

Urine-Derived Biomarkers for Immunotherapy Prediction Response in Urothelial Cancer (UrIPredict)

Rui Pedro Ribeiro Pereira

M

2024



Rui Pedro Ribeiro Pereira. Urine-Derived Biomarkers for
Immunotherapy Prediction Response in Urothelial Cancer
(UrIPredict)

M.ICBAS **2024**



Urine-Derived Biomarkers for Immunotherapy Prediction Response in Urothelial
Cancer (UrIPredict)
Rui Pedro Ribeiro Pereira



Rui Pedro Ribeiro Pereira

**Urine-Derived Biomarkers for Immunotherapy Prediction Response in
Urothelial Cancer (UrIPredict)**

Dissertação de Candidatura ao grau de **Mestre em Oncologia –**
Especialização em Oncologia Laboratorial submetida ao Instituto de Ciências
Biomédicas de Abel de Salazar da Universidade do Porto

Orientadora: **Professora Doutora Carmen de Lurdes Fonseca Jerónimo**

Professora Catedrática Convidada do
Departamento de Patologia e Imunologia Molecular
Instituto de Ciências Biomédicas Abel Salazar – Universidade do Porto
Diretora do Centro de Investigação, Coordenadora e Investigadora Auxiliar do
Grupo de Epigenética e Biologia do Cancro do
Centro de Investigação
Instituto Português de Oncologia do Porto Francisco Gentil, E.P.E

Coorientadora: **Doutora Sara Raquel Monteiro dos Reis**

Investigadora Júnior
**Instituto de Ciência e Inovação em Engenharia Mecânica e Engenharia
Industrial**
Investigadora Convidada do Grupo de Epigenética e Biologia do Cancro do
Centro de Investigação
Instituto Português de Oncologia do Porto Francisco Gentil, E.P.E

“There is no way around hard work. Embrace it. You have to put in the hours because there’s always something which you can improve.”

Roger Federer



This study was funded by two projects of the Research Center of Portuguese Oncology Institute of Porto (PI184-CI-IPOP-14-2023; TRANSCAN3/0001/2021)

AGRADECIMENTOS

Com o culminar desta dissertação, termina mais uma fase desafiante e recompensadora do meu percurso académico. Foi sem dúvida um caminho atribulado, mas de muita aprendizagem que me pôs a prova em muitas ocasiões. Contudo, este trabalho nunca seria possível de ser concluído sem o apoio e carinho de várias pessoas fundamentais que sempre estiveram ao meu lado e que me deram a força necessária para continuar todos os dias.

Primeiramente, queria agradecer à minha orientadora, Professora Carmen Jerónimo, pela confiança depositada em mim desde o início deste projeto. A sua orientação metódica, críticas construtivas, constante incentivo e paixão pela investigação foram essenciais para a conclusão deste trabalho. Muito obrigado por tudo.

A ti Sara, o que seria de mim sem o teu apoio. Foste sem dúvida a melhor coorientadora e amiga que podia ter tido para me acompanhar durante este ano intenso. Muito paniquei e stressei com tantas coisas que agora olho para trás e penso que desnecessário, mas estiveste sempre lá a dar força e a acalmar-me nestes momentos de delírio. Tive a maior sorte do mundo e não trocava por nada, muito obrigado Sara.

Ao Rúben, obrigado por seres o melhor amigo e abrigo que podia ter durante estes últimos anos. Não sei o que seria de mim sem o teu apoio, sem as nossas conversas, sem a tua presença durante este ano. O que seria de mim sem o teu infindável conhecimento estatístico para me ajudar? Melhoraste em muito a minha vida e espero que esta amizade continue a florescer e fortalecer durante muitos anos.

A vocês Diana, Mariana, Ângela e Marta o meu eterno obrigado. Diana por alegrares-me sempre o meu dia, por todas as calinadas e atentados à língua portuguesa que oferecias, por me ajudares com digital sempre que estava apertado e aflito e por seres uma amiga incrível que tornou este ano muito mais fácil e risonho. Marianinha, sem ti esta dissertação nunca ficaria finalizada. Obrigado por toda a ajuda e paciência a formatá-la. Eu juro que aprendi algumas coisas e para uma próxima já consigo ser mais independente e menos Ângela. Ângela, obrigado por toda a ajuda, os concelhos e os momentos risonhos que tivemos sem dúvida alguma tornaste esta fase mais entusiasmante e divertida. Por último, Marta muito obrigado pela pessoa que és e por tornares cada dia no laboratório uma aventura divertida, acolhedora e risonha.

Estou imensamente agradecido a todos os membros do GEBC. Não só me ensinaram muito, mas também me fizeram sentir parte de uma equipe e ambiente incrível. A alegria e o carinho que transmitiram durante esta jornada foi sem dúvida marcante. De uma forma especial obrigado à Aninhas por toda a ajuda, transmissão de conhecimento e apoio. Sem

ti este trabalho não era possível de ser realizado E a ti Cláudia, muito obrigado pelas palavras de motivação, pelo apoio incondicional, pelas risadas e por me aturares nos meus desvaneios pessimistas. Do fundo do meu coração estou-vos eternamente grato.

Focando agora em todas as outras pessoas tão especiais a nível pessoal que, me incentivam todos os dias a seguir os meus sonhos e que sem eles não estaria onde estou hoje.

Não poderia de deixar de agradecer a toda a minha família por todo o apoio e carinho dado durante esta fase, sem vocês nada disto seria possível. De uma forma particular, agradecer aos meus pais, o meu porto de abrigo e por sempre acreditarem em mim e deixarem me sempre perseguir os meus sonhos. O vosso amor e apoio incondicional foram a base de todas as minhas conquistas. A paciência, a compreensão e a força que sempre me transmitiram foram essenciais para que eu pudesse superar os desafios e alcançar meus objetivos. Dedico esta dissertação a vocês, como uma pequena demonstração da minha gratidão por tudo o que fizeram e se privaram de fazer por mim.

Duas das pessoas mais importantes que tenho comigo e que sem eles não sei se conseguiria ultrapassar com sucesso esta etapa e não poderia estar mais do que agradecido por tudo o que fizeram por mim, por isso, obrigado Carlos e Joana. Obrigado por estarem lá, por todas as conversas, por todo o apoio, por me ouvirem sempre mesmo quando não fazia mais nada a não ser incomodar-vos, por serem os melhores amigos e família que podia ter. Estou-vos eternamente grato do fundo do meu coração e nem tenho palavras suficientes para expressar o quão agradecido estou por terem-se mantido sempre ao meu lado durante este ano, significa muito não terem desistido de mim. Não conseguia ter terminado isto sem vocês e apenas mais posso pedir-vos desculpa por tudo o que vos fiz passar.

Por último, a ti Tatiana, nem tenho palavras nem sei como te agradecer. És a melhor pessoa que podia ter ao meu lado a partilhar a minha vida. Apenas te posso agradecer por tudo o teu apoio incondicional a qualquer altura, seres o meu abrigo a que posso chamar casa a qualquer momento, teres o abraço mais aconchegante que me acalma. Não consigo imaginar uma vida sem ti. Desculpa pelo meu feitio, a minha maneira de ser, de ser o maior pessimista que existe á face da terra, de nem sempre ser uma pessoa fácil mas obrigado por seres paciente e me ajudares a ultrapassá-las. Obrigado pelo teu carinho, o teu amor, a tua perseverança em nós, por cresceres comigo desde pequenos e por me ajudares cada dia a tornar-me uma pessoa melhor. Serei eternamente teu.

Para o Ildo e para a Graça

RESUMO

Atualmente, o cancro da bexiga (BICa) é o nono cancro mais incidente a nível mundial. No contexto do BICa, as lesões não músculo-invasivo (NMIBC) de alto risco são frequentemente tratadas através da técnica de ressecção transuretral (TURBT) seguida de instilações vesicais de *Bacillus Calmette-Guérin* (BCG). No entanto, estes doentes apresentam taxas de recidiva elevadas, com cerca de 30-40% a não responderem ou a não tolerarem a terapêutica num período de cinco anos. Assim, as comorbilidades associadas à doença impõem não só uma pressão económica desnecessária sobre os sistemas de saúde, como prognósticos reservados e pior qualidade de vida a estes doentes. Até à data nenhum biomarcador foi clinicamente aprovado para identificar *à priori* os doentes que não irão responder à terapia. Para resolver este problema, a investigação nesta área tem-se centrado na procura de biomarcadores não invasivos e específicos de doença que possam ser obtidos a partir de biópsias líquidas. Neste âmbito surgem os microRNAs, reguladores epigenéticos bem estabelecidos, que têm sido identificados como promotores da tumorigénese, progressão, desenvolvimento de metástases, estando também implicados na resposta a diversas terapêuticas antitumorais. Apesar disto, nenhum biomarcador baseado em microRNAs foi ainda recomendado por quaisquer diretrizes clínicas para orientar a terapia com BCG.

Assim, na Parte I desta dissertação, o objetivo centrou-se em analisar a literatura existente através de uma revisão sistemática para identificar possíveis biomarcadores de prognóstico e/ou preditivos de resposta à BCG em doentes com BICa. A Parte II centrou-se em proceder à validação de uma assinatura de quatro microRNAs descobertos anteriormente no âmbito do Projeto TRANSCAN UCIPredict.

Para atingir os objetivos delineados na Parte I, foi realizada uma pesquisa bibliográfica nas bases de dados PubMed, Scopus e Medline, com vista à recolha de registos relevantes publicados até 31 de Julho de 2024. Após uma triagem minuciosa, foram recolhidas as informações mais pertinentes e foi efetuada uma revisão sistemática de acordo com as diretrizes PRISMA. Paralelamente, na Parte II, realizámos um estudo de validação da assinatura de quatro microRNAs, através da técnica de *droplet digital PCR*, em sedimentos de urina de doentes de BICa tratados com BCG.

A revisão sistemática selecionou 2418 estudos, dos quais 63 foram incluídos na análise final. Destes, 53 investigaram marcadores de prognóstico, cinco focaram-se em marcadores preditivos e sete estudaram marcadores prognósticos e preditivos. As moléculas mais frequentemente estudadas foram o p53, o Ki-67 e o pRB. Vários estudos avaliaram moléculas derivadas de biópsias líquidas, que demonstram o seu poder em

captar melhor a heterogeneidade do tumor, bem como do microambiente tumoral, quando comparadas com as amostras de tecido. Adicionalmente, constatou-se que um número significativo dos biomarcadores estudados estava também relacionado com a imunologia. Relativamente ao estudo de validação, a assinatura de quatro microRNAs, constituída pelos microRNAs hsa-miR-483-3p, hsa-miR-579-3p, hsa-miR-874-5p e hsa-miR-4443, quando combinada em painel, foi capaz de discriminar eficazmente doentes que verdadeiramente responderam à terapia. Quando incorporado num nomograma, o modelo manteve a sua significância com uma sensibilidade de aproximadamente 58% e especificidade de 76%, demonstrando assim a capacidade de distinguir os doentes que responderam à terapia com BCG dos não-respondedores. Adicionalmente, os doentes que apresentavam uma expressão mais elevada de um determinado microRNA demonstraram um aumento significativo do risco de não resposta ao tratamento em comparação aos que apresentavam uma expressão mais baixa. A mesma tendência foi observada no modelo multivariado, ao comparar doentes de alto risco com doentes de baixo risco, com os primeiros a apresentarem um maior risco de não resposta.

Apesar de algumas limitações identificadas na revisão sistemática, tais como questões de reprodutibilidade e exequibilidade dos estudos, encontrámos uma gama diversificada de moléculas com forte potencial como marcadores de prognóstico e/ou preditivos que poderiam melhorar significativamente o tratamento dos doentes com BICa. Na Parte II desta dissertação, ao validar com êxito a assinatura de microRNAs, desenvolvemos um modelo prospetivo para selecionar doentes que melhor irão responder à terapia com BCG. Este modelo mostra-se promissor na melhoria da acuidade de identificação dos doentes, assegurando que aqueles que têm maior probabilidade de beneficiar da terapia são selecionados, e reduzindo assim a administração desnecessária de intervenções dispendiosas e invasivas. Consequentemente, este estudo destaca o papel crucial que a validação de biomarcadores em diversos contextos clínicos desempenha na otimização da terapia BCG para doentes com BICa e no avanço dos cuidados personalizados.

ABSTRACT

Bladder cancer (BlCa) ranks as the 9th most common cancer worldwide. High-risk non-muscle-invasive bladder cancer (NMIBC) is typically treated with *Bacillus Calmette-Guérin* (BCG) instillations following the resection of the bladder tumour through transurethral resection of the bladder tumour (TURBT). However, despite this treatment, high-risk patients still experience high relapse rates, with approximately 30-40% either not responding to or not tolerating the therapy within five years. This leads to increased comorbidities and places an unnecessary economic strain on healthcare systems. Currently, there is no clinically approved biomarker for identifying these non-responding patients in advance. To address this issue, research has focused on exploring non-invasive, disease-specific biomarkers that can be retrieved from liquid biopsies. MicroRNAs are well-established epigenetic regulators that can influence tumorigenesis, progression, metastasis, and response to treatment in BlCa. Nevertheless, no microRNA-based biomarker test has yet been recommended by clinical guidelines for guiding BCG therapy.

Part I of this dissertation aimed to investigate the existing literature through a systematic review to evaluate the promise of predictive and prognostic biomarkers for BCG failure in BlCa patients. Part II focused on validating a four-microRNA signature previously identified in the context of the UCIPredict Project.

To achieve the objectives outlined in Part I, a bibliographic search was conducted on PubMed, Scopus, and Medline databases, retrieving relevant records published up until July 31, 2024. After thorough screening and examination, the most pertinent information was collected, and a systematic review was performed following PRISMA guidelines. At the same time, in Part II, we conducted a validation study of a four-microRNA signature using droplet digital PCR (ddPCR) in urine sediments from BCG-treated BlCa patients.

The systematic review yielded 2418 studies, of which 63 were included in the final analysis. Of these, 53 investigated prognostic markers, five focused on predictive markers, and seven studied both prognostic and predictive markers. The most frequently studied molecules included p53, Ki-67, and pRB. Several studies also examined molecules derived from liquid biopsies, which may better capture the heterogeneity of the tumour and its microenvironment, compared to tissue biopsies. A significant number of the studied biomarkers were related to immunological cells.

Regarding our study, the four-microRNA signature, consisting of hsa-miR-483-3p, hsa-miR-579-3p, hsa-miR-874-5p, and hsa-miR-4443 was able to effectively discriminate true BCG responders from non-responders. When incorporated into a nomogram model, the multivariable model retained statistical significance achieving 58% sensitivity and 76%

specificity values in its ability to distinguish BCG responders from non-responders. Additionally, patients exhibiting higher expression of a specific microRNA within the panel were less responsive to BCG therapy compared to those with lower expression. The same tendency was verified when comparing high-risk and low-risk patients, with the first ones presenting a higher risk of non-response to the treatment.

Despite some limitations identified in the systematic review, such as issues with reproducibility and study feasibility, we found a diverse range of molecules with strong potential as prognostic and/or predictive markers that could significantly improve the clinical management of BICa. In Part II of this dissertation, by successfully validating the microRNA signature, we developed a prospective model for selecting patients prior to BCG therapy. This model shows promise in improving the precision of patient identification, ensuring that those who are more likely to benefit from BCG therapy are accurately selected, thus reducing the unnecessary administration of costly and invasive interventions. Consequently, this study highlights the crucial role that biomarker validation across diverse clinical settings plays in optimising BCG therapy for BICa patients and advancing personalised care.

