophages infiltration within stroma and tumor areas in the context of BCG treatment outcome, counts were stratified based on percentiles (25th, 50th, 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 other hand we observed that only CD163<sup>+</sup> stroma counts falling within the 25th percentile (>19 macrophages) presented a trend association with treatment outcome; CD163<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> LS phenotype (associated with LT) presented BCG treatment response rates similar to the cases with HS and high tumor CD163<sup>+</sup> counts (HT; >75th percentile – Fig. 1B). Based on these observations, we decided to merge these two groups (LS/LT and HS/HT) and compare it with the cases presenting HS but LT CD163<sup>+</sup> counts. Taking into consideration the low CD163<sup>+</sup> 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<sup>+</sup> macrophage group (HSP, Fig. 1C).

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

In order to estimate the influence of higher CD163<sup>+</sup> 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<sup>+</sup> macrophages (mean

RFS: 126 vs 92 months; log rank,  $p=0.052$ ; Fig.3A). Moreover, patients with HSP CD163<sup>+</sup> macrophages counts presented a different behavior in terms of RFS (log rank,  $p=0.008$ ; Fig.3B) and a lower RFS (mean: 85 months) than all the others (mean:123 months).

Univariate Cox Regression analysis revealed that patients with tumors presenting CD163<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> and HSP CD163<sup>+</sup> counts had more than a 2-fold increased risk of recurrence (respectively, 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).

#### CD163<sup>+</sup> Macrophages and expression of HIF-1 $\alpha$

The association between CD163<sup>+</sup> macrophages counts within tumor and hypoxia was evaluated based on HIF-1 $\alpha$  expression. The expression of HIF-1 $\alpha$  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<sup>+</sup> macrophages expressed high amounts of HIF-1 $\alpha$  (Fig. 4A&B). On the other hand, tumor areas with low CD163<sup>+</sup> macrophages counts, independently of the counts in the stroma, presented lower expression of HIF-1 $\alpha$  (Fig. 4C&D). In resume, while 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.

## **Discussion**

Although BCG immunotherapy is the primary treatment option for intermediate/high risk bladder tumors, the failure rate is over 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 prior to BCG treatment, although more studies are needed to validate its relevance [10-12, 21, 22]. On the other hand, the marker used was CD68, a lineage marker found in both M1 and M2 macrophages [7]. 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 [23-26]. To address this subject we investigated the influence of TAMs (CD68<sup>+</sup>) and also the M2-polarized macrophage phenotype (CD163<sup>+</sup>), 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, mBCG). Therefore, these variables were considered potential confounders and were taken into account in multivariate analysis models to assess the TAMs influence in BCG outcome. We also observed that CD68<sup>+</sup> and CD163<sup>+</sup> macrophages counts in both the stroma and 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<sup>+</sup> 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 high-risk of recurrence/progression bladder tumors.

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<sup>+</sup> macrophage counts in stroma and in tumor nests and the outcome. Even though contradictory these results may stem from the fact that two of this 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<sup>+</sup> 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.

Contrastingly, 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 favourable 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 influencing treatment outcome in different ways possibly due to the influence of differentiated micro environmental stimuli in the stroma and the tumor.

Since TAMs may be found in vascularised 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 the result of hypoxia. This was confirmed by the association between high tumor CD163<sup>+</sup> macrophage counts and high expression of the hypoxia marker HIF-1 $\alpha$  within tumor areas; conversely, in specimens with High stroma-predominant CD163<sup>+</sup> counts (and respectively low tumor counts), HIF-1 $\alpha$  expression within

