The role of functional polymorphisms in immune response genes as biomarkers of BCG Immunotherapy outcome in bladder cancer: Establishment of a predictive profile in a Southern Europe population

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ABSTRACT

Objective: To evaluate the predictive value of genetic polymorphisms in the context of BCG immunotherapy outcome and create a predictive profile that may allow discriminating the risk of recurrence.

Material and Methods: In a dataset of 204 patients treated with BCG, we evaluate 42 genetic polymorphisms in 38 genes involved in the BCG mechanism of action, using Sequenom MassARRAY technology. Stepwise multivariate Cox Regression was used for data mining.

Results: In agreement with previous studies we observed that gender, age, tumor multiplicity and treatment scheme were associated with BCG failure. 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 (SNPs in *TNFA-1031T/C* (rs1799964), *IL2RA* rs2104286 T/C, *IL17A-197G/A* (rs2275913), *IL17RA-809A/G* (rs4819554), *IL18R1* rs3771171 T/C, *ICAM1* K469E (rs5498), *FASL-844T/C* (rs763110) and *TRAILR1-397T/G* (rs79037040) in association with clinicopathological variables. This risk score allows the categorization of patients into risk groups: patients within the Low Risk group have a 90% chance of successful treatment, whereas patients in the High Risk group present 75% chance of recurrence after BCG treatment.

Conclusion: We have established the first predictive score of BCG immunotherapy outcome combining clinicopathological characteristics and a panel of genetic polymorphisms. Further studies using an independent cohort are warranted. Moreover, the inclusion of other biomarkers may help to improve the proposed model.

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 intravesical instillation of bacillus Calmette-Guerin (BCG) has delayed the 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 and in such cases the tumor may become more aggressive [4, 5]. Therefore, it is important to find biomarkers that may 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 indicate that the effective establishment of a Th1-cytokine profile is crucial for an effective antitumor activity [6, 10, 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 evidence 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 also reinforce the notion that alterations in these pathways may influence treatment outcome.

Several markers associated with 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]. In agreement with these observations, we have recently presented evidence 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, 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 (T1, high grade, multiple Ta/T1, *CIS*) 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). All the patients in the iBCG group completed the 6 instillation scheme and includes patients treated before the implementation of European Association of Urology guidelines recommending the mBCG scheme [19] or patients showing significant intolerance to prolonged BCG treatment. 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 new tumor in the bladder, after treatment, confirmed by pathology, with at least one tumor-free cystoscopy in-between. The end point of the study, termed recurrence-free survival (RFS), was defined as the period between the beginning of BCG treatment until the date of the most recent cystoscopy or recurrence. Since only 9% of the patients presented progression by stage/grade, this endpoint was not considered in this work. 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 [20] 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 SequenomiPLEX 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. This technique was carried-out by the Genomics Unit from the Genotyping Service of Instituto Gulbenkian de Ciência (IGC) (Oeiras, Portugal).

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. Genotyping of approximately 10% of the evaluated SNPs were repeated using TaqMan allelic discrimination assays for real time PCR and presented 100% concordance with the Sequenom MassARRAY genotyping.

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 [21].

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 to 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 for 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.

Genetic polymorphisms and BCG treatment outcome

Antigen presentation

In this study we evaluated five polymorphisms in genes involved in antigen presentation. From the studied 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 have demonstrated that *IL2RA* rs2104286 C/T, *IL17A* rs2275913 (-197G/A), *TNFA* rs1799964 (-1031T/C) and *CCR2* rs391835 G/A (V64I) were 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 as well as the 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).

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 [21]. The predictive model using 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).

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).

Risk groups stratification was performed based on the best cut-off values to obtain the highest sensitivity and specificity (approximately 90%). 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 within the two mentioned cut-off values were placed in the Intermediate Risk group, which have a 42% chance of treatment failure.

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).

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 [4]. Several clinicopathological characteristics influence the course of treatment, however there is no consensus regarding its predictive value [22]. 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, 22].