TABLE OF CONTENTS

GENERAL INTRODUCTION	1
Bladder Cancer	3
Epidemiology of Bladder Cancer	3
Risk Factors for Bladder Cancer	3
Bladder Cancer Staging and Grading.....	5
Bladder Cancer Diagnosis	6
Biomarkers through Liquid Biopsies	7
Current Treatment Options.....	8
Limitations in BlCa Management.....	10
Epigenetics	10
Epigenetic Alterations.....	10
Non-Coding RNAs	12
MicroRNAs in Bladder Cancer	14
MicroRNAs as Cancer Biomarkers.....	14
AIMS.....	23
PART I	27
Predictive and prognostic biomarkers of BCG failure in bladder cancer patients:	
A systematic review.....	29
Abstract.....	31
Introduction	31
Methods	33
Results	35
Discussion	53
Conclusion.....	54
PART II	65
Predicting BCG Failure in Bladder Cancer Patients: a microRNA-Driven	
Biomarker Approach.....	67
Abstract.....	69
Introduction	69

Methods	70
Results	74
Discussion	78
Conclusion.....	80
GENERAL DISCUSSION	85
CONCLUSIONS AND FUTURE PERSPECTIVES.....	95
APPENDIX	99

INDEX OF FIGURES

General Introduction

Figure 1: Incidence of Bladder Cancer worldwide in 2022. In 2022, BICa was reported as the ninth most incident worldwide with 614,000 new cases. In Portugal, was the thirteen most incident one with around 472,000 new cases. Retrieved from Global Cancer Statistics 2022 (1). 3

Figure 2: Main risk factors responsible for the development of Bladder Cancer. Created with BioRender.com. 4

Figure 3: Graphical representation of bladder wall layers and cancer staging under the TMN system. The NMIBC comprises Ta, T1 and Cis and accounts for 70-80% of the cases. The MIBC includes T2, T3 and T4 and represents about 25% of the diagnosed cases. 6

Figure 4: Therapeutic Scheme for NMIBC patients. Following a complete TURBT and the delivery of a single post-operative chemotherapy instillation, all patients with NMIBC should be placed under active surveillance. The decision regarding further treatment will be based on the stratification of risk groups. Created with BioRender.com. 9

Figure 5: Epigenetic Alterations. The aberrant alterations in the epigenetic mechanisms, including DNA methylation, modifications to histones (methylation and acetylation), and non-coding RNAs, result in the activation of oncogenes and/or the suppression of tumour suppressor genes, which impacts critical signalling pathways for maintaining, repairing, and regulating cellular balance. Created with BioRender.com. 11

Figure 6: Canonical microRNAs Biogenesis. miRNAs are transcribed as pri-miRNAs by RNA polymerase II. Following processing by the Drosha/DGCR8 complex, pre-miRNAs are exported to the cytoplasm by Exportin 5 (XPO5). Mature miRNAs are produced by Dicer and TRBP and loaded into the RISC. miRNAs function through degrading mRNA, repressing translation or even increasing translation to regulate cancer. Created with BioRender.com. 13

Part I

Figure 1: Flowchart of the literature selection process for the included records, according to the PRISMA guidelines. 36

Figure 2: Quality assessment of the included prognosis studies using the QUAPAS-2 tool. (A) Graphical illustration depicting the risk of bias in the prognosis studies.

(B) Graphical illustration depicting the risk of concerns regarding the applicability of the prognosis studies.....50

Figure 3: Quality assessment of the included predictive studies using the PROBAST tool. (A) Graphical illustration depicting the risk of bias in the predictive studies. (B) Graphical illustration depicting the risk of concerns regarding applicability in the predictive studies. 51

Figure 4: Distribution of prognostic and predictive biomarker studies for BCG of failure by source and molecules. Created with BioRender.com.....52

Part II

Figure 1: Variation of microRNA levels at the beginning and end of BCG treatment in both treatment response groups. ns: non-significant, Ti: initial timepoint, Tf: final timepoint. 74

Figure 2: hsa-miR-483-3p, hsa-miR-579-3p, has-miR-874-5p and miR-4443 levels in BCG treatment patients. The microRNA levels in the urine samples from the 79 NMIBC patients were distributed according to their response status: Responders (n=42) and Failure (n=37). 75

Figure 3: Performance and Survival Analysis of the Panel. (A) ROC Curve evaluating the performance of the Panel. (B) Kaplan-Meier Survival Curve for DFS based on the hsa-miR-483-3p/hsa-miR-579-3p/hsa-miR-4443 levels.....76

Figure 4: (A) Nomogram representative of the Risk of the Model, with the relevant clinicopathological variables: age at diagnosis (0: ≤68, 1: >68 years), smoke status (0: non-smoker, 1: active smoker, 2: former smoker), previous treatment (0: no previous treatment, 1: Mitomycin C, 2: BCG) and grade (1: low grade, 2: high grade). **(B) ROC curve to evaluate the performance of the nomogram model.** Panel categorisation- 0: high levels, 1: low levels. 77

Figure 5: Kaplan-Meier Survival Curve for DFS based on patient stratification risk model. FU: follow-up. 78

Appendix

Part I

Figure S1: Venn Diagram of the studied biomarker molecules by source. Created with BioRender.com. 104

Part II

Figure S1: Variation of microRNA levels at the beginning and end of BCG treatment in both treatment response groups. ns: non-significant, Ti: initial timepoint, Tf: final timepoint.....	104
Figure S2: Respective Receiver Operator Curves (ROC) from each individual microRNA.....	105
Figure S3: Performance and Survival Analysis of the Panel. (A) ROC Curve evaluating the performance of the Panel. (B) Kaplan-Meier Survival Curve for DFS based on the levels of the Panel microRNAs.....	106
Figure S4: Performance and Survival Analysis of the Panel. (A) ROC Curve evaluating the performance of the Panel. (B) Kaplan-Meier Survival Curve for DFS based on the expression levels of the Panel microRNAs.....	106
Figure S5: (A) Nomogram representative of the Risk Model, with the relevant clinicopathological variables: age at diagnosis (0: ≤ 68 , 1: > 68 years), smoke status (0: non-smoker, 1: active smoker, 2: former smoker), previous treatment (0: no previous treatment, 1: Mitomycin C, 2: BCG) and grade (1: low grade, 2: high grade). (B) ROC curve to evaluate the performance of the nomogram model. Panel categorisation - 0: high levels, 1: low levels.	107
Figure S6: (A) Nomogram representative of the Risk of the Model, with the relevant clinicopathological variables: age at diagnosis (0: ≤ 68 , 1: > 68 years), smoke status (0: non-smoker, 1: active smoker, 2: former smoker), previous treatment (0: no previous treatment, 1: Mitomycin C, 2: BCG) and grade (1: low grade, 2: high grade). (B) ROC curve to evaluate the performance of the nomogram model. Panel categorisation - 0: high levels, 1: low levels.	108

INDEX OF TABLES

Part I

Table 1: Information regarding Prognosis on Studies in bladder cancer patients treated with <i>Bacillus Calmette-Guérin</i>	37
---	----

Table 2: Information regarding Predictive Studies in bladder cancer patients treated with <i>Bacillus Calmette-Guérin</i>	47
---	----

Part II

Table 1: Demographic and clinicopathological features of the NMIBC patients in the retrospective cohort.....	71
--	----

Table 2: Panel performance in distinguishing BCG response in BICa Patients. .	76
---	----

Table 3: Summary of the logistic model representative of the Risk Model.	76
---	----

Table 4: Model Performance combining the different microRNAs and clinicopathological variables of interest in distinguishing BCG response in BICa Patients.....	78
---	----

Appendix

Part I

Table S1: Quality Assessment of each included study through Quality Assessment of Prognosis Accuracy Studies 2 (QUAPAS-2).	ci
---	----

Table S2: Quality Assessment of each included study through Prediction model Risk Of Bias Assessment (PROBAST).	ciii
--	------

Part II

Table S1: Summary of the relevant parameters of the tested Panels.....	105
--	-----

Table S2: Performance of the panels combining the different microRNAs in distinguishing BCG response in BICa Patients.	106
---	-----

Table S3: Summary of the logistic model representative of the Risk Model.....	107
---	-----

Table S4: Summary of the logistic model representative of the Risk Model.....	108
---	-----

Table S5: Performance of the models combining the different microRNAs and clinicopathological variables of interest in distinguishing BCG response in BICa Patients.....	109
--	-----

LIST OF ABBREVIATIONS

3

3'-UTR – 3 Untranslated Region

5

5'-UTR – 5 Untranslated Region

A

Ago – Argonaute

AUA – American Urology Association

AUC – Area under the curve

B

BCG - *Bacillus Calmette-Guérin*

BICa – Bladder Cancer

C

cDNA – complementary Deoxyribonucleic Acid

CDS – Coding Sequences

CE – Conformité Européene

CI – Confidence Interval

circRNAs – circular Ribonucleic Acids

CIS – Carcinoma *in situ*

CSS – Cancer-Specific Survival

CT – Computed Tomography

CTLA-4 – Cytotoxic T-lymphocyte associated protein 4

D

ddPCR – droplet digital Polymerase Chain Reaction

DFS – Disease-Free Survival

DGCR8 – DiGeorge Syndrome Critical Region 8

DNA – Deoxyribonucleic Acid

DSS – Disease-Specific Survival

E

EAU – European Association of Urology

EFS – Event-Free Survival

Exp5 – Exportin 5

F

FDA – Food and Drug Administration

FFPE – Formalin-Fixed Paraffin-Embedded

FISH – Fluorescence *in situ* Hybridisation

FN – False Negative

FP – False Positive

G

G1 – Grade 1

G2 – Grade 2

G3 – Grade 3

H

HG – High Grade

HR – Hazard Ratio

I

iBCG – induction *Bacillus Calmette-Guérin* scheme

ICIs – Immune Checkpoint Inhibitors

ISUP – International Society of Urological Pathology

IVU – Intravenous Urography

L

LG – Low Grade

lncRNAs – long non-coding Ribonucleic Acids

LoB – Limit of Blank

LoD – Limit of Detection

M

mBCG – maintenance *Bacillus Calmette-Guérin* scheme

MIBC – Muscle Invasive Bladder Cancer

miRNAs – micro-ribonucleic Acids

MitC – Mitomycin C

MMC – Mitomycin C

mos - months

MRI – Magnetic Resonance Imaging

mRNA – messenger Ribonucleic Acid

MVAC – methotrexate, vinblastine, adriamycin, cisplatin

N

NAC – Neoadjuvant Chemotherapy

ncRNAs – non-coding Ribonucleic Acids

NMIBC – Non-Muscle Invasive Bladder Cancer

NMP-22 – Nuclear Matrix Protein 22

NPV – Negative Predictive Value

O

OR – Odds Ratio

OS – Overall Survival

P

PBL – Peripheral Blood Leukocyte

PBS – Phosphate-Buffered Saline

PCa – Prostate Cancer

PD-1 – Programmed Cell Death Protein 1

PFS – Progression-Free Survival

piRNAs – PIWI-interacting Ribonucleic Acids

PPV – Positive Predictive Value

PRISMA – Preferred Reporting Items for Systematic Reviews and Meta-Analyses

PROBAST – Prediction Model Risk of Bias Assessment

PROSPERO – International Prospective Register of Systematic Reviews

PUNLMP – Papillary Urothelial Neoplasm of Low Malignant Potential

Q

QUAPAS – Quality Assessment of Prognostic Accuracy Studies

R

RC – Radical Cystectomy

RCa – Renal Cancer

RFS – Recurrence-Free Survival

RISC – Ribonucleic Acid-induced silencing complex

RNA – Ribonucleic Acid

ROC – Receiver Operating Characteristics

S

SE – Standard Error

SN – Sensitivity

SP – Specificity

T

Tis – Tumour *in situ*

TN – True Negative

TNM – Tumour-Node-Metastasis

TP – True Positive

TURBT – Transurethral Resection of the
Bladder Tumour

U

UCC – Urothelial Cell Carcinoma

UTI – Urinary Tract Infection

Y

yr – year

W

WHO – World Health Organisation

GENERAL INTRODUCTION

Bladder Cancer

Epidemiology of Bladder Cancer

In 2022, Bladder Cancer (BICa) was reported as the ninth most commonly diagnosed cancer worldwide, with approximately 614,000 new cases (Figure 1), and the thirteenth leading cause of death with nearly 220,000 deaths (1). Moreover, BICa presents a higher incidence rate in European countries and North America than in the countries from other geographies. Conversely, countries in Africa show the lowest incidence (1). Better diagnosis methods are more easily accessible in developed countries, which may explain these differences (2). In addition, lower exposure to some specific risk factors may also be a contributing factor (1). Particularly, in Portugal, BICa was the thirteen most incident cancer with 3,517 newly diagnosed cases in 2022 (Figure 1) (1). For men, BICa constitutes the sixth most frequent cancer, being four times more prevalent than in females (1). Around 80% of all cases are detected in individuals of older age, with a mean age of diagnosis of approximately 70 years old (3, 4).

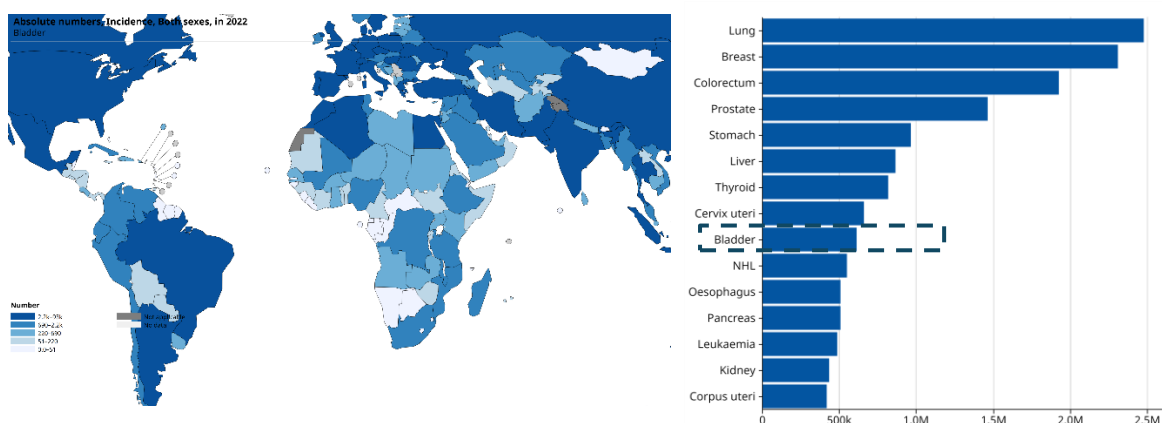


Figure 1: Incidence of Bladder Cancer worldwide in 2022. In 2022, BICa was reported as the ninth most incident worldwide with 614,000 new cases. In Portugal, was the thirteen most incident one with around 472,000 new cases. Retrieved from Global Cancer Statistics 2022 (1).

Risk Factors for Bladder Cancer

Several risk factors can increase the probability of developing BICa (Figure 2). Tobacco smoking is widely acknowledged as the most significant risk factor for this malignancy, responsible for around 50% of all cases (5). Indeed, smokers are two to three times more likely to develop BICa at some point, when compared with non-smokers (6). The second most significant risk factor is occupational carcinogen exposure, responsible for 5-10% of all cases. Those who work with dyes, rubber, paint, leather, and metals, in the oil industry or as hairdressers are at a higher risk due to exposure to certain compounds, mainly

aromatic amines (5). Moreover, pathogen infections, such as *Schistosoma*, are directly associated with the prevalence of squamous cell carcinoma, a subtype of BICa, which varies significantly worldwide (7). This infection is prevalent in certain regions of Africa and the Middle East and is a major contributing factor to the increased development of BICa (8). As mentioned before, age is also identified as a risk factor, since older individuals are typically diagnosed at a more advanced stage, indicating a considerable delay between the cancer's initial development and its diagnosis (6). There is also a correlation between gender and the likelihood of developing BICa, as it has been observed a difference in BICa incidence between males and females. Furthermore, it is also commonly acknowledged that smoking tobacco is different between genders, being more prevalent among men than women (4). Moreover, certain enzymes, differentially expressed between genders, have been identified as responsible for breaking down aromatic amines and detoxifying foreign substances linked to the metabolism of carcinogens leading to BICa (8). It should be noted that differences in sex hormone production can contribute to gender-based variations in the development of BICa (7). Moreover, the male genome is characterised by the presence of a single X and a single Y chromosome. A frequent genetic alteration observed in BICa is the loss of the Y chromosome, which has been correlated with an elevated risk of cancer-related mortality (3). Importantly, the occurrence of other chronic or benign pathological conditions, such as urinary tract infections (UTI), and bladder or kidney stones, also contribute to a higher probability of developing BICa (5). Other different factors, although not so frequent, can also increase the possibility of developing BICa, including certain dietary patterns, family history, alcohol consumption, localised pelvic radiotherapy, and diabetes. However, some of these factors may be disputed or not universally accepted (7, 9).

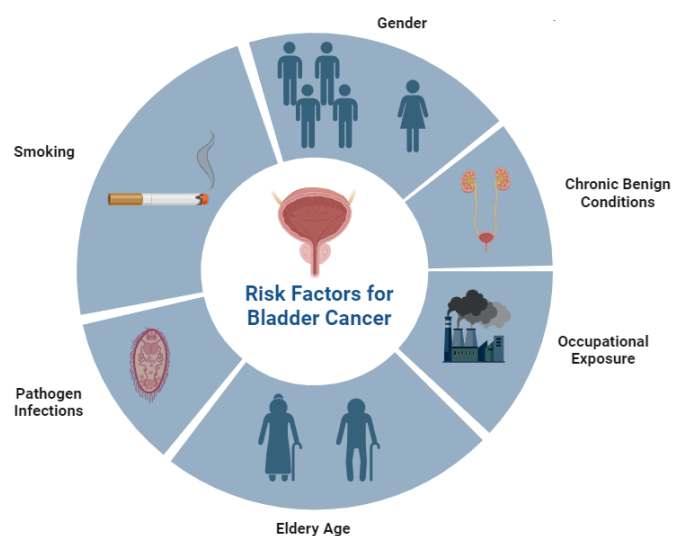


Figure 2: Main risk factors responsible for the development of Bladder Cancer. Created with BioRender.com.