tumor areas was low. These observations suggest that hypoxic conditions may dictate the accumulation of CD163<sup>+</sup> 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 VEGF and other proinflammatory cytokines like TNF- $\alpha$ , IL-1 $\beta$ , MIF and COX2 that act as promoters of a Th1 mediated response known to favour BCG action [19]. On the other hand, normoxia may favour the M2 immunosuppressive phenotype and the downregulation of molecules implicated in immunological activation such as IL-12, IL-18, IL-1 $\beta$  and TNF $\alpha$  [5]. This selective pressure also upregulates the expression of Th2-type cytokines, as well as IL-10, IL-1RA and TGF- $\beta$ , 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 favour the accumulation of M2-polarized macrophages in the tumor and also promote their angiogenic phenotype, ultimately leading to a better treatment outcome. Conversely, non-hypoxic or low-hypoxic conditions (low HIF-1 $\alpha$ ) 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 high stroma-predominant CD163<sup>+</sup> macrophage counts is a good predictor of recurrence after BCG treatment, some limitations need to be overcome in order 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, specially involving image acquisition and automatic counting software in order to create a standard technique and cut-off values. Also, 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<sup>+</sup>) 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 due to its immunosuppressive phenotype.

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Figure 1  
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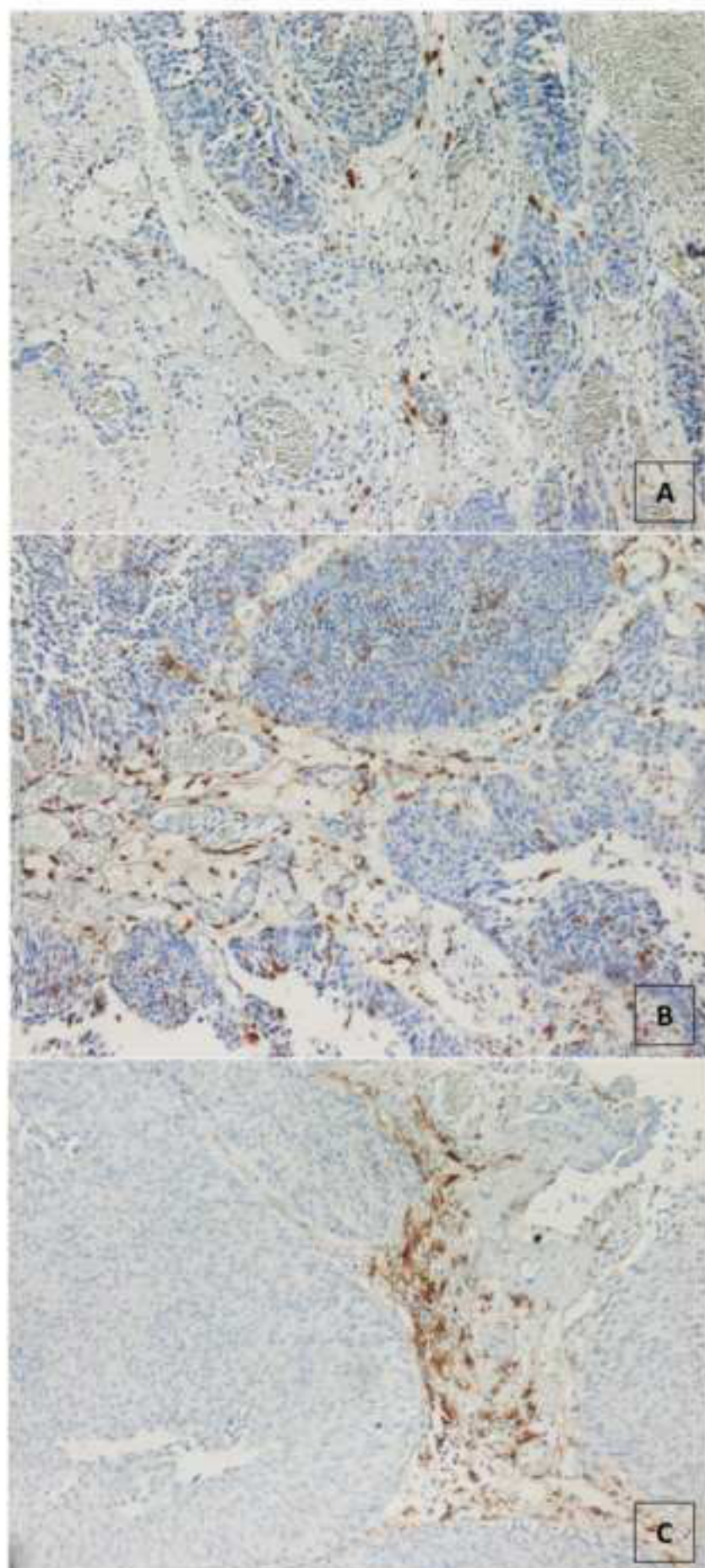