Our previous reports using a smaller dataset have demonstrated that age, tumor multiplicity and the treatment scheme are associated with treatment failure [18, 23-25]. Herein, with a larger sample, we confirmed that patients either with age over 65 years, or with multiple

tumors, or treated only with induction scheme were 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, 22-25] 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. Nevertheless, only few polymorphisms have been studied, individually and in small sample sets, mainly using non-integrative approaches [16, 26-28]. In an attempt to establish a predictive model of treatment response we have evaluated 42 functional SNPs in 38 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, that upon binding enhances proinflammatory signals such as inflammatory leukocyte recruitment [29]. Interestingly, circulating forms of ICAM-1 (sICAM1) were found to inhibit the interaction between T cells and tumors [30], and block NK cell-mediated toxicity [31]. 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 [32]. 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, 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 [33]. The presence of AA genotype of *IL17-197G/A* polymorphism has been associated to reduced the levels of this molecule [34], 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 polymorphisms 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 [35]. 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 this population, 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 SNP has been evaluated in a previous study in the context of BCG treatment outcome [26], however showing contradicting results. According to the authors the CC genotype was associated with a protective effect whereas we observed an association with increased risk of recurrence. These differences may be explained by either the different ethnicities of the populations; a bias of the sample number, since the number of patients that we evaluated was significantly higher (204 vs 70); or the inclusion of patients treated with mBCG in our study, since the treatment scheme was not considered in referred study.

We and others have reported that macrophages play a key role in BCG mechanism of action [6, 23, 36]. 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 [37] and its receptor (CCR2). Among the studied SNPs only the *CCR2-960T/A* polymorphism demonstrated an association with treatment outcome. This SNP is located within the functional promoter [38] and patients carrying A allele have been described to present reduced monocyte migration activity [39]. Moreover, our results showed that patients carrying this allele had an increased risk of recurrence possible due to a deficient monocyte migration. This would result in decreased macrophage attraction which would ultimately influence the immunological activation promoted by BCG therapy.

Another line of evidence indicates that neutrophils play 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 effectors of BCG antitumor action [14]. Therefore we evaluated genetic polymorphisms that could influence TRAIL expression as well as its receptor. Our results demonstrated that patients carrying the G allele of *TRAILR1-397T/G* polymorphism had an increased risk of recurrence after BCG treatment. According to Wang *et al*. the presence of this allele increases the transcriptional activity of *TRAILR1* promoter, possibly leading to higher expression of the receptor [40]. Although an increased expression of *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 [41]. 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 [41]. 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 work of Leibovici *et al.,* in 2005, was the first to evaluate genetic polymorphisms in the context of BCG treatment response [42]. This study screened 123 patients treated with BCG

for polymorphisms in *TNFA*, *IL6* and *IL8* genes and found that only the *IL6-174G/C* polymorphism was associated with an higher risk of recurrence after BCG immunotherapy [42]. Moreover, Ahirwar and colleagues found that *IL6-174G/C* and *IL8-251T/A* polymorphisms were associated with a reduced risk of recurrence in their population [27, 43]. In the present study we also evaluated these SNPs but did not found a significant association with recurrence. These differences may arise from different genetic backgrounds in the studied populations (American, European and Asian populations).

In resume, the effects exerted by the SNPs associated with treatment outcome 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. At the moment, the probability of recurrence is calculated based on the EORTC risk tables, which stratifies patients according to the risk of recurrence. Based on these tables, patients at intermediate and high risk of recurrence perform BCG immunotherapy. Moreover, the CUETO group created risk tables for BCG treated patients based in clinical and pathological characteristics [44]. Herein, we propose a predictive model and a risk score of recurrence after BCG treatment based in clinicopathological variables and the genetic variability of the host. This is a preliminary study, since no independent cohort was used for validation, but is important to highlight that the

proposed model could identify patients with 90% probability of successful treatment and 75% chance of recurrence, which is more accurate than the actual models. 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 alone or in combination with the existing risk scores. However it should be noticed that 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 an independent dataset of patients 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 in association with clinical and pathological characteristicscapable of stratifying patients according to the risk of recurrence after BCG immunotherapy. The established risk groups identify patients with poor prognosis and may constitute a helpful tool improve clinical decision.