Bladder Cancer Staging and Grading

BICa usually develops in the inner lining of the urinary tract, known as urothelium, and it is typically referred to as urothelial cell carcinoma (UCC) which accounts for about 90% of all cases (10). Squamous cell carcinoma, small cell carcinoma, adenocarcinoma and sarcoma are the less common types of BICa subtypes (10). The bladder wall consists of four primary layers: urothelium, lamina propria, detrusor muscle and adventitia (11, 12). The tumour-node-metastasis (TNM) system is used to determine the pathological stage. TNM stands for tumour size (T), lymph node involvement (N) and distant metastasis (M). This system can classify tumours from Ta to T4 based on the extent of bladder wall invasion (Figure 3) (13). Considering the tumour stage, *carcinoma in situ* (CIS) represents flat non-invasive neoplasia that is confined to the urothelium, Ta describes a non-invasive papillary tumour that is also narrowed in the urothelium, T1 shows a tumour that invades the lamina propria, T2 corresponds to tumour invading the muscle tiers, T3 comprises tumours capable of invade the perivesical tissue and T4 are malignancies that migrate into adjacent organs (13). The most common organs where BICa usually metastasises are bone, lung, liver, lymph nodes and the peritoneum (14).

Bladder cancer can be clinically divided into two primary subgroups, namely non-muscle invasive bladder cancer (NMIBC) and muscle invasive bladder cancer (MIBC). These subgroups have distinct prognoses and require different types of treatments as they originate from different molecular pathways, which leads to differentiated patient management (15, 16). NMIBC, which includes Ta, T1 and Cis, represents the majority of diagnosed tumours, accounting for about 70-80% of all cases, and usually do not represent a survival threat. However, about 50% to 70% of these tumours may recur after treatment (Figure 3). Moreover, 10-20% of patients with NMIBC may progress to MIBC (9, 17). The former group of tumours, comprising T2, T3 and T4, represents about 25% all diagnosed cases, and consist of clinically more aggressive cases that can rapidly progress and metastasize, being also responsible for the most BICa-related deaths (Figure 3) (9, 16, 18). The process of histological classification holds paramount importance in determining the optimal course of treatment for neoplasms of this type. In 1973, the World Health Organisation (WHO) and the International Society of Urological Pathology (ISUP) classified the tumours as grade 1 (G1) for well-differentiated tumours, grade 2 (G2) for moderately differentiated neoplasms and grade 3 (G3) for poorly differentiated lesions (9). More recently and according to the updated classification system of the WHO/ISUP 2004/2016, these neoplasms were categorised as Papillary Urothelial Neoplasm of Low Malignant Potential (PUNLMP), Low-Grade papillary carcinoma (LG), and High-Grade carcinoma (HG) based on the tumour's morphological features and cellular atypia (9, 19). Nonetheless, CIS is

always considered a high-grade tumour, even though it is a flat non-invasive lesion, due to its higher capability to spread to other bladder wall sections. Ta and T1 tumours can be either LG or HG, while T2, T3 and T4 are always designated as HG tumours (9).

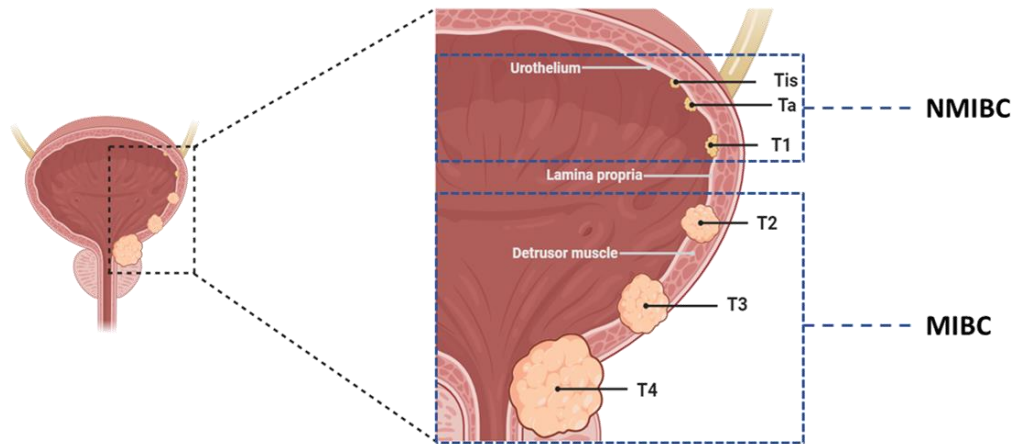


Figure 3: Graphical representation of bladder wall layers and cancer staging under the TMN system. The NMIBC comprises Ta, T1 and Cis and accounts for 70-80% of the cases. The MIBC includes T2, T3 and T4 and represents about 25% of the diagnosed cases.

Bladder Cancer Diagnosis

The presence of blood in the urine – Haematuria – is the most common symptom of BICa, being found in about 80% of BICa patients (20, 21). This can be microscopic or gross haematuria and can establish the type of lesion, with visible haematuria usually associated with more advanced higher stages of the disease (12). Other less frequent indicators of the disease include irritative symptoms, such as dysuria and/or polyuria, and increased frequency/urgency to urinate; and/or constitutional symptoms, such as fatigue, weight loss, and a pelvic mass (12, 20). Individuals with this kind of presentation, usually are required to be submitted for further evaluation.

There are several methods used to diagnose BICa, such as imaging techniques like ultrasound, which is the most used modality for primary assessment, intravenous urography (IVU), computed tomography (CT) used to understand the extent of tumour invasion, and multiparametric magnetic resonance imaging (MRI), capable of diagnose and stage tumours, as well as cystoscopy and biopsy procedures (12, 21). Nonetheless, the current standard assessment for BICa diagnosis consists of urinary cytology and cystoscopy/biopsy evaluation of the bladder (also used for disease monitoring) and histological evaluation of abnormal lesions acquired either with cold-cup biopsy or transurethral resection of the bladder tumour (TURBT) (9, 20). Urine cytology is used for BICa diagnosis as it can detect the aberrant morphology of the malignant cells in the urine. However, the results can be

influenced by an unsatisfactory number of exfoliated cells, UTIs, bladder stones or infusion therapy (21). On the same note, cystoscopy is a procedure that allows visualisation of the interior of the bladder; however, it can be a very invasive and costly technique and comes with potential risks such as bleeding, pain, urothelial injuries and UTIs (22, 23).

To perform an early and accurate diagnosis of BICa, several molecular urine-based tumour markers have been developed based on different targets, such as the nuclear matrix protein 22 (NMP22), bladder antigen tests, like BTA-STAT and BTTA-TRAK, immunoassay urinalysis, UroVysion, which is based on fluorescence in situ hybridisation (FISH) (21, 24), and Bladder EpiCheck, which detects specific methylation signatures of urothelial malignant cells (25). However, most of these markers have not been successfully implemented in current routine practice, mainly because of their low specificity (21). For that reason and until improvements of better molecular assays, urine cytology and cystoscopy followed by histological evaluation will remain the best and standard procedure for diagnosing and monitoring BICa (21).

Biomarkers through Liquid Biopsies

The diagnosis and management of BICa can impose a significant and stressful physical and psychological burden on the patient's life, as they often undergo multiple invasive biopsies to monitor the malignancy, significantly impacting their quality of life (26). Liquid biopsies offer the potential to replace invasive procedures by extracting tumour-derived material from bodily fluids such as blood, urine, saliva, ascites, cerebrospinal fluid, and pleural fluid. This minimal invasive approach can provide biomarkers for various clinical purposes, including early diagnosis, monitoring, prognosis, and predictive assessments (27, 28). These biomarkers are highly valuable due to their stability in bodily fluids and the availability of relatively cost-effective detection methods (29). The use of liquid biopsies can reduce patient morbidity by enabling more precise and frequent monitoring of neoplasia, helping to prevent unnecessary treatments that offer no benefit (30).

Urine is a commonly used biofluid in BICa biomarker tests and has proven to be a highly advantageous option for diagnostic and prognostic monitoring. Unlike blood, urine is fully accessible, collected non-invasively without discomfort for the patient, and easily processed (29). Furthermore, due to its proximity to the tumour and direct contact with the tumour microenvironment, urine is enriched with biomarkers, making it especially valuable in urological cancers like BICa (31). Both voided urine and its fractions (supernatant and sediment) have been established as viable liquid biopsy sources in BICa; nonetheless, the most suitable option may depend on the specific biomarker of interest (32).

Despite their potential, liquid biopsies have some limitations. Factors such as hydration status and medication levels can cause fluctuations that affect test reliability. Additionally, many biomarker tests lack the specificity needed for clinical use, leading to false positives, often due to benign conditions such as inflammation, UTIs, cystitis, or trauma. Another challenge, particularly for RNA-based biomarkers, is its relatively low abundance in urine, making extraction more difficult (33).

While several FDA-approved and commercially available markers exist for diagnosing and monitoring BICa, most of them have not been recommended for routine clinical practice by any clinical guidelines. Moreover, there are still no approved markers for the assessment of the prognosis of the disease (24).

Current Treatment Options

The TURBT procedure for NMIBC aims to completely remove all the visible malignant lesions, constituting the standard of care for this group of tumours, except for CIS (9, 34). Therefore, the quality of the TURBT specimen plays a critical role in determining the recurrence and/or progression of the disease (35). Following TURBT, adjuvant therapy is crucial for complete ablation of possible residual disease (9). In this regard, a single postoperative intravesical instillation is typically performed within 24 hours using a chemotherapy agent, such as Mitomycin C (MMC), epirubicin or pirarubicin (9). MMC is an alkylating agent responsible for causing DNA damage, it crosslinks both single or double-strand breaks, inhibiting DNA replication and, therefore, transcription (36). It is proven that post-TURBT intravesical instillation with such agents significantly reduces the risk of recurrence (37).

The NMIBC 2021 scoring model, as per the European Association of Urology (EAU), combines two classification systems, WHO 2004/2016 and WHO 1973, and divides patients into four distinct risk groups: low, intermediate, high, and very high-risk (9). Active surveillance is typically recommended as the best treatment option after TURBT for low-risk NMIBC patients, as they usually do not benefit from any adjuvant therapy (38). Intermediate-risk patients, on the other hand, are usually treated with either adjuvant intravesical chemotherapy with MMC for one-year (39), or intravesical immunotherapy *Bacillus Calmette-Guérin* (BCG) instillations for one to two years of treatment in some cases, particularly if chemotherapy was previously administered. BCG, an immunotherapy agent, is usually directly applied to the bladder lining. This treatment stimulates the immune system to attack and destroy cancer cells in the bladder. While BCG instillations are known to be more effective in preventing recurrences in BICa patients, they also come with greater

side effects (40). Moreover, BCG instillations are used as a maintenance treatment for high and very high-risk patients for up to three years to reduce the risk of relapse. Nevertheless, research shows that about 60% of patients do not respond to or tolerate the treatment within 5 years, leading to overtreatment and worse prognosis (41). In such cases, performing a Radical Cystectomy (RC) is the standard and recommended course of treatment (Figure 4) (9).

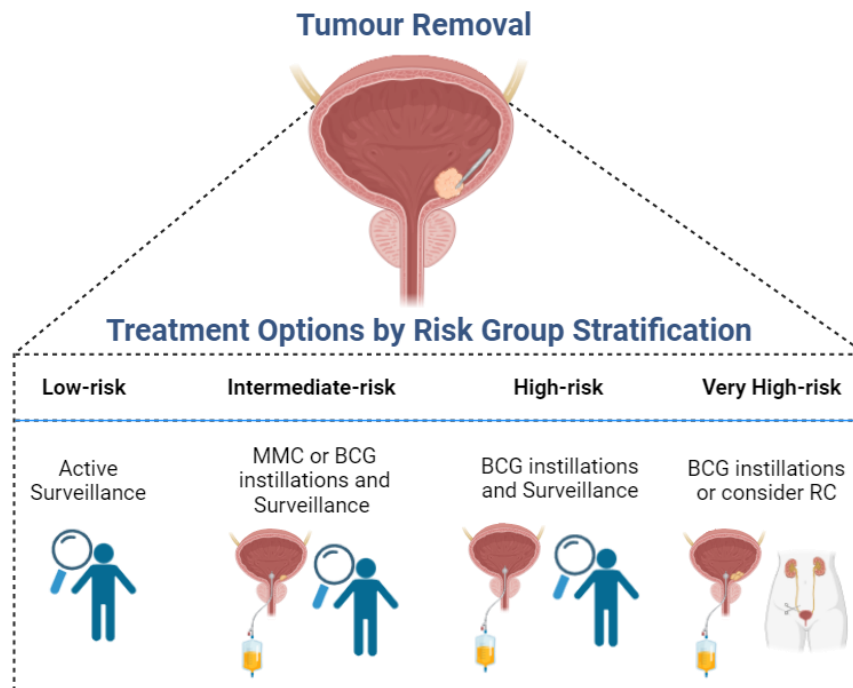


Figure 4: Therapeutic Scheme for NMIBC patients. Following a complete TURBT and the delivery of a single post-operative chemotherapy instillation, all patients with NMIBC should be placed under active surveillance. The decision regarding further treatment will be based on the stratification of risk groups. Created with BioRender.com.

Concerning MIBC tumours (T2-T4), both for primary and progression disease, the standard approach is the performance of an RC. Radical cystectomy includes the removal of the whole bladder and the nearby regional lymph nodes (42). In some cases, neoadjuvant chemotherapy (NAC) with a cisplatin-based combination, such as gemcitabine and cisplatin or MVAC (methotrexate, vinblastine, adriamycin, cisplatin) (43), is recommended before cystectomy for patients with non-metastatic or locally advanced BICa. This helps to reduce the tumour size and improve overall survival (OS) and cancer-specific survival (CSS) (44). Recently, immunotherapy with immune checkpoint inhibitors (ICIs), such as cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1), is being tested alone or in combination with chemotherapy in multiple ongoing clinical trials for individuals who recur after treatment and possess metastatic disease at diagnosis (45). For these patients, a palliative-intend treatment based on cisplatin chemotherapy is usually

the first line of treatment, followed by approved ICIs combinations, such as pembrolizumab, atezolizumab or nivolumab, as a second line of treatment for resistant patients or cisplatin-illegible individuals (46, 47).

Limitations in BICa Management

Regrettably, various issues can be pinpointed concerning the management of BICa. As mentioned, cystoscopy and cytology are the gold standard course of treatment for diagnosis and surveillance of BICa, however, they hamper some disadvantages. For instance, cystoscopy is a highly invasive and expensive procedure, since it requires instrumentalisation, which can cause comorbidities such as haematuria, frequent painful urine voiding and urine infections (24, 48). Moreover, it is an operator-dependent technique that may not detect smaller flat tumours, such as Ta and Cis (48). Urine cytology is used as a complement to cystoscopy, and it is still very useful, especially for detecting Cis, due to its high sensitivity for detecting high-grade tumours. However, the ability of the test to detect low-grade tumours is limited, with sensitivity ranging from 4% to 31%. Indeed, urine cytology has a 12% false-positive rate, indicating that it is ineffective at distinguishing patients with inflammation, atypical urothelial cells, or a history of other cancer treatments (24, 49). Therefore, both procedures may lead to misdiagnosed and, consequently, untreated Ta and Cis tumours, which may cause disease progression (50). Nevertheless, cystoscopy and urine cytology have been routinely performed in BICa patients as part of their surveillance since no other test has been yet approved to assess response to treatment through different timepoints of follow-up. Moreover, as the current follow-up scheme involves costly resources, high financial pressure is being put on the health institutions, making the management of BICa one of the most lifetime expenses per patient among all cancers (51). Hence, it is of the utmost urgency to discover non-invasive biomarkers for identifying patients who truly may benefit from current treatment options, substantially decreasing the cost of treatment and the invasive procedures performed during the patient's follow-up schemes, ultimately ensuring a better quality of patient care.

Epigenetics

Epigenetic Alterations

Epigenetics was first described in 1942 by the embryologist Conrad Waddington. He introduced the term “epigenetics” into modern biology and defined it as the “entire complex of developmental processes” in which the genotype's genes produce the phenotype's

effects (52). Since then, this definition suffered an evolution, and nowadays the term Epigenetics refers to a reversible event, less focused on genotype, and more related to mechanisms that control and regulate gene expression without entailing alterations in the primary DNA sequence (53). The significant role of epigenetics in embryonic development has been firmly established for several years since epigenetic mechanisms are responsible for regulating essential cell functions, which are crucial for differentiation and organogenesis (54). Additionally, in normal cells, it has been established that epigenetics plays important roles in chromatin organisation, inactivation of X-chromosome-associated genes and imprinting (55). Therefore, and for all the reasons mentioned above, in the latest revision of the Hallmarks of Cancer, Hannah D. proposed the inclusion of “Nonmutational Epigenetic Reprogramming” as one of the key biological pathways that lead to cancer development (54). Indeed, epigenetic molecular alterations might occur through several mechanisms, which comprise DNA Methylation, Histone Modifications, chromatin remodelling complexes and non-coding RNAs, as demonstrated in Figure 5 (56).