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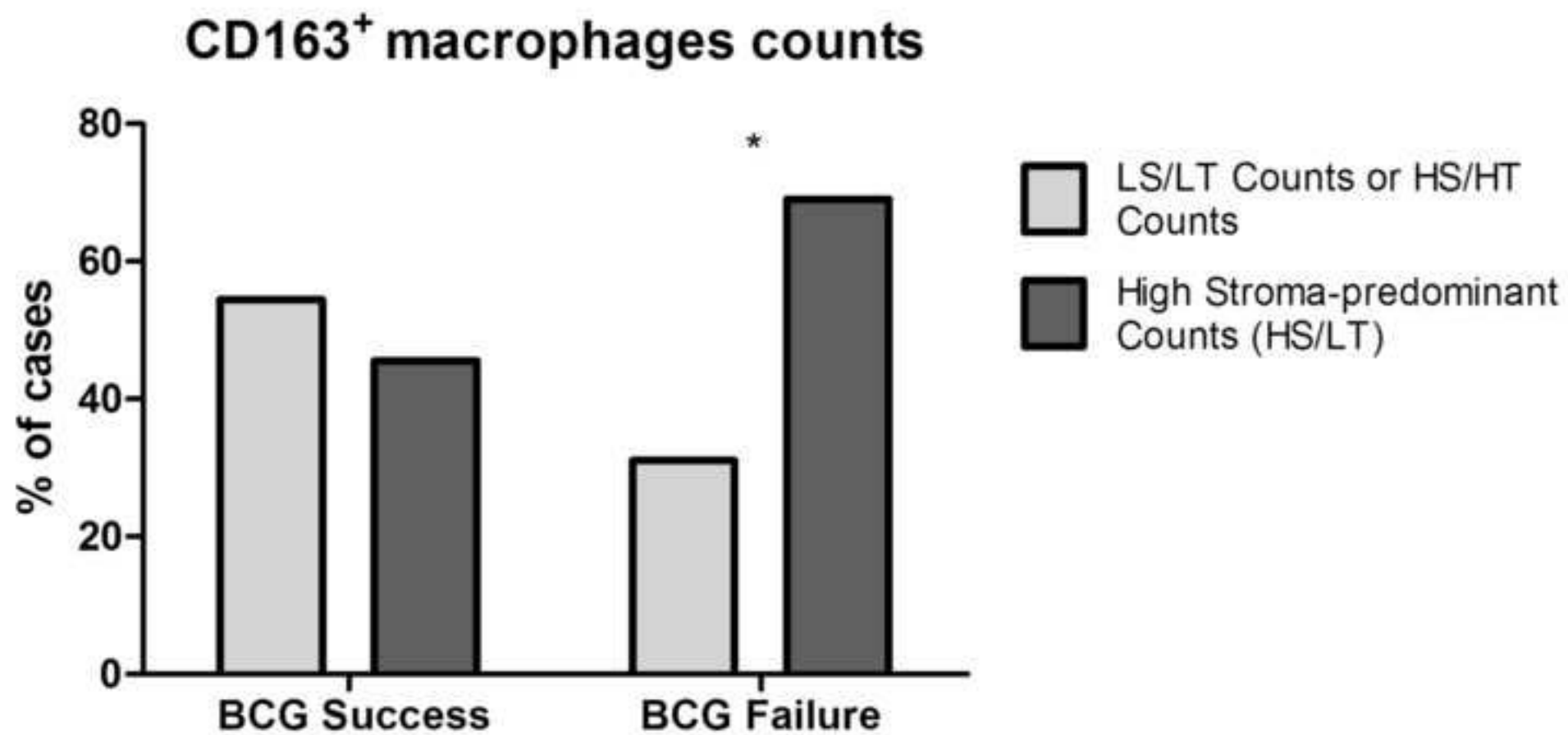