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REFERENCES

[1] Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 2010 Dec 15: **127**:2893-917

[2] Sievert KD, Amend B, Nagele U, et al. Economic aspects of bladder cancer: what are the benefits and costs? World J Urol. 2009 Jun: **27**:295-300

[3] Babjuk M, Burger M, Zigeuner R, et al. EAU Guidelines on Non-Muscle-invasive Urothelial Carcinoma of the Bladder: Update 2013. Eur Urol. 2013 Oct: **64**:639-53

[4] Ehdaie B, Sylvester R, Herr HW. Maintenance Bacillus Calmette-Guerin Treatment of Non-muscle-invasive Bladder Cancer: A Critical Evaluation of the Evidence. Eur Urol. 2013 May 18:

[5] Witjes JA. Management of BCG failures in superficial bladder cancer: a review. Eur Urol. 2006 May: **49**:790-7

[6] Alexandroff AB, Nicholson S, Patel PM, Jackson AM. Recent advances in bacillus Calmette-Guerin immunotherapy in bladder cancer. Immunotherapy. 2010 Jul: **2**:551-60

[7] Kuroda K, Brown EJ, Telle WB, Russell DG, Ratliff TL. Characterization of the internalization of bacillus Calmette-Guerin by human bladder tumor cells. J Clin Invest. 1993 Jan: **91**:69-76

[8] Bevers RFM, Kurth K-H, Schamhart DHJ. Role of urothelial cells in BCG immunotherapy for superficial bladder cancer. British Journal of Cancer. 2004: **91**:607-12

[9] Ikeda N, Toida I, Iwasaki A, Kawai K, Akaza H. Surface antigen expression on bladder tumor cells induced by bacillus Calmette-Guerin (BCG): A role of BCG internalization into tumor cells. Int J Urol. 2002 Jan: **9**:29-35

[10] Bohle A, Brandau S. Immune mechanisms in bacillus Calmette-Guerin immunotherapy for superficial bladder cancer. J Urol. 2003 Sep: **170**:964-9

[11] Askeland EJ, Newton MR, O'Donnell MA, Luo Y. Bladder Cancer Immunotherapy: BCG and Beyond. Adv Urol. 2012: **2012**:181987

[12] Luo Y. Blocking IL-10 enhances bacillus Calmette-Guerin induced T helper Type 1 immune responses and anti-bladder cancer immunity. Oncoimmunology. 2012 Oct 1: **1**:1183-5

[13] Shah G, Zhang G, Chen F, Cao Y, Kalyanaraman B, See WA. iNOS expression and NO production contribute to the direct effects of BCG on urothelial carcinoma cell biology. Urol Oncol. 2013 Sep 17:

[14] Rosevear HM, Lightfoot AJ, O'Donnell MA, Griffith TS. The role of neutrophils and TNF-related apoptosis-inducing ligand (TRAIL) in bacillus Calmette-Guerin (BCG) immunotherapy for urothelial carcinoma of the bladder. Cancer Metastasis Rev. 2009 Dec: **28**:345-53

[15] Brandau S, Suttmann H, Riemensberger J, et al. Perforin-mediated lysis of tumor cells by Mycobacterium bovis Bacillus Calmette-Guerin-activated killer cells. Clin Cancer Res. 2000 Sep: **6**:3729-38

[16] Lima L, Dinis-Ribeiro M, Longatto-Filho A, Santos L. Predictive biomarkers of bacillus calmette-guerin immunotherapy response in bladder cancer: where are we now? Adv Urol. 2012: **2012**:232609

[17] Gu J, Grossman HB, Dinney CP, Wu X. The pharmacogenetic impact of inflammatory genes on bladder cancer recurrence. Pharmacogenomics. 2005 Sep: **6**:575-84

[18] Lima L, Ferreira JA, Tavares A, et al. FASL polymorphism is associated with response to bacillus Calmette-Guerin immunotherapy in bladder cancer. Urol Oncol. 2013 Aug 12:

[19] Oosterlinck W, van der Meijden A, Sylvester R, et al. Guidelines on TaT1 (Non-muscle invasive) Bladder Cancer. In Office EG ed, EAU Guidelines 2006. Arnhem, The Netherlands EAU Guidelines Office, 2006