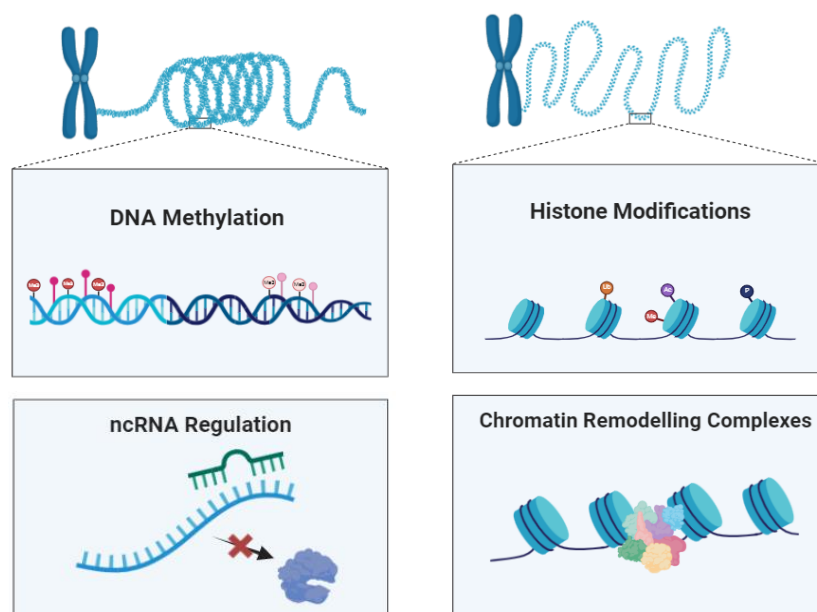


Figure 5: Epigenetic Alterations. The aberrant alterations in the epigenetic mechanisms, including DNA methylation, modifications to histones (methylation and acetylation), and non-coding RNAs, result in the activation of oncogenes and/or the suppression of tumour suppressor genes, which impacts critical signalling pathways for maintaining, repairing, and regulating cellular balance. Created with BioRender.com.

DNA methylation is one of the most extensively studied and stable epigenetic modifications in cancer. However, recently, attention has shifted towards non-coding RNAs, which are emerging as valuable tools for exploration. Similar to DNA methylation, non-coding RNAs are highly stable, but they play a particularly crucial role as regulators at the post-transcriptional level. They are known to directly influence gene expression and interact

with other epigenetic mechanisms, forming complex regulatory networks with various molecules (57, 58). Furthermore, their unique properties make them promising candidates as drug targets in a wide range of diseases.

Non-Coding RNAs

Extensive research and sequencing have shown that approximately 75% of the human genome is transcribed into messenger RNA, yet only about 3% encodes protein-coding genes, with the majority ultimately transcribed as non-coding RNAs (ncRNAs) (59). These ncRNAs can be categorised into different classes according to their length, shape and location. The four major ncRNA types are microRNAs (miRNAs), long ncRNAs (lncRNAs), circular RNAs (circRNAs) and PIWI-interacting RNAs (piRNAs), each of them having different functions in cancer (59). miRNAs are characterised as small sequences of 17-25 nucleotides that interact with mRNAs' 3'untranslated region (3'-UTRs), leading to target degradation or translation inhibition (60, 61). Recently, miRNAs have been found to target not only the 3'-UTR but also other regions, such as the coding sequences (CDS) or 5'-UTR (61). A lncRNA is a highly diverse and extensive category of ncRNAs characterised by transcripts greater than 200 nucleotides in length. These transcripts do not encode proteins but play crucial roles in interacting with other RNA molecules, such as mRNAs, circRNAs and miRNAs (61, 62). Contrarily, circRNAs are not linear RNA molecules; instead, they consist of a single circular RNA strand that is covalently connected at the 3' and 5' ends. This structure makes circRNAs less susceptible to the activity of exonucleases and more stable compared to linear RNAs (61-63). piRNAs play pivotal roles in gene regulation and genome stability, with significant functions in the suppression of transposable elements and epigenetic regulation. Furthermore, they exhibit the capacity to interact with other non-coding RNAs (64). miRNAs have emerged as promising candidates for clinical application due to their inherent stability in biological fluids and the availability of relatively inexpensive and straightforward detection methods. These characteristics render miRNAs attractive as non-invasive liquid biopsy biomarkers. Recent research has highlighted the potential of miRNAs as diagnostic and prognostic indicators for various cancers, including BICa (65, 66).

As shown in Figure 6, canonical miRNAs are transcribed as long primary transcripts by RNA polymerase II in the nucleus. Multiple miRNAs can be transcribed as clusters on polycistronic transcripts, which are subsequently processed into individual miRNAs. This process generates immature miRNAs, known as pri-miRNAs, which undergo enzymatic maturation by the microprocessor complex, consisting of the Drosha endonuclease and the

double-stranded RNA binding protein DGCR8. This complex cleaves the long precursor transcript into a shorter, single hairpin structure called pre-miRNA. Following nuclear processing, the pre-miRNA is transported to the cytoplasm by Exportin 5 (Exp5). Once in the cytoplasm, Dicer further cleaves the pre-miRNA into a mature miRNA molecule. The mature double-stranded miRNA is then loaded onto an Argonaute (Ago) protein, forming the RNA-induced silencing complex (RISC). Within RISC, one strand of the miRNA duplex is selected as the guide strand, while the passenger strand dissociates. After dissociation from Dicer, the AGO protein-guide strand complex binds to complementary sequences in the 3' UTR of target mRNAs. (67, 68). As a result, mRNA undergoes decay, leading to inhibition of translation and subsequent reduction in protein expression levels. Moreover, recent findings suggest that miRNAs may potentially bind to the 5'UTR of target genes, thereby enhancing translation, or to the CDS of target genes, promoting either miRNA degradation or translation repression (61, 62).

This particular class of ncRNAs is considered promiscuous due to the well-established fact that individual miRNAs can target multiple mRNAs, while conversely, a single mRNA can be targeted by hundreds of different miRNAs (62, 69). MicroRNAs undoubtedly play critical roles in numerous biological processes, including cell proliferation, differentiation, embryonic development, and apoptosis (62, 70).

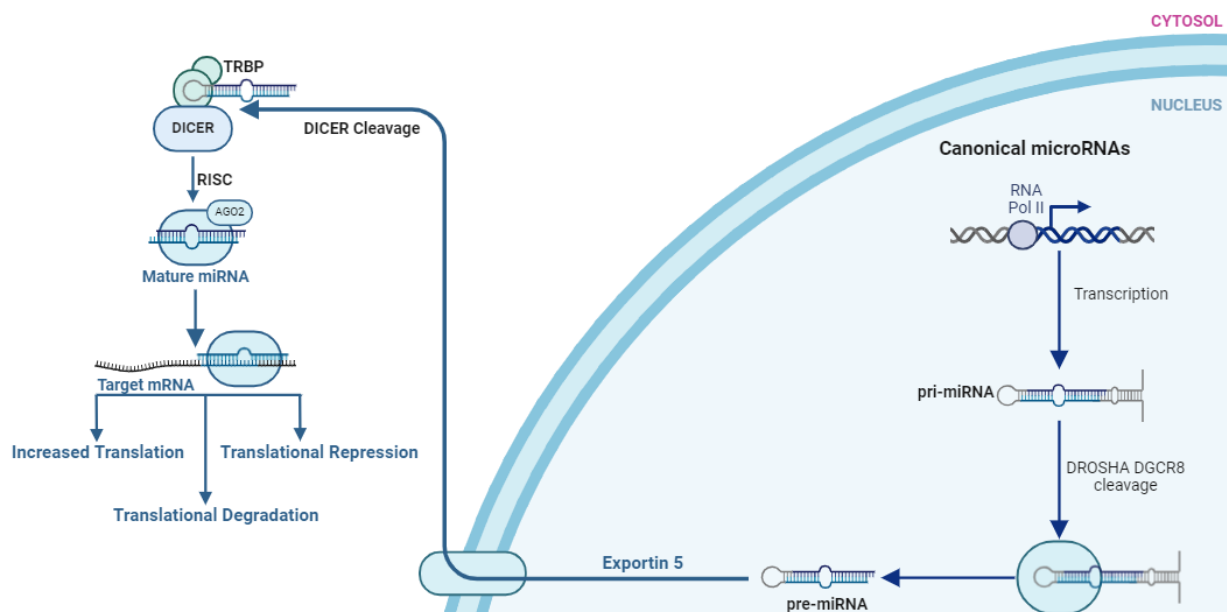


Figure 6: Canonical microRNAs Biogenesis. miRNAs are transcribed as pri-miRNAs by RNA polymerase II. Following processing by the Drosha/DGCR8 complex, pre-miRNAs are exported to the cytoplasm by Exportin 5 (XPO5). Mature miRNAs are produced by Dicer and TRBP and loaded into the RISC. miRNAs function through degrading mRNA, repressing translation or even increasing translation to regulate cancer. Created with BioRender.com.

MicroRNAs in Bladder Cancer

MiRNAs are recognised as significant and well-established factors influencing tumorigenesis, progression, metastasis, and treatment response of several cancers including bladder carcinomas. Their dysregulation is frequently associated with various aspects of BICa development. Numerous studies have demonstrated that miRNA levels correlate with different stages and grades of BICa, reflecting the tumour's heterogeneity. Furthermore, the aberrant expression of miRNAs can act as either oncogenic or tumour-suppressive, being upregulated or downregulated in various BICa-related signalling pathways. For instance, miR-29c exerts its tumour-suppressive function by targeting CDK6, thereby inhibiting cell proliferation, migration, and inducing G1 phase arrest. Additionally, miR-203 has been implicated in enhancing sensitivity to cisplatin by promoting apoptosis through the downregulation of Bcl2. Moreover, miR-203 can act as a tumour suppressor miRNA by negatively targeting Twist1, a transcription factor involved in BICa progression (70, 71). These expression patterns have been extensively characterised in clinical specimens such as blood, urine, and tissue. Consequently, profiling miRNA expression is emerging as a promising approach for non-invasive diagnosis, prognosis, and potential therapeutic targeting in BICa. However, extensive independent validation studies are still needed to confirm miRNA's clinical value in bladder (68, 70, 71).

MicroRNAs as Cancer Biomarkers

Over the last decade, miRNAs have emerged as a significant area of research for their potential use as biomarkers in various diseases, including different forms of cancer. They serve different roles in terms of both detection and prognosis (31, 72). In the case of BICa, many miRNAs have been extensively researched, and growing evidence indicates that individual miRNAs or miRNA panels have the potential to serve as markers for predicting recurrence and progression. For instance, a study on the miR-200 family highlighted the complex nature of its role in BICa. Tumours originating in the urothelium were found to exhibit increased expression of miR-200 family members. Nonetheless, elevated levels of BMI1 and/or EZH2 contributed to the downregulation of miR-200, which in turn facilitated the recurrence of NMIBC. The heightened expression of EZH2 and BMI1 further suppressed miR-200, leading to the upregulation of transcription factors that promote EMT, ultimately driving NMIBC progression (73). However, due to inconsistent outcomes across various publications and the absence of testing in separate independent cohorts and clinical trials, the current findings cannot be confidently adopted in everyday clinical decision-

making (32, 74). Moreover, there is limited knowledge about the changes in miRNA patterns during treatment with intravesical drugs and how they may change during treatment.

Therefore, there is an urgent need for new biomarkers insights on, and validation to improve patient classification and treatment prediction.

References

1. Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I, Jemal A. 2024 Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*.
 2. Wong MCS, Fung FDH, Leung C, Cheung WWL, Goggins WB, Ng CF. 2018 The global epidemiology of bladder cancer: a joinpoint regression analysis of its incidence and mortality trends and projection. *Sci Rep*;8(1):1129.
 3. Doshi B, Athans SR, Woloszynska A. 2023 Biological differences underlying sex and gender disparities in bladder cancer: current synopsis and future directions. *Oncogenesis*;12(1):44.
 4. Mushtaq J, Thurairaja R, Nair R. 2019 Bladder cancer. *Surgery (Oxford)*;37(9):529-37.
 5. Lobo N, Afferi L, Moschini M, Mostafid H, Porten S, Psutka SP, et al. 2022 Epidemiology, Screening, and Prevention of Bladder Cancer. *Eur Urol Oncol*;5(6):628-39.
 6. Halaseh SA, Halaseh S, Alali Y, Ashour ME, Alharayzah MJ. 2022 A Review of the Etiology and Epidemiology of Bladder Cancer: All You Need To Know. *Cureus*;14(7):e27330.
 7. Jubber I, Ong S, Bukavina L, Black PC, Compérat E, Kamat AM, et al. 2023 Epidemiology of Bladder Cancer in 2023: A Systematic Review of Risk Factors. *Eur Urol*;84(2):176-90.
 8. Karagas MR, Park S, Warren A, Hamilton J, Nelson HH, Mott LA, Kelsey KT. 2005 Gender, smoking, glutathione-S-transferase variants and bladder cancer incidence: a population-based study. *Cancer Letters*;219(1):63-9.
 9. Babjuk M, Burger M, Capoun O, Cohen D, Compérat EM, Dominguez Escrig JL, et al. 2022 European Association of Urology Guidelines on Non-muscle-invasive Bladder Cancer (Ta, T1, and Carcinoma in Situ). *Eur Urol*;81(1):75-94.
 10. Saginala K, Barsouk A, Aluru JS, Rawla P, Padala SA, Barsouk A. 2020 Epidemiology of Bladder Cancer. *Med Sci (Basel)*;8(1).
 11. Bolla SR, Odeluga N, Amraei R, Jetti R. 2024 Histology, Bladder. StatPearls. Treasure Island (FL): StatPearls Publishing
- Copyright © 2024, StatPearls Publishing LLC. p.
12. Kaseb H, Aeddula NR. 2024 Bladder Cancer. StatPearls. Treasure Island (FL): StatPearls Publishing

13. Paner GP, Stadler WM, Hansel DE, Montironi R, Lin DW, Amin MB. 2018 Updates in the Eighth Edition of the Tumor-Node-Metastasis Staging Classification for Urologic Cancers. *European Urology*;73(4):560-9.
14. Shinagare AB, Ramaiya NH, Jagannathan JP, Fennessy FM, Taplin M-E, Abbeele ADVd. 2011 Metastatic Pattern of Bladder Cancer: Correlation With the Characteristics of the Primary Tumor. *American Journal of Roentgenology*;196(1):117-22.
15. Dyrskjöt L, Hansel DE, Efsthathiou JA, Knowles MA, Galsky MD, Teoh J, Theodorescu D. 2023 Bladder cancer. *Nat Rev Dis Primers*;9(1):58.
16. Kamoun A, de Reynies A, Allory Y, Sjodahl G, Robertson AG, Seiler R, et al. 2020 A Consensus Molecular Classification of Muscle-invasive Bladder Cancer. *Eur Urol*;77(4):420-33.
17. Mar N, Dayyani F. 2019 Management of Urothelial Bladder Cancer in Clinical Practice: Real-World Answers to Difficult Questions. *J Oncol Pract*;15(8):421-8.
18. Jin H, Ying X, Que B, Wang X, Chao Y, Zhang H, et al. 2019 N(6)-methyladenosine modification of ITGA6 mRNA promotes the development and progression of bladder cancer. *EBioMedicine*;47:195-207.
19. Miyamoto H, Miller JS, Fajardo DA, Lee TK, Netto GJ, Epstein JI. 2010 Non-invasive papillary urothelial neoplasms: the 2004 WHO/ISUP classification system. *Pathol Int*;60(1):1-8.
20. Powles T, Bellmunt J, Comperat E, De Santis M, Huddart R, Loriot Y, et al. 2022 Bladder cancer: ESMO Clinical Practice Guideline for diagnosis, treatment and follow-up. *Ann Oncol*;33(3):244-58.
21. Dong X, Song G, Guan K, Wang T, Feng X, Liu Y, et al. 2023 Clinical practice guideline on bladder cancer (Part I). *UroPrecision*;1(1):20-30.
22. Engelskjerd JS, Deibert CM. 2024 Cystoscopy. StatPearls. Treasure Island (FL): StatPearls Publishing

23. Soubra A, Risk MC. 2015 Diagnostics techniques in nonmuscle invasive bladder cancer. *Indian J Urol*;31(4):283-8.
24. Lee HH, Kim SH. 2020 Review of non-invasive urinary biomarkers in bladder cancer. *Transl Cancer Res*;9(10):6554-64.