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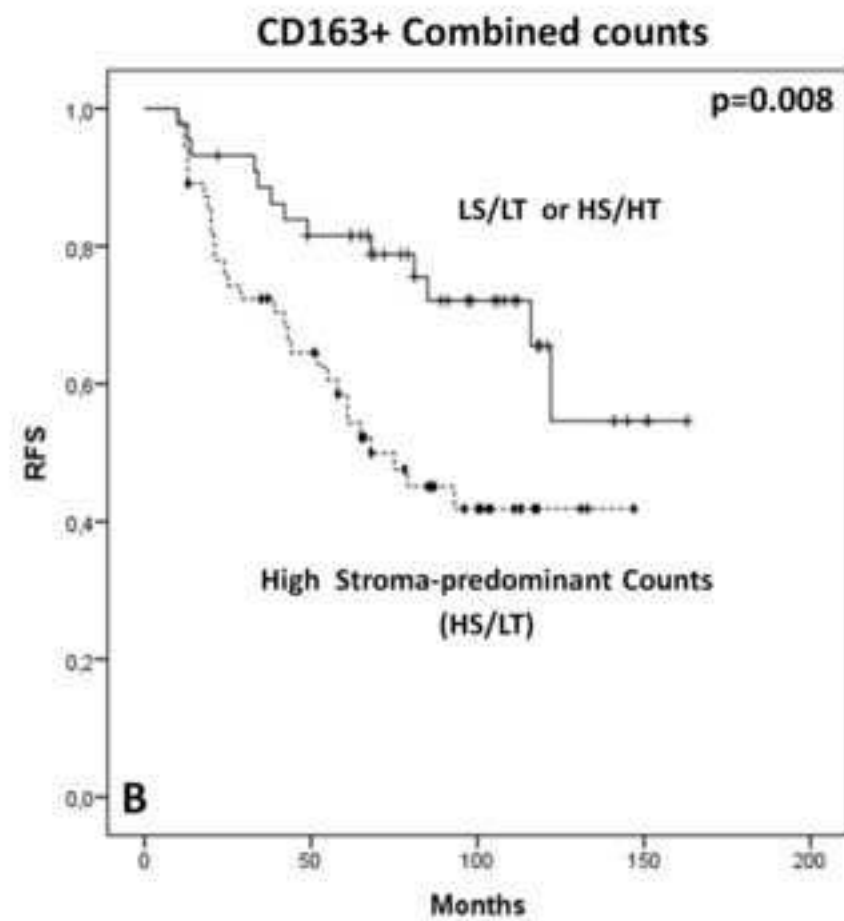