[20] Lima L, Morais A, Lobo F, Calais-da-Silva FM, Calais-da-Silva FE, Medeiros R. Association between FAS polymorphism and prostate cancer development. Prostate cancer and prostatic diseases. 2008: **11**:94-8

[21] Harrell FE, Jr., Lee KL, Mark DB. Multivariable prognostic models: issues in developing models, evaluating assumptions and adequacy, and measuring and reducing errors. Stat Med. 1996 Feb 28: **15**:361-87

[22] Zuiverloon TCM, Nieuweboer AJM, Ve´kony H, Kirkels WJ, Bangma CH, Zwarthoff EC. Markers Predicting Response to Bacillus Calmette-Gue´rin Immunotherapy in High-Risk Bladder Cancer Patients: A Systematic Review. European Urology. 2011:1-18

[23] Lima L, Oliveira D, Tavares A, et al. The predominance of M2-polarized macrophages in the stroma of low-hypoxic bladder tumors is associated with BCG immunotherapy failure Urol Oncol. 2013: **in press**

[24] Lima L, Severino PF, Silva M, et al. Response of high-risk of recurrence/progression bladder tumours expressing sialyl-Tn and sialyl-6-T to BCG immunotherapy. Br J Cancer. 2013 Oct 15: **109**:2106-14

[25] Lima L, Silva J, Amaro T, et al. IL-4 and TNF-alpha polymorphisms are associated with risk of multiple superficial tumors or carcinoma in situ development. Urol Int. 2011: **87**:457-63

[26] Ahirwar DK, Mandhani A, Dharaskar A, Kesarwani P, Mittal RD. Association of tumour necrosis factor-alpha gene (T-1031C, C-863A, and C-857T) polymorphisms with bladder cancer susceptibility and outcome after bacille Calmette-Guerin immunotherapy. BJU Int. 2009 Sep: **104**:867-73

[27] Ahirwar DK, Mandhani A, Mittal RD. IL-8 -251 T > A polymorphism is associated with bladder cancer susceptibility and outcome after BCG immunotherapy in a northern Indian cohort. Arch Med Res. 2010 Feb: **41**:97-103

[28] Jaiswal PK, Singh V, Srivastava P, Mittal RD. Association of IL-12, IL-18 variants and serum IL-18 with bladder cancer susceptibility in North Indian population. Gene. 2013 Apr 25: **519**:128-34

[29] Witkowska AM, Borawska MH. Soluble intercellular adhesion molecule-1 (sICAM-1): an overview. Eur Cytokine Netw. 2004 Apr-Jun: **15**:91-8

[30] Becker JC, Termeer C, Schmidt RE, Brocker EB. Soluble intercellular adhesion molecule-1 inhibits MHC-restricted specific T cell/tumor interaction. J Immunol. 1993 Dec 15: **151**:7224-32

[31] Becker JC, Dummer R, Hartmann AA, Burg G, Schmidt RE. Shedding of ICAM-1 from human melanoma cell lines induced by IFN-gamma and tumor necrosis factor-alpha. Functional consequences on cell-mediated cytotoxicity. J Immunol. 1991 Dec 15: **147**:4398-401

[32] Bielinski SJ, Reiner AP, Nickerson D, et al. Polymorphisms in the ICAM1 gene predict circulating soluble intercellular adhesion molecule-1(sICAM-1). Atherosclerosis. 2011 Jun: **216**:390-4

[33] Takeuchi A, Dejima T, Yamada H, et al. IL-17 production by gammadelta T cells is important for the antitumor effect of Mycobacterium bovis bacillus Calmette-Guerin treatment against bladder cancer. Eur J Immunol. 2011 Jan: **41**:246-51

[34] Li N, Zhu Q, Li Z, et al. IL17A gene polymorphisms, serum IL-17A and IgE levels, and hepatocellular carcinoma risk in patients with chronic hepatitis B virus infection. Mol Carcinog. 2012 Dec 31:

[35] Knevel R, de Rooy DP, Zhernakova A, et al. Association of variants in IL2RA with progression of joint destruction in rheumatoid arthritis. Arthritis Rheum. 2013 Jul: **65**:1684-93