25. Fiorentino V, Pizzimenti C, Franchina M, Rossi ED, Tralongo P, Carlino A, et al. 2023 Bladder Epicheck Test: A Novel Tool to Support Urothelial Carcinoma Diagnosis in Urine Samples. *Int J Mol Sci*;24(15).
26. Alix-Panabières C, Marchetti D, Lang JE. 2023 Liquid biopsy: from concept to clinical application. *Scientific Reports*;13(1):21685.
27. Franceschini T, Giunchi F, Montironi R, Scarpelli M, Lopez-Beltran A, Cheng L, Fiorentino M. 2020 Liquid biopsies in urological cancers: what we need to know before starting using them. *Expert Rev Mol Diagn*;20(2):135-9.
28. Nikanjam M, Kato S, Kurzrock R. 2022 Liquid biopsy: current technology and clinical applications. *J Hematol Oncol*;15(1):131.
29. Sequeira-Antunes B, Ferreira HA. 2023 Urinary Biomarkers and Point-of-Care Urinalysis Devices for Early Diagnosis and Management of Disease: A Review. *Biomedicines*;11(4).
30. Adhit KK, Wanjar A, Menon S, K S. 2023 Liquid Biopsy: An Evolving Paradigm for Non-invasive Disease Diagnosis and Monitoring in Medicine. *Cureus*;15(12):e50176.
31. Lodewijk I, Duenas M, Rubio C, Munera-Maravilla E, Segovia C, Bernardini A, et al. 2018 Liquid Biopsy Biomarkers in Bladder Cancer: A Current Need for Patient Diagnosis and Monitoring. *Int J Mol Sci*;19(9).
32. Kutwin P, Konecki T, Borkowska EM, Traczyk-Borszynska M, Jablonowski Z. 2018 Urine miRNA as a potential biomarker for bladder cancer detection - a meta-analysis. *Cent European J Urol*;71(2):177-85.
33. Oshi M, Murthy V, Takahashi H, Huyser M, Okano M, Tokumaru Y, et al. 2021 Urine as a Source of Liquid Biopsy for Cancer. *Cancers (Basel)*;13(11).
34. Roumigué M, Wong A, Belanger E, Wang G, Black PC. 2023 Detection and resection of carcinoma in situ of the bladder: Implications for clinical trial design. *Urol Oncol*;41(5):254.e9-.e15.
35. Kim LHC, Patel MI. 2020 Transurethral resection of bladder tumour (TURBT). *Transl Androl Urol*;9(6):3056-72.
36. Gad SE. 2014 Mitomycin C. In: Wexler P, editor. *Encyclopedia of Toxicology* (Third Edition). Oxford: Academic Press. p. 354-6.
37. Halstuch D, Lotan P, Karchever I, Rubinshtein D, Kedar D, Baniel J, Golan S. 2023 Single-Dose Post-Office Fulguration Mitomycin C Instillation Appears to Improve

Recurrence-Free Survival in Patients With Low-Grade Noninvasive Bladder Cancer. *Clinical Genitourinary Cancer*;21(5):e320-e5.

38. Parrao D, Lizana N, Saavedra C, Larrañaga M, Lindsay CB, San Francisco IF, Bravo JC. 2024 Active Surveillance in Non-Muscle Invasive Bladder Cancer, the Potential Role of Biomarkers: A Systematic Review. *Current Oncology*;31(4):2201-20.
39. Tan WS, Steinberg G, Witjes JA, Li R, Shariat SF, Roupret M, et al. 2022 Intermediate-risk Non-muscle-invasive Bladder Cancer: Updated Consensus Definition and Management Recommendations from the International Bladder Cancer Group. *Eur Urol Oncol*;5(5):505-16.
40. Decaestecker K, Oosterlinck W. 2015 Managing the adverse events of intravesical bacillus Calmette-Guérin therapy. *Res Rep Urol*;7:157-63.
41. Witjes JA. 2021 Follow-up in non-muscle invasive bladder cancer: facts and future. *World J Urol*;39(11):4047-53.
42. Aminoltejari K, Black PC. 2020 Radical cystectomy: a review of techniques, developments and controversies. *Transl Androl Urol*;9(6):3073-81.
43. Zargar H, Shah JB, van Rhijn BW, Daneshmand S, Bivalacqua TJ, Spiess PE, et al. 2018 Neoadjuvant Dose Dense MVAC versus Gemcitabine and Cisplatin in Patients with cT3-4aN0M0 Bladder Cancer Treated with Radical Cystectomy. *J Urol*;199(6):1452-8.
44. Witjes JA, Bruins HM, Cathomas R, Compérat EM, Cowan NC, Gakis G, et al. 2021 European Association of Urology Guidelines on Muscle-invasive and Metastatic Bladder Cancer: Summary of the 2020 Guidelines. *Eur Urol*;79(1):82-104.
45. Okobi TJ, Uhomoibhi TO, Akahara DE, Odoma VA, Sanusi IA, Okobi OE, et al. 2023 Immune Checkpoint Inhibitors as a Treatment Option for Bladder Cancer: Current Evidence. *Cureus*;15(6):e40031.
46. Dobruch J, Oszczudłowski M. 2021 Bladder Cancer: Current Challenges and Future Directions. *Medicina (Kaunas)*;57(8).
47. Mori K, Pradere B, Moschini M, Mostafaei H, Laukhtina E, Schuettfort VM, et al. 2021 First-line immune-checkpoint inhibitor combination therapy for chemotherapy-eligible patients with metastatic urothelial carcinoma: A systematic review and meta-analysis. *European Journal of Cancer*;151:35-48.
48. Zhu CZ, Ting HN, Ng KH, Ong TA. 2019 A review on the accuracy of bladder cancer detection methods. *J Cancer*;10(17):4038-44.

49. Yafi FA, Brimo F, Steinberg J, Aprikian AG, Tanguay S, Kassouf W. 2015 Prospective analysis of sensitivity and specificity of urinary cytology and other urinary biomarkers for bladder cancer. *Urol Oncol*;33(2):66.e25-31.
50. Guldhammer CS, Vásquez JL, Kristensen VM, Norus T, Nadler N, Jensen JB, Azawi N. 2024 Cystoscopy Accuracy in Detecting Bladder Tumors: A Prospective Video-Confirmed Study. *Cancers*;16(1):160.
51. Sievert KD, Amend B, Nagele U, Schilling D, Bedke J, Horstmann M, et al. 2009 Economic aspects of bladder cancer: what are the benefits and costs? *World J Urol*;27(3):295-300.
52. Deichmann U. 2016 Epigenetics: The origins and evolution of a fashionable topic. *Dev Biol*;416(1):249-54.
53. Wu C, Morris JR. 2001 Genes, genetics, and epigenetics: a correspondence. *Science*;293(5532):1103-5.
54. Hanahan D. 2022 Hallmarks of Cancer: New Dimensions. *Cancer Discov*;12(1):31-46.
55. Dupont C, Armant DR, Brenner CA. 2009 Epigenetics: definition, mechanisms and clinical perspective. *Semin Reprod Med*;27(5):351-7.
56. Jerónimo C, Henrique R. 2014 Epigenetic biomarkers in urological tumors: A systematic review. *Cancer Letters*;342(2):264-74.
57. Pathania AS. 2023 Crosstalk between Noncoding RNAs and the Epigenetics Machinery in Pediatric Tumors and Their Microenvironment. *Cancers (Basel)*;15(10).
58. Yamamura S, Imai-Sumida M, Tanaka Y, Dahiya R. 2018 Interaction and cross-talk between non-coding RNAs. *Cell Mol Life Sci*;75(3):467-84.
59. Yan H, Bu P. 2021 Non-coding RNA in cancer. *Essays Biochem*;65(4):625-39.
60. Beermann J, Piccoli MT, Viereck J, Thum T. 2016 Non-coding RNAs in Development and Disease: Background, Mechanisms, and Therapeutic Approaches. *Physiol Rev*;96(4):1297-325.
61. Zhang Y, Zhan L, Jiang X, Tang X. 2024 Comprehensive review for non-coding RNAs: From mechanisms to therapeutic applications. *Biochem Pharmacol*;224:116218.
62. Nemeth K, Bayraktar R, Ferracin M, Calin GA. 2024 Non-coding RNAs in disease: from mechanisms to therapeutics. *Nat Rev Genet*;25(3):211-32.

63. Ashwal-Fluss R, Meyer M, Pamudurti NR, Ivanov A, Bartok O, Hanan M, et al. 2014 circRNA biogenesis competes with pre-mRNA splicing. *Mol Cell*;56(1):55-66.
64. Uppaluri KR, Challa HJ, Gaur A, Jain R, Krishna Vardhani K, Geddam A, et al. 2023 Unlocking the potential of non-coding RNAs in cancer research and therapy. *Transl Oncol*;35:101730.
65. Peng Y, Croce CM. 2016 The role of MicroRNAs in human cancer. *Signal Transduct Target Ther*;1:15004.
66. Sequeira JP, Lobo J, Constancio V, Brito-Rocha T, Carvalho-Maia C, Braga I, et al. 2022 DigiMir Test: Establishing a Novel Pipeline for MiR-371a Quantification Using Droplet Digital PCR in Liquid Biopsies From Testicular Germ Cell Tumor Patients. *Front Oncol*;12:876732.
67. Hynes C, Kakumani PK. 2024 Regulatory role of RNA-binding proteins in microRNA biogenesis. *Front Mol Biosci*;11:1374843.
68. Li Y, Li G, Guo X, Yao H, Wang G, Li C. 2020 Non-coding RNA in bladder cancer. *Cancer Lett*;485:38-44.
69. Bartel DP. 2018 Metazoan MicroRNAs. *Cell*;173(1):20-51.
70. Parizi PK, Yarahmadi F, Tabar HM, Hosseini Z, Sarli A, Kia N, et al. 2020 MicroRNAs and target molecules in bladder cancer. *Med Oncol*;37(12):118.
71. Taheri M, Shirvani-Farsani Z, Ghafouri-Fard S, Omrani MD. 2020 Expression profile of microRNAs in bladder cancer and their application as biomarkers. *Biomed Pharmacother*;131:110703.
72. Padrao NA, Monteiro-Reis S, Torres-Ferreira J, Antunes L, Leca L, Montezuma D, et al. 2017 MicroRNA promoter methylation: a new tool for accurate detection of urothelial carcinoma. *Br J Cancer*;116(5):634-9.
73. Martínez-Fernández M, Dueñas M, Feber A, Segovia C, García-Escudero R, Rubio C, et al. 2015 A Polycomb-mir200 loop regulates clinical outcome in bladder cancer. *Oncotarget*;6(39):42258-75.
74. Li Q, Wang H, Peng H, Huang Q, Huyan T, Huang Q, et al. 2019 MicroRNAs: Key Players in Bladder Cancer. *Mol Diagn Ther*;23(5):579-601.

AIMS

Aims

Over the years, the role of microRNAs in bladder cancer, particularly their potential as non-invasive biomarkers for predicting treatment response has been explored. However, current clinical guidelines lack any approved microRNA-based biomarkers for predicting response to BCG treatment in bladder cancer patients. In this regard, identifying new biomarkers to predict treatment outcomes could reduce the need for invasive, costly, and aggressive therapies in patients who would not benefit from them.

Therefore, the main objectives of this dissertation are:

- Identify the most promising biomarkers of prognosis and response to BCG treatment through a systematic review of the literature.
- Validate molecular signatures from urine samples using a reliable and reproducible laboratory method for predicting BCG response in bladder cancer patients.
- Analyze target miRNA expression levels in liquid biopsies from a retrospective cohort of BCG immunotherapy patients and evaluate their potential as predictive biomarkers for BCG response.

PART I

Predictive and prognostic biomarkers of BCG failure in bladder cancer patients: A systematic review

Rui Ribeiro-Pereira, Ana Teixeira-Marques, Sara Monteiro-Reis, Rui Henrique, Carmen Jerónimo

Manuscript Under Submission

Abstract

High-risk non-muscle invasive bladder cancer (NMIBC) is a common urological malignancy with a high propensity for recurrence despite treatment with *Bacillus Calmette-Guérin* (BCG) instillations. Identifying patients who are likely to respond to BCG therapy prior to treatment initiation would significantly improve patient outcomes and reduce unnecessary treatments. This systematic review aimed to evaluate the existing literature on the potential of non-invasive biomarkers to predict and prognosticate BCG failure in high-risk NMIBC patients.

A comprehensive bibliographic search of the PubMed, Scopus, and Medline databases was conducted to identify relevant studies published up to July 31, 2024. After a thorough screening and selection process, 63 studies were included in the final analysis. Of these, 53 focused on prognostic markers, five on predictive markers, and seven examined both. The most frequently studied biomarkers were p53, Ki-67, and pRB. Several records focused on liquid biopsy-derived biomarkers, particularly due to their ability to reflect the dynamic characteristics of the tumour and its microenvironment. Additionally, several immunological markers have also been explored, highlighting the critical role of immune responses in BCG therapy.

Despite its valuable insights, this systematic review faced limitations, including challenges related to retrieved studies reproducibility, feasibility, and the small sample sizes. Nonetheless, the findings here suggest that a wide array of molecules might serve as prognostic and/or predictive markers, which could greatly enhance the clinical management of this complex disease.

Introduction

Bladder Cancer Epidemiology

Bladder cancer (BICa) is the 9th most common malignancy diagnosed worldwide, depicting approximately 614,000 new cases and 220,000 deaths in 2022 (1). Despite being one of the most expensive over a lifetime, BICa remains underprioritised in policy and research, earning the title of the “Cinderella” of cancers, due to its overlooked impact on patients, healthcare systems, and economies (2, 3).

Most types of bladder tumours originate in the bladder urothelium, consequently, being the urothelial carcinoma, the most frequent BICa type, comprising 90% of the total diagnosed cases (4). According to the tumour's capability of invading the muscle layer, this

malignancy can be stratified into non-muscle invasive BICa (NMIBC, comprising 75-80% of cases), and muscle-invasive BICa (MIBC, comprising 20-25% of cases) (4).

Bladder cancer management and treatment

The majority of BICa is detected based on a transurethral resection of the bladder tumour (TURBT), a procedure that excise visible lesions detected upon cystoscopy evaluation (5). However, the final diagnosis is only attained after histopathologic evaluation of the tissue obtained upon TURBT (5). Thereafter, patients are generally stratified according to EAU/ AUA prognostic groups according to the histopathologic features of the tumour, as well as multifocality, stage, size, previous recurrences and the occurrence of concomitant *in situ* tumour (TIS) (6, 7). The risk stratification allows for clinical management of the disease, particularly for helping to decide the best adjuvant treatment option and follow-up scheme, depending on the probability of recurrence and/or progression (6, 7). In this regard, intermediate-risk patients are often treated with intravesical chemotherapy (e.g. Mitomycin C), to avoid recurrences (8). *Bacille Calmette-Guérin* (BCG) is also an option for intermediate-risk patients, although these patients may present more therapy-associated side effects than to be more common in these patients than the ones subjected to intravesical chemotherapy (6, 7, 9). Importantly, high-risk patients are usually treated with BCG, which is proven to delay disease recurrence and progression (6, 7).

Limitations in *Bacille Calmette-Guérin* treatment management

Although prognostic tables allow stratification of patients for treatment guidance, they do not truly mirror the molecular heterogeneity and complexity nature of BICa, consequently leading to high relapse rates. Nonetheless, BCG prevents progressions and recurrences, while still associated with a high risk of relapse (30-40%) (10, 11). Moreover, no FDA/CE-approved biomarker is available to identify patients who might benefit from this treatment, leading to higher mortality and associated comorbidities, and a significant economic burden on the healthcare system. Importantly, the identification of predictive and prognostic biomarkers for BCG immunotherapy failure may considerably improve a patient's prognosis and avoid ineffective treatment.

Given the lack of approved biomarkers for therapy guidance, this systematic review aims to compile and explore published studies on predictive and prognostic biomarkers for BCG failure in BICa patients.

Methods

Protocol and Registration

This systematic review followed the most recent guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) (12). The protocol for the study was registered with the International Prospective Register of Systematic Reviews (PROSPERO) and assigned the registration ID CRD42024560005.

Retrieval Methodology

To perform this comprehensive systematic review, an extensive search was conducted across three major scientific databases, namely PubMed, Scopus, and Medline. The search aimed to identify all published studies investigating the potential of different molecules as biomarkers of prognostic and predictive of failure to BCG treatment in patients with BlCa. The search was limited to studies published until the 31st of July 2024.