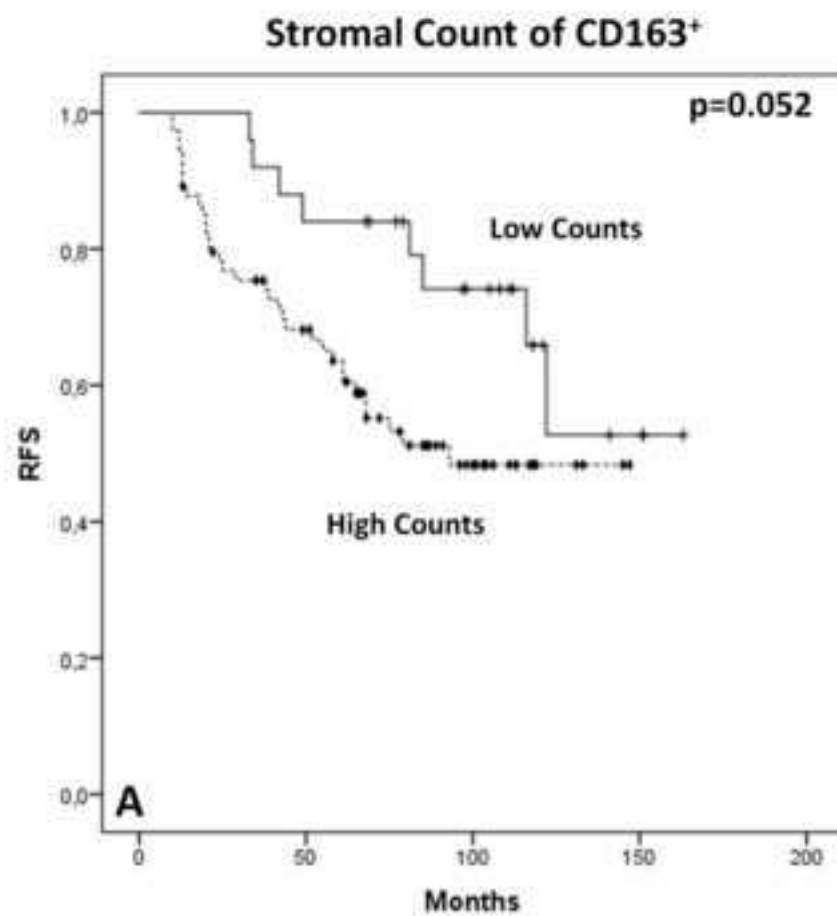
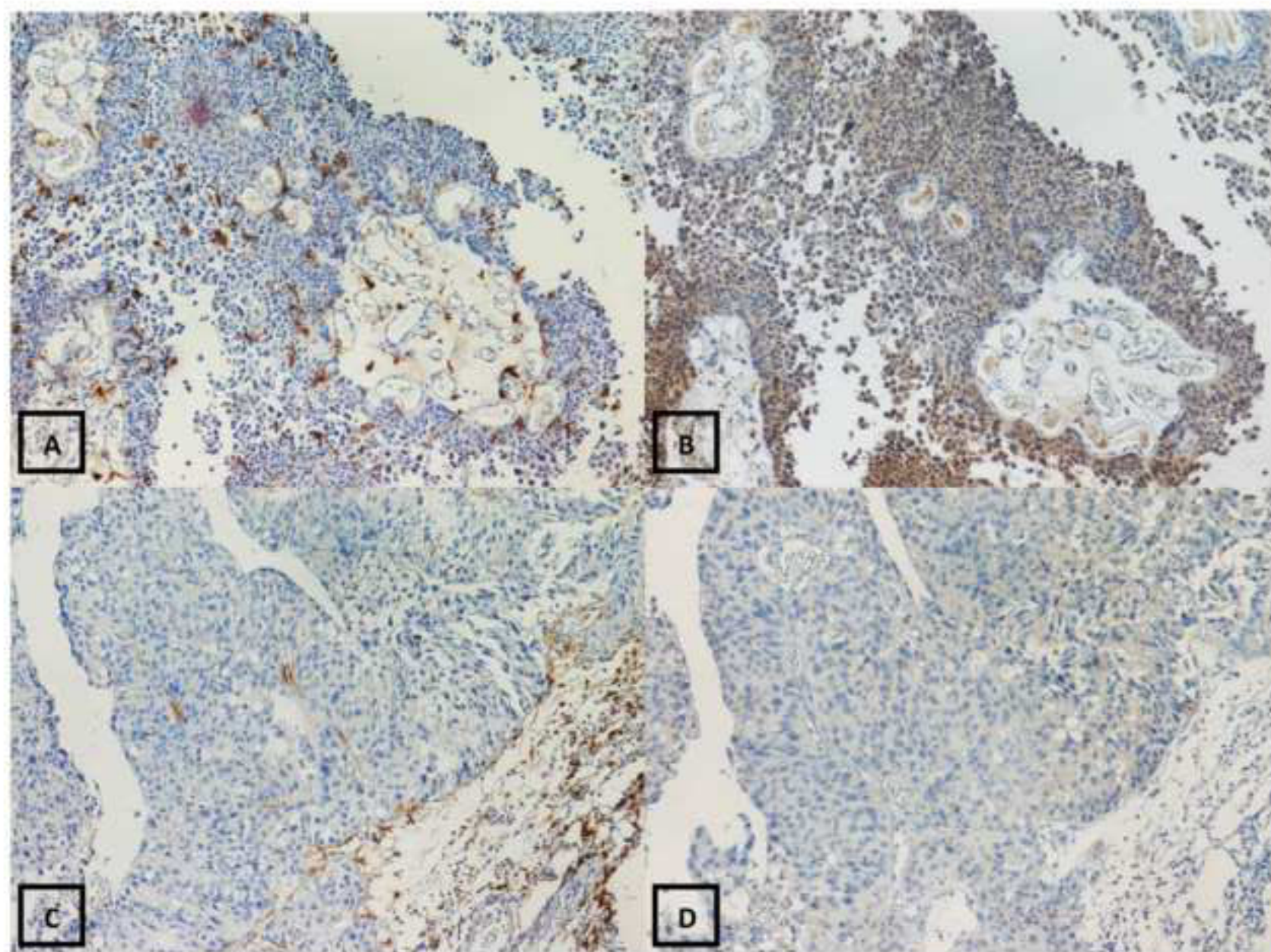