[36] Luo Y, Yamada H, Evanoff DP, Chen X. Role of Th1-stimulating cytokines in bacillus Calmette-Guerin (BCG)-induced macrophage cytotoxicity against mouse bladder cancer MBT-2 cells. Clin Exp Immunol. 2006 Oct: **146**:181-8

[37] Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. J Interferon Cytokine Res. 2009 Jun: **29**:313-26

[38] Yamamoto K, Takeshima H, Hamada K, et al. Cloning and functional characterization of the 5'-flanking region of the human monocyte chemoattractant protein-1 receptor (CCR2) gene. Essential role of 5'-untranslated region in tissue-specific expression. J Biol Chem. 1999 Feb 19: **274**:4646-54

[39] Rittig K, Peter A, Baltz KM, et al. The CCR2 promoter polymorphism T-960A, but not the serum MCP-1 level, is associated with endothelial function in prediabetic individuals. Atherosclerosis. 2008 Jun: **198**:338-46

[40] Wang M, Cheng G, Zhang Z, Fu G. Genetic variants in the death receptor 4 gene contribute to susceptibility to bladder cancer. Mutat Res. 2009 Feb 10: **661**:85-92

[41] Collison A, Foster PS, Mattes J. Emerging role of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) as a key regulator of inflammatory responses. Clin Exp Pharmacol Physiol. 2009 Nov: **36**:1049-53

[42] Leibovici D, Grossman HB, Dinney CP, et al. Polymorphisms in inflammation genes and bladder cancer: from initiation to recurrence, progression, and survival. J Clin Oncol. 2005 Aug 20: **23**:5746-56

[43] Ahirwar D, Kesarwani P, Manchanda PK, Mandhani A, Mittal RD. Anti- and proinflammatory cytokine gene polymorphism and genetic predisposition: association with smoking, tumor stage and grade, and bacillus Calmette-Guerin immunotherapy in bladder cancer. Cancer Genet Cytogenet. 2008 Jul: **184**:1-8

[44] Fernandez-Gomez J, Madero R, Solsona E, et al. Predicting nonmuscle invasive bladder cancer recurrence and progression in patients treated with bacillus Calmette-Guerin: the CUETO scoring model. J Urol. 2009 Nov: **182**:2195-203

FIGURES LEGENDS

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.

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 clinicalpathological 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 (Clinicopathological variables alone). Model 1, AUC: 0.684; Model 2, AUC: 0.734; Model 3, AUC:0.820.

Figure 3 - Recurrence-free survival (RFS) of the patients within 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.

Table 1. Relation between patients clinical and tumour characteristics and recurrence after BCG treatment.

Variables	Total	BCG success	BCG failure	HR [95% CI]	p**
	n (%)	n (%)	n (%)		
Age					
<65 years	97 (47.5)	71 (53.0)	26 (37.1)	1.0	
≥65 years	107 (52.5)	63 (47.0)	44 (62.9)	1.973 [1.212-3.212]	**0.006**
Sex					
Female	29 (14.2)	24 (17.9)	5 (7.1)	1.0	
Male	175 (85.8)	110 (82.1)	65 (92.9)	2.533 [1.018-6.303]	**0.046**
Stage					
Ta	82 (40.2)	54 (40.3)	28 (40.0)	1.0	
T1	122 (59.8)	80 (59.7)	42 (60.0)	1.067 [0.661-1.723]	0.790
Grade					
Low	55 (27.0)	36 (26.9)	19 (27.1)	1.0	
High	149 (73.0)	98 (73.1)	51 (72.9)	1.188 [0.700-2.017]	0.524
Size (cm)					
<3	129 (67.9)	83 (66.4)	46 (70.8)	1.0	
≥3	61 (32.1)	42 (33.6)	19 (29.2)	0.828 [0.485-1.414]	0.490
Tumor number					
Unifocal	96 (47.1)	68 (50.7)	28 (40.0)	1.0	
Multifocal	108 (52.9)	66 (49.3)	42 (60.0)	1.554 [0.963-2.510]	0.071
CIS					
No	186 (91.2)	124 (92.5)	62 (88.6)	1.0	
Yes	18 (8.8)	10 (7.5)	8 (11.4)	1.238 [0.592-2.588]	0.570
Recurrence Status					
Primary	104 (51.0)	72 (53.7)	32 (45.7)	1.0	
Recurrent	100 (49.0)	62 (46.3)	38 (54.3)	1.304 [0.814-2.089]	0.270
BCG schedule					
mBCG	138 (67.6)	101 (75.4)	37 (52.9)	1.0	
iBCG	66 (32.4)	33 (24.6)	33 (47.1)	2.034 [1.270-3.256]	**0.003**