In the search for the most relevant studies, various combinations of terms were created, including "Bladder Cancer", "Biomarker" and "BCG", which were utilised to construct queries for searching in different databases. The query used for search in PubMed was the following: (((("Urinary Bladder Neoplasms"[MeSH Terms]) OR ("Non-muscle invasive bladder neoplasms"[MeSH Terms]) OR ("bladder cancer"[title/abstract]) OR ("bladder urothelial carcinoma"[title/abstract]) OR ("lower tract urothelial carcinoma"[title/abstract]) OR ("Papillary urothelial carcinoma"[title/abstract]) OR ("Transitional Cell Bladder Cancer"[title/abstract]))) AND ((("Biomarkers"[MeSH Terms]) OR ("Biomarkers, Tumor"[MeSH Terms]) OR ("Biomarker"[title/abstract]) OR ("Molecular Marker"[title/abstract]) OR ("Marker"[title/abstract]))) AND ((("BCG"[title/abstract]) OR ("Bacillus Calmette-Guérin"[title/abstract]) OR ("Immunotherapy"[title/abstract]) OR ("Immunotherapy"[MeSH Terms])))). The query used for search in Scopus was the following: TITLE-ABS-KEY(("Urinary Bladder Neoplasms") OR ("Non-muscle invasive bladder neoplasms") OR ("bladder cancer") OR ("bladder urothelial carcinoma") OR ("lower tract urothelial carcinoma") OR ("Papillary urothelial carcinoma") OR ("Transitional Cell Bladder Cancer")) AND ((("Biomarkers") OR ("Biomarker") OR ("Molecular Marker") OR ("Marker"))) AND ((("BCG") OR ("Bacillus Calmette-Guérin")))). The query used for search in Medline was the following: (((MH Urinary Bladder Neoplasms) OR (MH Non-muscle invasive bladder neoplasms) OR (AB bladder cancer) OR (AB bladder urothelial carcinoma) OR (AB lower tract urothelial carcinoma) OR (AB Papillary urothelial carcinoma) OR (AB Transitional Cell Bladder Cancer)) AND ((MH Biomarkers) OR (MH Biomarkers, Tumor) OR (AB Biomarker) OR (AB Molecular Marker) OR (AB Marker)) AND ((AB BCG) OR (AB *Bacillus Calmette-Guérin*))).

Eligibility Criteria

Following the elimination of duplicate records, the resulting publications underwent a rigorous review process based on their title and abstract, adhering to a set of predetermined eligibility criteria established by two independent authors (Ribeiro-Pereira R. and Teixeira-Marques A.) who worked in a blinded manner.

To conduct this systematic review inclusion criteria included molecular biomarker studies in patients with lower tract urothelial carcinoma treated with BCG immunotherapy and focusing on patients' prognosis and/or predictive of failure to treatment. Additionally, papers assessing prognostic and predictive molecular biomarkers with respective survival and/or performance analysis in patients who performed BCG were included.

Regarding exclusion criteria, studies that focused only on types of BlCa other than NMIBC were excluded, as well as publications performed on *in vivo/in vitro* models, records that did not reach statistical significance and studies that were not original, such as editorials, letters, case reports, meta-analyses and other reviews. Studies published in languages other than English or Portuguese were also excluded from the analysis.

Data Extraction

During the evaluation process, the remaining studies underwent a thorough assessment to determine their eligibility for further analysis. The two blinded independent authors extracted the relevant information from the selected records. The extracted information for both prognosis and predictive studies included the size of the cohort of interest, tumour stage, histological grade, treatment scheme, outcome (reported as the authors referred in the article), source of the material used, type of molecule and respective expression level. Additionally, for prognosis studies, survival analysis, clinical significance and hazard ratio/odds ratio were mentioned, while for predictive studies the biomarker performance parameters (sensitivity, specificity, and area under the curve (AUC)) were recorded when available.

Risk of bias assessment

Prognosis studies

To evaluate the quality of prognostic studies, the adapted QUAPAS tool (Quality Assessment of Prognostic Accuracy Studies) was utilised (13). Assessment of risk of bias included participants, index test, outcome, flow and timing, and analysis evaluation. Moreover, four domains, namely participants, index test, outcome, and flow and timing, were also assessed for concerns regarding applicability.

Predictive studies

To conduct a more thorough quality assessment of the selected predictive studies, we utilised adapted available criteria from the PROBAST (Prediction model Risk Of Bias Assessment) Tool (14). Thus, five key domains were covered: participants, predictors, outcome, flow and timing, and analysis. Concerns regarding applicability included participants, predictors, outcome, and flow and timing.

Results

Literature Overview

A total of 2418 records through our bibliographic search, which was sourced from three distinct public databases: PubMed (1166), Scopus (875), and Medline (377) were identified. Out of these records, 400 were identified as duplicates. After a comprehensive review of titles and abstracts, from the screened 2018 articles, 1855 were excluded, and 163 were retained articles for further analysis. Subsequently, 100 publications were excluded based on specific criteria: 22 manuscripts did not address BICa prognosis and/or predictive of failure, four focused on subtypes other than NMIBC, 11 were excluded due to the therapy scheme (BCG was not performed or was combined with other therapeutic agents), 40 publications did not conduct any survival or biomarker performance analysis, and nine did not reach statistical significance. Finally, 14 studies were excluded because they were not focused on molecular biomarkers. Hence, 63 articles were included in the final analysis. The flow diagram depicted in Figure 1 offers a more comprehensive insight into the criteria employed for the study selection process.

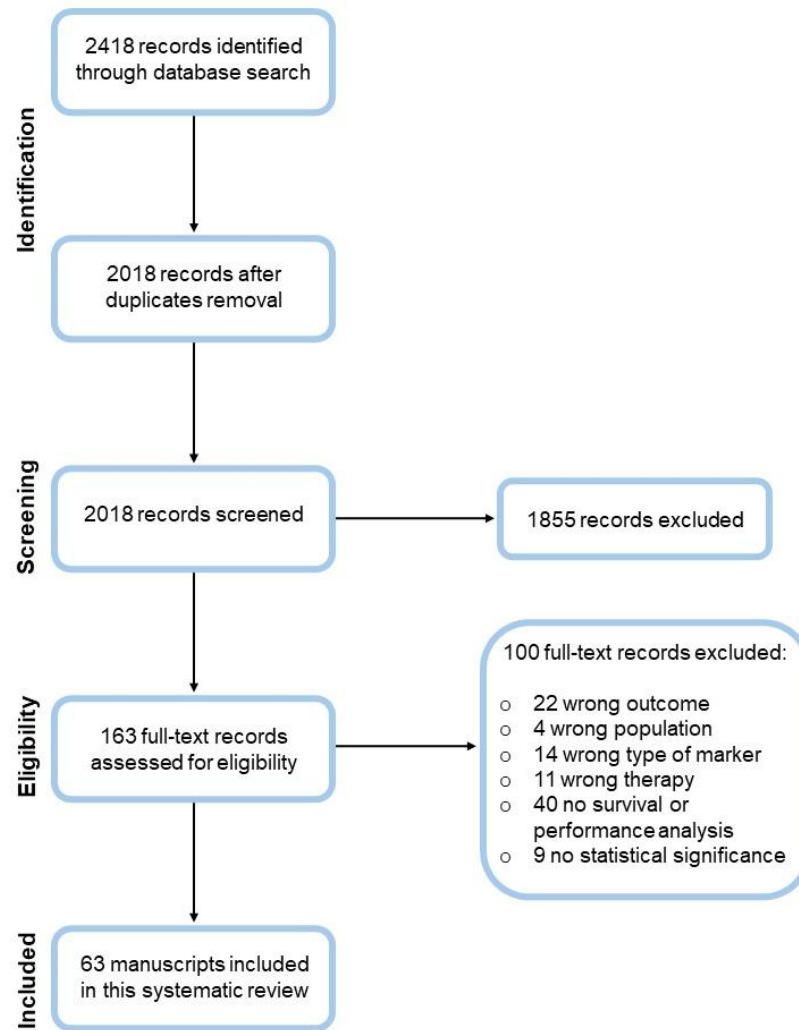


Figure 1: Flowchart of the literature selection process for the included records, according to the PRISMA guidelines.

Herein, over 30 years of research on BICa prognostic and predictive biomarkers, following BCG treatment were addressed, including studies from 1987 to 2023, with the majority originating from Asian and Western populations, representing a global perspective. The analysis included 54 cohort studies, eight case-control studies and one clinical trial. From the selected studies, 53 solely conducted survival analysis on the studied prognostic markers, five only evaluated markers' performance in predicting treatment failure, and seven focused on both prognostic and predictive of failure to BCG. A comprehensive summary for each study's information is shown in Tables 1 and 2.

Table 1: Information regarding Prognosis on Studies in bladder cancer patients treated with *Bacillus Calmette-Guérin*.

Study (Ref)	Year	Cohort	Stage (n)	Histologic Grade (n)	Outcome (n)	Treatment Scheme	Molecule	Level of Molecule	Clinical Significance	Survival Analyses: HR/OR
1. Tissue										
1.1. Protein										
V1 (15)	2024	106	Tis (3), Ta (20), T1 (83)	HG (85), LG (19)	Responder (47), Non-Responder s (59)	iBCG + mBCG	CD276 TIGIT PD-1 CTLA4 TNFRSF14	↑ BCG Non-Response ↑ BCG Response	–	–
U4 (16)	2023	67	Ta (26), T1 (41)	HG (54), LG (10)	Recurrence (38), Progression (7)	iBCG	HER-2	↑ Recurrence	RFS	2.686
U1 (17)	2023	454	Tis (21), Ta (215), T1 (218)	HG (403), LG (51)	Recurrence (89)	iBCG + mBCG	HER-2	↑ BCG Failure	RFS	2.36
								–	PFS	2.91
Q1 (18)	2019	22	T1	HG	Responder s (12), Non-Responder s (10)	iBCG + mBCG	Peritumoral T bet+/Lymphocyte	↑ Recurrence	RFS	5.3
							[GATA-3+/T-bet+]/[neutrophil/lymphocyte]	↓ Recurrence		0.18
							Peritumoral T bet+	↑ Recurrence	–	–
P2 (19)	2018	78	–	–	–	iBCG	PD-1	↑ BCG Failure	RFS	2.99
									PFS	8.27
P1 (20)	2018	54	T1	HG	Recurrence (25), Progression (14)	iBCG iBCG + mBCG	HSP60 > 65%	↑ BCG Failure	PFS	3.96
							HSP70 > 5%		PFS	0.33
								↑ BCG Response	RFS	0.29

O4 (21)	2017	41	Ta (12), T1 (28), Tis (1)	G2 (3), G3 (38)	–	iBCG + mBCG	IL-17	↑ BCG Response	EFS	0.2918
O2 (22)	2017	34	T1	G3	Recurrence (18), Progression (17)	iBCG + mBCG	HER-2	↓ Recurrence	DFS	2.7968
									PFS	2.8161
O3 (23)	2017	71	Tis, Ta, T1	LG, HG	Recurrence (40), Progression (17)	iBCG	Tregs (FOXP3+)	↑ BCG Failure	RFS	3.07
							TAM (CD204+)		PFS	3.43
									RFS	-
									PFS	-
N1 (24)	2016	40	Tis (10), Ta (9), T1 (21)	LG (7) and HG (33)	–	–	CD4/CD8 ratio	↓ BCG Failure	RFS	0.03
							CD68	↑ BCG Failure		3.34
							CD163	↑ BCG Failure		2.01
							CD163/CD68 ratio	↑ BCG Failure		2.38
							FOXP3	↑ BCG Failure		6.05
							CD25	↑ BCG Failure		4.27
							GATA3	↓ BCG Failure		0
							GATA3/T-bet ratio	↓ BCG failure		0
							CD4+	↓ BCG failure		0.13
M1 (25)	2015	134	T1 (134), Concomitant Tis (78)	–	–	iBCG + mBCG	ΔNp63	↑ Recurrence	OS	0.654
L1 (26)	2014	309	T1 + Concomitant Tis (106), T1 (202)	–	–	iBCG + mBCG	Ki-67	↑ Progression	PFS	2.8
							CK20	↑ Recurrence	RFS	5.89
L2 (27)	2014	99	Ta (40), T1 (59), Concomitant Tis (7)	LG (39) and HG (60)	Responder s (27), Non-Responder s (42)	iBCG + mBCG	CD163	↑ Recurrence	RFS	2.627

K2 (28)	2013	192	Ta (121), T1 (115), Concomitant Tis (10)	G1 (53), G2 (76), G3 (63)	Recurrence (111), Progression (20)	iBCG iBCG + mBCG	p53	↓ Recurrence	RFS	0.61
K4 (29)	2013	27	Ta (12), T1 (15)	LG (21), HG (6)	Responders (7), Non-Responders (12)	iBCG + mBCG	CD68	↑ Recurrence	RFS	0.148
J3 (30)	2012	28	Ta (19), T1(9)	LG (19), HG (9)	–	iBCG	Bcl-2 Bax	↑ Recurrence	RFS	3 9.9
J4 (31)	2012	28	Ta (19), T1 (9)	LG (19), HG (9)	–	iBCG	CD34	↑ Recurrence	RFS	67.2
I3 (32)	2011	87	T1 (87)	G2 (15), G3 (72)	–	–	AQP3	↓ Progression	PFS	7.58
I4 (33)	2011	42	Ta (17), T1 (25), Concomitant Tis (8)	G1 (6), G2 (23) and G3 (13)	–	iBCG	MMP9, Bcl2, CD1, and p21 MMP9 and p21	↑ Recurrence ↑ Progression	RFS PFS	– –
H2 (34)	2010	27	T1 (27)	G3 (27)	Recurrence (10), Progression (5)	iBCG + mBCG	pRB	–	PFS RFS	– –
H3 (35)	2010	77	Ta (48), T1 (29),	G1 (35), G2 (28), G3 (14)	Recurrence (77)	iBCG + mBCG	INF-α	Loss of Heterozygosity in Recurrence	RFS	4.09
G1 (36)	2009	27	T1 (27)	G3 (27)	Recurrence (10), Progression (5)	iBCG	pRB	–	PFS and RFS	–
G2 (37)	2009	92	T1 (91), Concomitant Tis (55)	G3 (91)	Recurrence (40), Progression (17)	iBCG	ezrin	↓ Progression and lower survival	DSS	–
G3 (38)	2009	66	Ta (30), T1 (23), Tis (13)	G1 (5), G2 (36), G3 (25)	–	iBCG + mBCG	CD68 expression and infiltration CD68 expression	↑ Recurrence	– –	4.64 3.81

F1 (39)	2007	83	T1 (31), Concomitant Tis (12)	–	No progression (52), Progression (31)	iBCG + mBCG	p53	↑ Progression	PFS	6.44
E1 (40)	2004	102	T1a (53), T1b (17), Concomitant Tis (18)	G1-G2 (64), G3 (38)	Recurrence (36), Progression (13), Without relapse (53)	iBCG	p53	↑ Recurrence	RFS	0.15
C2 (41)	1997	104	Ta (61), T1 (43)	G1 (30), G2 (63) and G3 (11)	Recurrence (60), Non- Recurrence (44)	–	Ki-67	↑ Recurrence	RFS	–
C1 (42)	1997	32	Papillary (26) Non- papillary (6), Concomitant Tis (2)	G2 (7), G3 (8)	Responder s (15), Non- Responder s (17)	iBCG	p53	↓ Non-Responders	DFS	3.8
B1 (43)	1996	98	Ta (22), T1 (19), Tis (57)	–	Responder s (62), Progression (29)	iBCG	p53	↓ Non-Responders	PFS	6.7
1.2. DNA										
S1 (44)	2021	42	T1 (42)	HG (42)	Responder s (17), Non- Responder s (25)	iBCG	<i>MLC1</i>	↑ BCG Response	RFS	0.77
R4 (45)	2020	35	Ta (10), T1 (25)	LG (5), HG (30)	Responder s (17), Non- Responder s (18)	iBCG + mBCG	<i>TMB</i>	↑ BCG Response	RFS	0.28
							<i>NAL</i>			0.30
							<i>DDR</i>			0.27
R3 (46)	2020	125	Ta (51) T1 (74)	LG (40), HG (85)	–	iBCG + mBCG	<i>TERTp</i> c.1-146G>A	↑ BCG Response	RFS	0.382

I2 (47)	2011	91	T1 (91)	G3 (91)	Recurrence (40), Progression (17)	iBCG	MSH6, TP73	↓ Progression	PFS	0.302
							RB1, THBS1			0.133
							RB1, PYCARD			0.149
							THBS1, PYCARD			0.211
							MSH6, THBS1, PYCARD			0.226
							MSH6, THBS1, TP73			0.226
							RB1, THBS1, PYCARD			0.134
							MSH6, THBS1, RB1			0.262
							RB1, MSH6, TP73, THBS1			0.226
							MSH6, THBS1			0.226
							RB1, MSH6, TP73, THBS1, PYCARD		0.226	
H4 (48)	2010	108	–	–	Recurrence (35), Progression (21)	iBCG + mBCG	Myopodin	Progression/Recurrence↑	RFS	2.541
									PFS	11.227
									DSS	7.552
H5 (49)	2010	28	Ta (6), T1 (15), T2 (4), Concomitant Tis (28)	G3 (28)	Tis Recurrence (9), Tis Progression (4)	iBCG + mBCG	Cyclin D3	Amplification in progression	PFS	61.503
1.3. RNA										
T5 (50)	2022	59	Ta (19), T1 (40)	LG (21) HG (38)	Recurrence (26), Non-Recurrence (33)	–	RPL9	↓ BCG Failure	RFS	2.649
T3 (51)	2022	100	T1 (100)	G3 (100)		iBCG + mBCG	miR-31	Downregulated	↓ RFS	0.05