Figure 4  
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**Figure 1. Immunohistochemical staining showing different grades of CD163<sup>+</sup> macrophages infiltration in bladder tumors (400x).** Representative images (A-C) of macrophages stained with anti-CD163 (brown). A- Low Stroma and Low Tumor Macrophages infiltration (LS/LT); B- High Stroma and High Tumor Macrophages infiltration (HS/HT); C- High Stroma and Low Tumor Macrophages infiltration (HS/LT) – High Stroma-predominant Macrophages Counts.

**Figure 2. Association between combined CD163<sup>+</sup> macrophages counts and BCG treatment failure.** Higher stroma-restricted CD163<sup>+</sup> macrophages counts were associated with non-response after BCG immunotherapy. LS/LT: Low Stromal and Low tumor; HS/HT: High Stromal and High Tumor; HS/LT: High Stromal and Low Tumor – High Stroma-predominant Counts. “\*” p=0.020 (Chi-square Test).

**Figure 3. Effect M2-polarized TAMs in recurrence-free survival (RFS).** Kaplan-Meier analysis to evaluate the association between RFS in the studied patients and: A- CD163<sup>+</sup> macrophages counts in stroma; B- CD163<sup>+</sup> macrophages combined counts. High Stroma-predominant counts (HS/LT: High Stromal but Low Tumor Counts) vs. LS/LT (Low Stromal and Low Tumor counts) or HT/HT (High Stromal with High Tumor counts). Comparison performed by log-rank test (A: p=0.052; B: p=0.008); + censored “Low Counts” or “LS/LT or HS/HT” cases; ♦ censored “High Counts” or “High Stroma-predominant Counts (HS/LT)” cases.

**Figure 4. Immunohistochemical staining showing different grades of CD163+ macrophages infiltration and HIF-1α in the tumor nest of bladder cancer (100x).** Representative images (A and C) of Macrophages stained with anti-CD163 (brown) and the same areas stained with anti-HIF-1α (brown - B and D). A- High infiltration of CD163+ Macrophages in tumor; B- High staining of HIF-1 α in tumor; C- Low infiltration of CD163+ Macrophages in tumor; D- Low staining of HIF-1 α in tumor;