HR: Hazard Ratio; CI: Confidence Interval

*: Wald test; Bold values indicate p<0.05

Table 2 – Multivariate analysis and risk estimation of the most significant polymorphisms

		HR[a]	95%CI	P value
ICAM1 **rs5498 A/G (K469E)**				
	AA	1.0	Referent	-
	AG	0.821	[0.440-1.532]	0.536
	GG	1.678	[0.865-3.256]	0.125
	AA+AG	1.0	Referent	-
	GG	1.759	[1.050-2.949]	**0.032**
IL2RA **rs2104286 C/T**				
	TT	1.0	Referent	-
	CT	2.007	[1.207-3.335]	0.007
	CC	1.245	[0.476-3.257]	0.655
	CT+CC	1.603	[1.001-2.567]	0.050
IL17A **rs2275913 (-197G/A)**				
	GG	1.0	Referent	-
	GA	0.856	[0.506-1.449]	0.562
	AA	1.946	[0.988-3.836]	0.054
	GG+GA	1.0	Referent	-
	AA	2.097	[1.118-3.933]	**0.021**
TNFA **rs1799964 (-1031T/C)**				
	TT	1.0	Referent	-
	TC	0.778	[0.458-1.324]	0.355
	CC	2.112	[0.970-4.598]	0.060
	TT+TC	1.0	Referent	-
	CC	2.427	[1.144-5.149]	**0.021**
CCR2 **rs391835 G/A (V64I)**				
	GG	1.0	Referent	-
	GA	0.816	[0.473-1.407]	0.055
	AA	0.410	[0.191-0.879]	**0.022**
	GA+AA	0.455	[0.232-0.893]	**0.022**
TRAILR1 **rs13278062 (-397T/G)**				
	TT	1.0	Referent	-
	TG	3.546	[1.477-8.513]	**0.005**
	GG	3.078	[1.251-7.573]	**0.014**
	TG+GG	3.195	[1.373-7.433]	**0.007**

HR: Hazard ratio; 95%CI: 95% Confidence Interval; [a]: adjusted to BCG scheme, age, gender and tumor multifocality. Bold values indicate p<0.05.

Table 3 – Predictive models of recurrence after BCG treatment obtain by Multivariate Stepwise Cox Regression