										↓ PFS	0.03		
										miR-let7a	Downregulated	↓ PFS	0.001
										miR-199a	Upregulated	↓ RFS	2.8
										ARID1A	Downregulated	↓ RFS	0.9
										NCOR1	Upregulated	↓ PFS	0.18
										UTX	Downregulated	↓ RFS	0.9
										miR-21	Upregulated	↓ RFS	0.03
										STAG2	Upregulated	↓ PFS	1.2
												↓ PFS	3.1
H1 (52)	2010	80	T1 (48)	G1 (6), G2 (33) and G3 (9)	Recurrence (22), Non-Recurrence (26), Progression (5), No Progression (43)	iBCG	Recurrence: MAEA, SEC24C, ZC3HC1, RBAF600, AP1G1, UBE2I, HLA-A, CEBPZ*, RNPS1, PTPRM, BTG2		↓ Recurrence/Progression, *↑ Recurrence/Progression	RFS	3.38		
							Progression: VPS37C*, UFC1*, POLR2A, STOML2, RNF20, XRCC5, SMARC4A4*, SEPX1, SUGT1*, TAX1BP3, LGMN, HEBP2*			PFS	10.49		
1.4. Glycan													
K3 (53)	2013	94	Ta (40), T1 (54), Concomitant Tis (6),	LG (38) and HG (56)	Recurrence (36)	iBCG + mBCG	sTn and/or s6T	↓ Recurrence	RFS	0.296			
2. Tissue and Blood													
2.2. Protein													

R1 (54)	2020	29	Tis (2), Ta (11), T1 (16)	HG (5)	Responder s (21), Non-Responder s (8)	iBCG	CD4+FOXP3- T cells	↑ BCG Response	RFS	9.54
							CD8+PD1+ T cells			9.96
2.3. DNA and RNA										
L3 (55)	2014	125	Ta (51), T1 (74), Concomitant Tis (13)	LG (26) and HG (99)	Responder (77), Non-Responder s (48)	iBCG iBCG + mBCG	FASL-844 (TT+TC vs CC)	CC Genotype ↓RFS	RFS	1.922
							FASL	↑ Recurrence	RFS	2.833
3. Blood										
3.1. Protein										
U2 (56)	2023	1709	Tis (317), Ta (468), T1 (924)	LG (101), HG (1557), Not Evaluated (51)	–	iBCG iBCG + mBCG	C-Reactive Protein	↑ BCG Failure	RFS	1.431
									PFS	1.660
									CSS	1.847
R2 (57)	2020	Discovery Cohort 13	T1 (50)	HG (9), LG (4)	Responder s (9), Non-Responder s (4)	iBCG + mBCG	CCL27	–	–	–
		Validation Cohort 37		HG (24), LG (13)	Responder s (28), Non-Responder s (9)				↑ BCG Response	RFS
N2 (58)	2016	1117 (MITc 48, BCG 300)	Ta (674), T1 (443), Concomitant Tis (66)	G1 (231), G2 (398), G3 (488)	–	iBCG	C-Reactive Protein	↑ Progression	Disease progression	3.51
3.2. DNA										
T1 (59)	2022	195	Ta (88), T1 (107)	G1 (10), G2 (44), G3 (140), NA (1)	Recurrence (43)	iBCG	HLA-B62	↑ Recurrence	RFS	5.73
							HLA-B07	↓ Recurrence	RFS	0.43

							HLA-B044			0.36
M2 (60)	2015	BCG (51), Non-treated (37)	Ta (16), T1 (63), Tis (9), Concomitant Tis (14)	G1/G2 (14) and G3 (28)	–	–	NOS3 (TT vs CT/CC)	↓ Progression	PFS	0.05
							NOS3 (GG vs GT/TT)	↓ Progression, Recurrence	RFS	0.29
									PFS	0.1
							NOS2 promoter microsatellite (CCTTT)n ≥13 repeats)	–	CSD	0.12
M3 (61)	2015	204	Ta (82), T1 (122), Concomitant Tis (186)	LG (55) HG (149)	BCG Success (134), BCG Failure (70)	iBCG iBCG + mBCG	TNF-α1031T/C (TT+TC vs CC)	CC genotype ↑ Recurrence	RFS	3.49
							IL4-33T/C (CT vs TT)	–		1.93
							IL2RA T/C (TT vs TC+CC)	TT genotype ↑ Recurrence		2.79
							IL17A-197G/A (GG+GA vs AA)	AA genotype ↑ Recurrence		2.65
							IL17RA-809A/G (AA+AG vs GG)	–		2.89
							IL18R1 T/C (CC vs TC+TT)	–		2.75
							FASL-844T/C (TT+TC vs CC)	–		1.7
							TRAILR1-397T/G (TT vs TG+GG)	TG+CG genotype ↑ Recurrence		5.19
							ICAM1 K469E (AA+AG vs GG)	GG genotype ↑ Recurrence		2.47
J2 (62)	2012	200 [Non- treated (71), BCG (78)]	Ta (64), T1 (85), T2 (51)	G1 (67), G2 (43), G3 (90)	Recurrence (65), Non- Recurrence (83)	iBCG + mBCG	Survivin 31G>C (GC vs GG)	GG genotype ↑ Recurrence	RFS	0.35
							Survivin 31G>C (CC vs GG)			0.22

I1 (63)	2011	99 [BCG (69), BCG+IFN- α (30)]	Tis (30), Ta (21), T1 (48), HC (85)	G1 (20), G2 (46) and G3 (33)	–	iBCG + mBCG	NRAMP1 (GA vs GG) NRAMP1 (GT)n allele 3	GG genotype $\uparrow$ Recurrence $\downarrow$ Recurrence	RFS	4.57 24.789	
H6 (64)	2010	200 BICa 265 Controls, BCG (78), Non-treated (71)	Ta (64), T1 (85), T2 (51)	G1 (64), G2 (43), G3 (90)	Recurrence (65), Non-Recurrence (84)	iBCG + mBCG	<i>p53R72P</i> G>C (GC vs GG)	GG genotype $\uparrow$ Recurrence	RFS	0.29	
4. Blood and Urine											
4.1. Protein											
O1 (65)	2017	PBL Group 51	Tis, Ta, T1		Recurrence (28), Non-Recurrence (23)	–	IL-8	$\uparrow$ BCG Failure	RFS	4.24	
		Urine Group 112	Tis/Ta (49), T1 (63)	–	Recurrence (48), Non-Recurrence (64)					4.25	
4.2. RNA											
T2 (66)	2022	204	T1 (204)	LG (16), HG (170)	Recurrence (16), Non-Recurrence (88)	iBCG + mBCG	IL-10	$\downarrow$ BCG Response	RFS PFS	15.4	
							CTLA-4			3.18	
							FOXP3			3.18	
							T-bet			3.18	
							GATA3/T-bet ratio		RFS	4.9	
									PFS	1.6	
5. Urine											
5.1. Protein											
T4 (67)	2022	64	Tis (5), Ta (30), T1 (29)	HG (64)	Recurrence (11), Non-Recurrence (53)	–	A1AT	$\uparrow$ Recurrence	RFS	–	
							APOE			–	
							ANG			42.89	
							CA9			3.44	
							IL8			–	

							MMP9				–
							MMP10				3.86
							PAI1				–
							SDC1				–
							VEGFA				–
							Panel				–
J1 (68)	2012	72	Ta (49) and T1 (23), HD (49)	G1 (8), G2 (26) and G3 (38)	–	iBCG	IL-6/IL-10 ratio	↑ Recurrence	RFS	4.09	
D1 (69)	2003	20	–	–	–	iBCG	IL-2	↓ Non-Responder	–	0.368	
5.2. DNA											
S2 (70)	2021	123	Tis (17), Ta (67), T1 (39)	LG (37), HG (86)	Recurrence (63)	iBCG iBCG + mBCG	Mycobacterium tuberculosis complex	↑ Recurrence/Progression	RFS	1yr (0.967), 5yr (0.817)	
									PFS	0.942	
5.3. Glycan and DNA											
A1 (71)	1987	66	–	–	–	iBCG	ABH reactivity, DNA ploidy	Favourable Prognosis	RFS PFS	–	

Abbreviations: BCG (*Bacillus Calmette-Guérin*), BICa (Bladder Cancer), CSS (Cancer-Specific Survival), DFS (Disease-Free Survival), DSS (Disease-Specific Survival), EFS (Event-Free Survival), G1 (Grade 1), G2 (Grade 2), G3 (Grade 3), HG (High Grade), HR (Hazard ratio), iBCG (induction BCG course), LG (Low Grade), mBCG (maintenance BCG course), MITc (mitomycin C), OR (Odds ratio), OS (Overall Survival), PBL (Peripheral Blood Leukocyte), PFS (Progression-Free Survival), RFS (Recurrence-Free Survival), Tis (*Carcinoma in Situ*), yr (year).

Table 2: Information regarding Predictive Studies in bladder cancer patients treated with *Bacillus Calmette-Guérin*.

Study (Ref)	Year	Cohort	Stage (n)	Histologic Grade (n)	Outcome (n)	Treatment Scheme	Molecule	Level of Molecule	Sensitivity	Specificity	AUC
1.1. Tissue											
1.2. Protein											
U3 (72)	2023	32	Tis (2), Ta (6), T1 (24)	LG (6), HG (26)	Responders (21), Non-Responders (11)	iBCG + mBCG	GATA-3/T-bet ratio	↑ Response	0.91	0.57	0.74
Q1 (18)	2019	22	T1	HG	Responders (12), Non-Responders (10)	iBCG + mBCG	Peritumoral T bet+/Lymphocyte	↑ Recurrence	0.75	0.80	–
							GTR/NLR	↓ Recurrence	0.75	0.80	
							Peritumoral T bet+	↑ Recurrence	0.667	0.70	
P4 (73)	2018	105	Tis (26), Ta (23), T1 (35), T1 + Concomitant Tis (21)	LG (32), HG (73)	Responders (89), Non-Responders (16)	iBCG + mBCG	NEDD9	↑ Recurrence/Progression	0.836	0.642	–
								↓ BCG Response	0.913	0.512	
									0.841	0.809	
O2 (22)	2017	34	T1	G3	Recurrence (18), Progression (17)	iBCG + mBCG	HER-2	↓ Recurrence/Progression	0.58	0.83	–
									0.65	0.65	
N1 (24)	2016	40	Tis (10), Ta (9) and T1 (21)	LG (7) and HG (33)	–	–	CD163+ TAM density	–	–	–	1
							CD68+ TAM density	–	–	–	1
							total FOXP3+ cell density	–	–	–	1
							CD25+ cell density	–	–	–	0.92
							GATA3+ T-cell density	–	–	–	0.99
							total T-bet+ T-cell density	–	–	–	0.72
							CD4+ T-cell count	–	–	–	0.8
L4 (74)	2014	38	Tis (38)	–	Responders (20), Non-Responders (18)	iBCG	Ratio of GATA-3+ lymphocytes/ T-bet+ lymphocytes	↑ Responders	1	0.8	–
1.3. DNA											

R5 (75)	2020	Discovery Cohort 53	Tis, Ta, T1	HG (48), Not Specified (5)	Responders (26), Failures (27)	–	–	↑ BCG failure	–	–	–	
		Internal Validation Cohort 37			Responders (19), Failures (18)		<i>KLF8, C12orf42, FLT1, WDR44, CHST11</i>				0.633 to 68.1	
							<i>GPR158</i>				0.809	
2. Blood												
2.1. Protein												
R2 (57)	2020	Discovery Cohort 13	T1 (50)	HG (9), LG (4)	Responders (9), Non-Responders (4)	iBCG + mBCG	CCL27	–	–	–	–	
		Validation Cohort 37			HG (24), LG (13)			Responders (28), Non-Responders (9)	↑ BCG Response	Pre-BCG treatment (0.67)	Pre-BCG treatment (0.78)	Pre-BCG treatment (0.730)
										Post-BCG treatment (0.89)	Post-BCG treatment (0.68)	Post-BCG treatment (0.726)
										–	–	Combined timepoints GSCCL27 (0.897)
2.2. DNA												
M3 (61)	2015	204	Ta (82), T1 (122), Concomitant Tis (186)	LG (55), HG (149)	BCG Success (134), BCG Failure (70)	iBCG+ mBCG	<i>TNF-α</i> 1031T/C (TT+TC vs CC)	CC genotype ↑ Recurrence	–	–	0.82	
							<i>IL4</i> -33T/C (CT vs TT)	–				
							<i>IL2RA</i> T/C (TT vs TC+CC)	TT genotype ↑ Recurrence				
							<i>IL17A</i> -197G/A (GG+GA vs AA)	AA genotype ↑ Recurrence				
							<i>IL17RA</i> -809A/G (AA+AG vs GG)	–				
							<i>IL18R1</i> rs3771171 T/C (CC vs TC+TT)	–				

								FASL-844T/C (TT+TC vs CC)	–			
								TRAILR1-397T/G (TT vs TG+GG)	TG+CG genotype ↑ Recurrence			
								ICAM1 K469E (AA+AG vs GG)	GG genotype ↑ Recurrence			
3. Urine												
3.1. Protein												
T4 (67)	2022	64	Tis (5), Ta (30), T1 (29)	HG (64)	Recurrence (11), Non-Recurrence (53)	–		A1AT	↑ Recurrence	0.818	0.566	0.6364
								APOE		0.818	0.509	0.6724
								ANG		0.636	0.811	0.7444
								CA9		0.636	0.698	0.6878
								IL8		0.364	0.774	0.5146
								MMP9		0.909	0.264	0.5420
								MMP10		0.727	0.679	0.7238
								PAI1		0.636	0.660	0.6261
								SDC1		0.545	0.887	0.6106
								VEGFA		0.273	0.981	0.5935
Panel									0.818	0.8490	0.8971	
N3 (76)	2016	130	Ta (62), T1 (61)	LG (34), HG (96)	Recurrence (44), No Recurrence (86)	iBCG + mBCG	Panel: IL-2, IL-8, IL-6, IL-1ra, IL-10, IL-12[p70], IL-12[p40], TRAIL, and TNF-a	–	0.8	0.774	0.86	
3.2. DNA												
S2 (70)	2021	123	Tis (17), Ta (67), T1 (39)	LG (37), HG (86)	Recurrence (63)	iBCG iBCG + mBCG	Mycobacterium tuberculosis complex	↑ Recurrence	0.75	1	0.868	
								↑ Progression	0.833	0.915	0.875	

Abbreviations: AUC (Area under the curve), BCG (*Bacillus Calmette-Guérin*), G1 (Grade 1), G2 (Grade 2), G3 (Grade 3), HG (High Grade), iBCG (induction BCG course), LG (Low Grade), mBCG (maintenance BCG course), Tis (*Carcinoma in Situ*).

Quality Assessment

The QUAPAS-2 and Probast tools were used to assess the quality of prognostic and predictive studies, respectively (Figures 2 and 3). The signalling questions were tailored according to the objectives and were answered for each study (Tables S1 and S2). Regarding prognostic marker studies, most exhibited low or possible risk of bias, as many signalling questions were unanswered. This was especially evident in the analysis domain, where 62% of the manuscripts showed potential bias since it was challenging to determine whether the information was missing or, in cases of data censoring due to unclear data handling and censoring methods (Figure 2A). Another domain associated with a high risk of bias, evident in 31% of the studies, relates to participant selection (Figure 2A). This is mainly due to the lack of random or consecutive patient sampling and the incomplete description of participant criteria. Regarding applicability concerns, the participants' domain had the highest rate of concern (36%). This was primarily because of the exclusion of important BICa stages from the records, particularly patients with *carcinoma in situ* (Tis) or concomitant Tis (Figure 2B). For predictive studies, applicability concerns and risk of bias in the participants' domain mirrored those seen in prognosis studies, with 33% exhibiting a high risk of bias (Figures 3A and 3B).