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

Variables	Total	Responders	Non- Responders	HR [95% CI]	p <sup>*</sup>
	n (%)	n (%)	n (%)		
Age					
<65 years	45 (45,5)	32 (56,1)	13 (31,0)	1.0	0,002
≥65 years	54 (54,5)	25 (43,9)	29 (69,0)	2,763 [1,431-5,336]	
Sex					
Male	84 (84,8)	46 (80,7)	38 (90,5)	1.0	0,223
Female	15 (15,2)	11 (19,3)	4 (9,5)	0,526 [0,187-1,478]	
Stage					
Ta	40 (40,4)	22 (38,6)	18 (42,9)	1.0	0,899
T1	59 (59,6)	35 (61,4)	24 (57,1)	0,961 [0,521-1,773]	
Grade					
Low	39 (39,4)	24 (42,1)	15 (35,7)	1.0	0,287
High	60 (60,6)	33 (57,9)	27 (64,3)	1,410 [0,749-2,654]	
Size (cm)					
<3	64 (65,3)	36 (63,2)	28 (68,3)	1.0	0,416
≥3	34 (34,7)	21 (36,8)	13 (31,7)	0,760 [0,393-1,470]	
Tumor number					
Unifocal	45 (45,5)	29 (50,9)	16 (38,1)	1.0	0,087
Multifocal	54 (54,5)	28 (49,1)	26 (61,9)	1,729 [0,924-3,235]	
CIS					
No	92 (92,9)	53 (93,0)	39 (92,9)	1.0	0,923
Yes	7 (7,1)	4 (7,0)	3 (7,1)	0,944 [0,291-3,056]	
Recurrence Status					
Primary	51 (51,5)	33 (57,9)	18 (42,9)	1.0	0,153
Recurrent	48 (48,5)	24 (42,1)	24 (57,1)	1,562 [0,847-2,881]	
BCG schedule					
iBCG	41 (41,4)	18 (31,6)	23 (54,8)	1.0	0,026
mBCG	58 (58,6)	39 (68,4)	19 (45,2)	2,002 [1,088-3,684]	

HR: Hazard Ratio; CI: Confidence Interval; CIS: Carcinoma *in situ*.

\*: Wald test

Table 2

**Table 2.** Correlation between clinical parameters and CD68<sup>+</sup> and CD163<sup>+</sup> macrophages counts, in tumor stroma and tumor nest.

	CD68 <sup>+</sup> macrophages counts				CD163 <sup>+</sup> macrophages counts				CD163 <sup>+</sup> /CD68 <sup>+</sup> macrophage ratio			
	Tumor Stroma		Tumor Nest		Tumor Stroma		Tumor Nest		Tumor Stroma		Tumor Nest	
	Correlation coefficient	P value	Correlation Coefficient	P value	Correlation Coefficient	P value	Correlation Coefficient	P value	Correlation Coefficient	P value	Correlation Coefficient	P value
Age	0,026	0,802	-0,030	0,770	0,012	0,906	0,034	0,735	-0,015	0,884	0,014	0,897
Sex	-0,046	0,649	0,129	0,204	-0,066	0,519	0,166	0,101	-0,028	0,786	0,074	0,477
Stage	0,371	<b>0,000</b>	0,271	<b>0,007</b>	0,274	<b>0,006</b>	0,300	<b>0,003</b>	0,116	0,253	-0,012	0,907
Grade	0,284	<b>0,004</b>	0,194	0,054	0,232	<b>0,021</b>	0,278	<b>0,005</b>	0,135	0,184	0,126	0,223
Size	0,263	<b>0,009</b>	0,290	<b>0,004</b>	0,172	0,090	0,284	<b>0,005</b>	0,220	<b>0,029</b>	0,109	0,294
Tumor Number	-0,081	0,424	-0,023	0,818	0,004	0,972	0,012	0,903	-0,062	0,544	0,093	0,369
CIS	-0,176	0,082	0,072	0,481	-0,137	0,178	0,046	0,655	0,105	0,302	0,280	<b>0,006</b>
Primary / Recurrent	-0,120	0,239	-0,273	<b>0,006</b>	-0,037	0,718	-0,229	<b>0,023</b>	0,023	0,824	0,020	0,850

CIS: Carcinoma *in situ*.  
P value : Chi-square test

**Table 2.** Multivariate analysis and risk estimation of CD163<sup>+</sup> macrophages influence on BCG therapy outcome.

CD163 <sup>+</sup> macrophages		HR <sup>a</sup>	95%CI	p value
Stromal Counts				
	Low (≤19)	1.0	Referent	0.012
	High (>19)	2,402	1.211-4.763	
Combined Counts				
	LS/LT or HS/HT	1.0	Referent	0.005
	High Stroma-predominant Counts (HS/LT)	2.627	1.340-5.150	

LS/LT: Low Stromal and Low tumor counts;  
HS/HT: High Stromal and High Tumor counts ;  
HS/LT: High Stromal but Low Tumor counts;  
HR: Hazard Ratio; CI: Confidence Interval  
<sup>a</sup> adjusted for age and BCG schedule