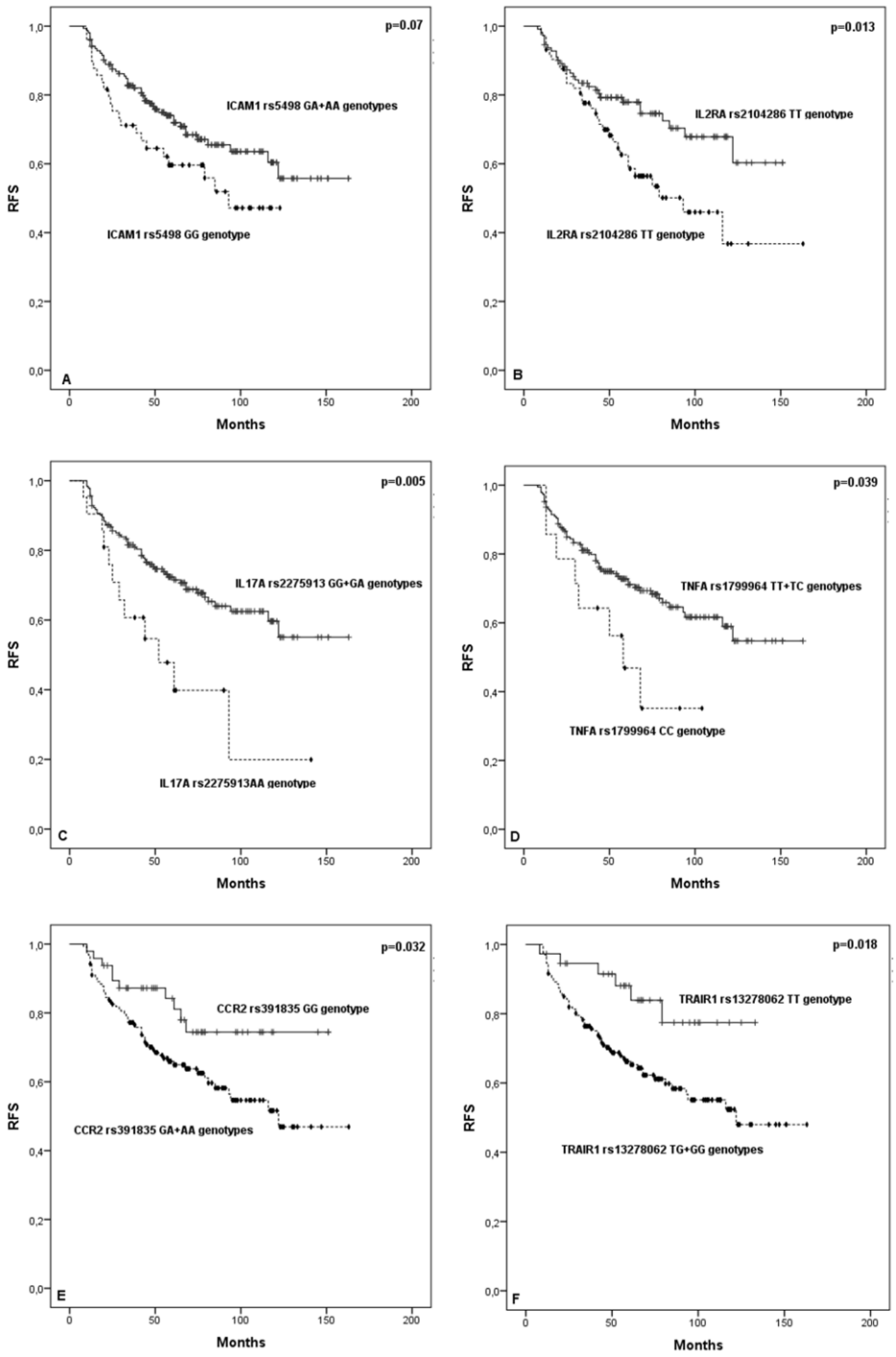
	HR	95%CI	P value	c-index
Model 1				0.698
Age (<65 vs. ≥65)	1.78	[1.06-3.01)	0.031	
Gender (female vs. male)	2.72	[1.09-6.81]	0.033	
Tumor Number (unifocal vs. multifocal)	1.64	[0.97-2.76]	0.063	
Treatment Scheme (mBCG vs. iBCG)	2.01	[1.22-3.32]	0.006	
Model 2				0.735
TNFA-1031T/C (rs1799964) (TT+TC vs. CC)	2.45	[1.31-5.33]	0.023	
IL4-33T/C (rs2070874) (CT vs. TT)	1.72	[0.86-3.38]	0.116	
IL2RA rs2104286 T/C (TT vs. TC+CC)	1.80	[1.08-2.99]	0.023	
IL17A-197G/A (rs2275913) (GG+GA vs. AA)	2.27	[1.16-4.45]	0.017	
IL17RA-809A/G (rs4819554) (AA+AGvs. GG)	2.19	[1.19-4.01]	0.011	
IL18R1 rs3771171 T/C (CC vs. TC+TT)	2.88	[0.88-9.47]	0.081	
IL6R Asp358Ala (rs8192284) (CC vs. CA+AA)	2.00	[0.85-4.67]	0.110	
ICAM1 K469E (rs5498) (AA+AG vs. GG)	1.87	[1.08-3.26]	0.027	
FASL-844T/C (rs763110) (TT+TC vs. CC)	1.71	[1.02-2.86]	0.041	
TRAILR1-397T/G (rs79037040) (TT vs. TG+GG)	2.59	[1.11-6.06]	0.028	
Model 3				0.820
Gender (female vs. male)	4.45	[1.71-11.6]	0.002	
Tumor Number (unifocal vs. multifocal)	2.29	[1.37-3.84]	0.002	
Treatment Scheme (mBCG vs. iBCG)	3.57	[2.03-6.28]	<0.001	
TNFA-1031T/C (rs1799964) (TT+TC vs. CC)	3.49	[1.58-7.70]	0.002	
IL4-33T/C (rs2070874) (CT vs. TT)	1.93	[0.96-3.86]	0.065	
IL2RA rs2104286 T/C (TT vs. TC+CC)	2.79	[1.63-4.78]	<0.001	
IL17A-197G/A (rs2275913) (GG+GA vs. AA)	2.65	[1.32-5.32]	0.006	
IL17RA-809A/G (rs4819554) (AA+AG vs. GG)	2.89	[1.49-5.63]	0.002	
IL18R1 rs3771171 T/C (CC vs. TC+TT)	2.75	[0.82-9.18]	0.101	
ICAM1 K469E (rs5498) (AA+AG vs. GG)	2.47	[1.42-4.29]	0.001	
FASL-844T/C (rs763110) (TT+TC vs. CC)	1.70	[1.02-2.84]	0.042	
TRAILR1-397T/G (rs79037040) (TT vs. TG+GG)	5.19	[2.05-13.1]	0.001	

HR: Hazard ratio; 95%CI: 95% Confidence Interval.

Table 4. Patients distribution among the risk groups and risk assessment of each group.

	Total	BCG success	BCG failure	HR	95% CI	p*
	n (%)	n (%)	n (%)			
Low RisK	90 (44.1)	83 (61.9)	7 (10.0)	1.0	Referent	-
Intermediate Risk	69 (33.8)	40 (29.9)	29 (41.4)	6.58	[2.88-15.1]	<0.001
High Risk	45 (22.1)	11 (8.2)	34 (48.6)	18.7	[8.24-42.6]	<0.001

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



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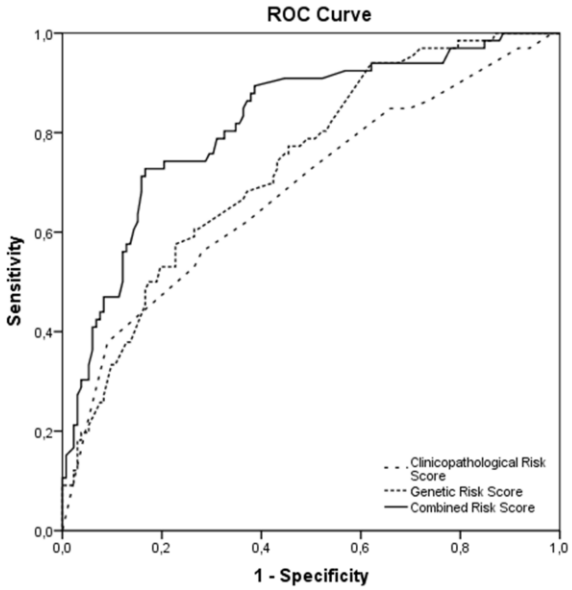

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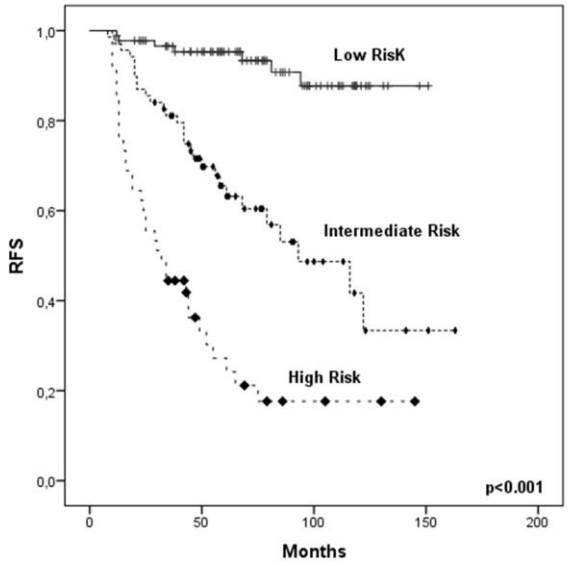

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