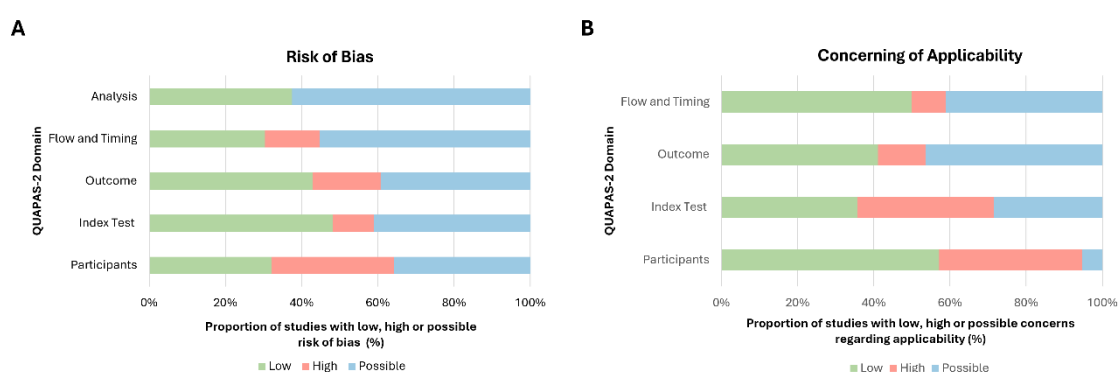


Figure 2: Quality assessment of the included prognosis studies using the QUAPAS-2 tool. (A) Graphical illustration depicting the risk of bias in the prognosis studies. **(B)** Graphical illustration depicting the risk of concerns regarding the applicability of the prognosis studies.

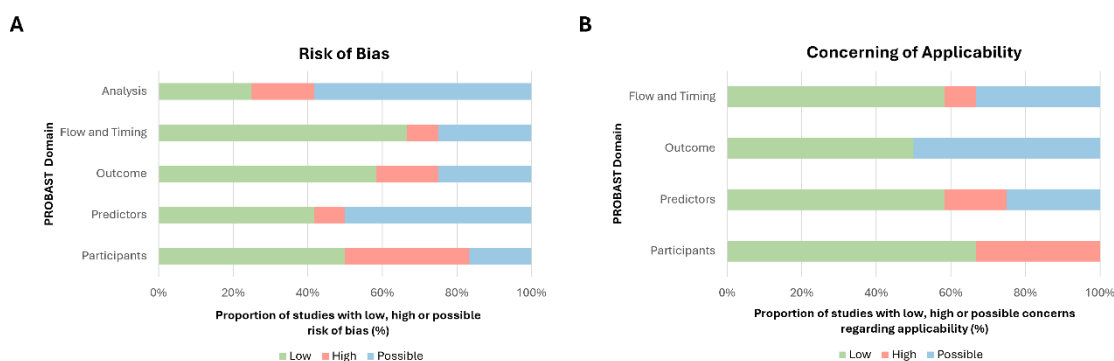


Figure 3: Quality assessment of the included predictive studies using the PROBAST tool. (A) Graphical illustration depicting the risk of bias in the predictive studies. **(B)** Graphical illustration depicting the risk of concerns regarding applicability in the predictive studies.

Prognostic Biomarkers

Regarding the biomarker biological sources, 40 studies used tissues, mostly Formalin-Fixed Paraffin-Embedded (FFPE), 11 assessed blood and five research articles assessed urine (Figure 4). Interestingly, most studies evaluated protein biomarkers, followed by DNA, RNA and glycans. Proteins were the most evaluated molecules in tissues and urines, while DNA was the most investigated biomarker in blood.

Of the original articles assessing tissue-based biomarkers, five evaluated the potential of p53 protein in tissue using immunohistochemistry (28, 39, 40, 42, 43), where this protein was reported as an independent factor of progression-free survival (PFS), disease-specific survival (DSS) and/or recurrence-free survival (RFS) in a multivariate analysis in BCG-treated patients. Additionally, two manuscripts have investigated the value of Ki-67. Indeed, Asakura et al (41) and Bertz et al (26) found that Ki-67 overexpression was associated with higher disease recurrence and progression probability. Two additional studies evaluated the behaviour of CD4 in the response to BCG treatment. In this regard, Pichler and colleagues (24) demonstrated that a high CD4/CD8 ratio was associated with a high RFS, while Lim et al (54) observed the same tendency when using a CD4⁺/FOXP3⁺ ratio, which was higher in responders, and also predicting longer RFS after BCG treatment. Moreover, Fukumoto and colleagues showed that higher PD-1 expression in BCG-relapsing tumours was independently associated with subsequent tumour recurrence (RFS=2.99), and disease progression (PFS=8.27) (19).

A limited number of studies focused on biomarkers analysed in liquid biopsies such as urine or blood; Elsayy and colleagues observed that higher IL-10 levels in urinary reliably forecasted recurrence and progression following BCG treatment (66). Similarly, Cai et al analysed pre-TURBT urine samples and found that a high IL6/IL10 ratio was linked to an increase in recurrent patients, with an RFS of 4.09 (68). Furthermore, Qu et al found that

higher levels of the protein IL-8 in urine and peripheral blood leukocytes were significantly linked to an increased risk of recurrence compared to patients with lower IL-8 levels (65).

Predictive Biomarkers

Overall, seven focused on tissue-based markers, while four investigated liquid biopsies, specifically using urine or blood. Regarding the analyte addressed, proteins and DNA were the mostly investigated (Figure 4). Protein-based biomarkers AUCs ranged from 0.64-1, with the maximum value reported for CD163, CD68, and FOXP3 analysed in FFPE tissues by immunohistochemistry (24). Moreover, the Oncuria™ test, a multiplex immunoassay that measures MMP9, VEGFA, CA9, SDC1, PAI1, APOE, A1AT, ANG, and MMP10 levels, predicted disease recurrence with 81.8% sensitivity, 84.9% specificity and 0.89 AUC in patients' urines pre-BCG treatment (67). A nomogram score for changes in the levels of nine urinary cytokines (IL-2, IL-8, IL-6, IL-1ra, IL-10, IL-12(p70), IL-12(p40), TRAIL, and TNF- α) predicted recurrence in patients under BCG treatment, with 80% sensitivity, 77.4% specificity and 0.855 AUC (76). A study evaluating CCL27 levels in pre- and post-treatment serum samples predicted response to BCG treatment with 0.897 AUC (57).

Furthermore, the AUC ranged from 0.63 to 0.868 in the reports that investigated DNA-based biomarkers. Particularly, the detection of Mycobacterium tuberculosis complex DNA using PCR in pre-treatment urine, predicted disease recurrence and progression with 0.868 and 0.875 AUC, respectively (70). Moreover, *GPR58*, *KLF8*, *C12orf42*, *WDR44*, *FLT1* and *CHST11* promoter hypermethylation levels were identified and validated in FFPE tissues, being *GPR58* the most promising BCG failure predictor, achieving 0.809 AUC (75).

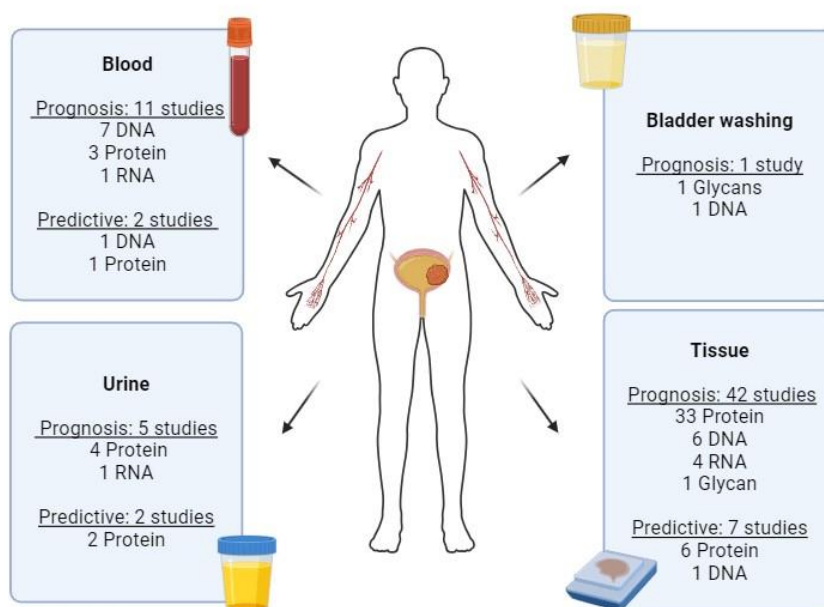


Figure 4: Distribution of prognostic and predictive biomarker studies for BCG of failure by source and molecules. Created with BioRender.com.

Discussion

BCG therapy is commonly offered to BICa patients stratified into intermediate and high-risk (6, 7). Despite being the first immunotherapy ever applied for cancer treatment, biomarkers that allow for the identification of patients who might benefit from this type of therapy are still lacking. Indeed, to this date, there are still no FDA/CE-approved biomarkers to identify patients who might benefit from this therapy (77). Thus, this systematic review aimed to evaluate studies on biomarkers for prognostic assessment and prediction of BCG therapy failure in BICa patients.

Our analysis revealed a considerable number of studies investigating prognostic and predictive biomarkers for BCG failure in BICa. Of those, the majority assessed protein markers primarily in paraffin-embedded tissues. Notably, several proteins such as *p53*, *Ki-67*, and *pRB*, were evaluated across multiple works, indicating their potential clinical value (26, 28, 34, 36, 39, 40, 42, 43, 78) (Figure S1). Additionally, some biomarkers were investigated in more than one biological source. For instance, *FASL*, *FOXP3*, *GATA3*, *IL17*, *CTLA-4* and *P53* were found in both tissue and blood, while *IL2*, *IL8* and *IL10* were studied in both blood and urine (28, 39, 40, 42, 43, 54, 61, 64-69) (Figure S1). *MMP9* was the only marker found both in tissue and urine (33, 67) (Figure S1). These results support the dual potential of these biomarkers across different types of biological material sources.

Indeed, non- or minimally invasive liquid biopsies, such as urine and blood, may better capture tumour and microenvironment heterogeneity compared with tissue samples (79). Urine is particularly valuable in BICa, because it directly contacts tumour cells and their surrounding microenvironment, offering a comprehensive analysis of potential prognostic and predictive markers for failure to BCG treatment (80). This is supported by the CyPRIT clinical trial (NCT01007058, completed in 2016) which evaluated a panel of nine cytokines in urine supernatant to predict recurrence in BCG-treated patients with promising results (76).

This systematic review found that many of the studied biomarkers were related to immunology, including interleukins, immune cell markers, and immune checkpoint inhibitors such as *PD-1* or *CTLA-4*. This suggests their high potential as biomarkers, especially since BCG instillations, the primary treatment for high-risk NMIBC is immunotherapy. The immune system's activation, along with the presence or absence of immune-related cells may indicate which patients are likely to respond to the treatment (81, 82).

However, the reviewed studies presented several limitations. A significant concern pertains to applicability due to the inadequate reporting of the key variables, hindering the reproducibility of the tests. Additionally, most studies were based on small patient cohorts,

raising bias concerns about representativeness. Moreover, there were few studies on predictive biomarkers, as most were preliminary, requiring validation in larger independent cohorts.

As per the EAU guidelines, optimal BCG efficacy requires an induction schedule (iBCG) of 6-weekly instillations, followed by a maintenance schedule (mBCG) (83). The studies in this systematic review showed considerable variation in the BCG schedules, with about 46% involving patients who underwent both induction and maintenance. This variability might affect patient outcomes and bias biomarker performance.

Further research is essential to enhance the performance, reliability, and robustness of biomarkers, to establish them as prognostic and/or predictive tools for clinical use. Despite these challenges, the studies reviewed here support the idea that the integration of a panel of molecular biomarkers could significantly improve the accuracy of predicting BCG treatment outcomes. In this regard, future studies should focus on combining the most promising biomarkers to advance this field.

Conclusion

Our comprehensive review identified a diverse range of molecules with strong potential as prognostic and/or predictive biomarkers for BCG therapy failure. Importantly, these biomarkers may significantly improve the clinical management of BlCa patients undergoing BCG treatment, leading to improved patient care and outcomes.

References

1. Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I, Jemal A. 2024 Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*.
2. Van Hemelrijck M, Patel P, Mouw KW. 2020 Editorial: Bladder Cancer – A Cinderella Cancer: Advances and Remaining Research Questions. *Frontiers in Oncology*;10.
3. Richters A, Aben KKH, Kiemeny L. 2020 The global burden of urinary bladder cancer: an update. *World J Urol*;38(8):1895-904.
4. Minoli M, Kiener M, Thalmann GN, Julio MKD, Seiler R. 2020 Evolution of urothelial bladder cancer in the context of molecular classifications. *International Journal of Molecular Sciences*;21(16):1-25.
5. Sharma P, Callahan MK, Bono P, Kim J, Spiliopoulou P, Calvo E, et al. 2016 Nivolumab monotherapy in recurrent metastatic urothelial carcinoma (CheckMate 032): a multicentre, open-label, two-stage, multi-arm, phase 1/2 trial. *The Lancet Oncology*;17(11):1590-8.
6. Sylvester RJ, Rodríguez O, Hernández V, Turturica D, Bauerová L, Bruins HM, et al. 2021 European Association of Urology (EAU) Prognostic Factor Risk Groups for Non-muscle-invasive Bladder Cancer (NMIBC) Incorporating the WHO 2004/2016 and WHO 1973 Classification Systems for Grade: An Update from the EAU NMIBC Guidelines Panel. *European Urology*;79(4):480-8.
7. Ritch CR, Velasquez MC, Kwon D, Becerra MF, Soodana-Prakash N, Atluri VS, et al. 2020 Use and Validation of the AUA/SUO Risk Grouping for Nonmuscle Invasive Bladder Cancer in a Contemporary Cohort. *Journal of Urology*;203(3):505-11.
8. Powles T, Bellmunt J, Comperat E, De Santis M, Huddart R, Loriot Y, et al. 2022 Bladder cancer: ESMO Clinical Practice Guideline for diagnosis, treatment and follow-up. *Ann Oncol*;33(3):244-58.
9. Böhle A, Jocham D, Bock PR. 2003 Intravesical bacillus Calmette-Guerin versus mitomycin C for superficial bladder cancer: a formal meta-analysis of comparative studies on recurrence and toxicity. *J Urol*;169(1):90-5.
10. Zlotta AR, Fleshner NE, Jewett MA. 2009 The management of BCG failure in non-muscle-invasive bladder cancer: an update. *Can Urol Assoc J*;3(6 Suppl 4):S199-205.

11. Kikuchi E, Hayakawa N, Fukumoto K, Shigeta K, Matsumoto K. 2020 Bacillus Calmette-Guérin-unresponsive non-muscle-invasive bladder cancer: Its definition and future therapeutic strategies. *Int J Urol*;27(2):108-16.
12. Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. 2021 The PRISMA 2020 statement: An updated guideline for reporting systematic reviews. *PLoS Med*;18(3):e1003583.
13. Lee J, Mulder F, Leeflang M, Wolff R, Whiting P, Bossuyt PM. 2022 QUAPAS: An Adaptation of the QUADAS-2 Tool to Assess Prognostic Accuracy Studies. *Ann Intern Med*;175(7):1010-8.
14. 2019 PROBAST: A Tool to Assess the Risk of Bias and Applicability of Prediction Model Studies. *Annals of Internal Medicine*;170(1):51-8.
15. Zucca LER, Laus AC, Sorroche BP, Paro E, Sussuchi L, Marques RF, et al. 2024 Immune-checkpoint gene expression and BCG response in non-muscle invasive bladder cancer. *Translational Oncology*;46:102003.
16. Nam W, Chae HK, Jung Y, Kang H, Park M, Choi A, et al. 2023 HER2 positivity predicts BCG unresponsiveness and adaptive immune cell exhaustion in EORTC risk-stratified cohort of bladder cancer. *Frontiers in Immunology*;14.
17. Tan X, Liu Z, Cai T, Wang Y, Wu Z, Qin Z, et al. 2023 Prognostic Significance of HER2 Expression in Patients with Bacillus Calmette-Guérin-exposed Non-muscle-invasive Bladder Cancer. *European urology oncology*.
18. Martínez R, Tapia G, De Muga S, Hernández A, Cao MG, Teixidó C, et al. 2019 Combined assessment of peritumoral Th1/Th2 polarisation and peripheral immunity as a new biomarker in the prediction of BCG response in patients with high-risk NMIBC. *OncolImmunology*;8(8).
19. Fukumoto K, Kikuchi E, Mikami S, Hayakawa N, Matsumoto K, Niwa N, Oya M. 2018 Clinical Role of Programmed Cell Death-1 Expression in Patients with Non-muscle-invasive Bladder Cancer Recurring After Initial Bacillus Calmette-Guérin Therapy. *Annals of surgical oncology*;25(8):2484-91.
20. Mano R, Zilber S, Di Natale R, Kedar D, Lifshitz D, Yossepowitch O, et al. 2018 Heat shock proteins 60 and 70 are associated with long-term outcome of T1-stage high-grade urothelial tumors of the bladder treated with intravesical Bacillus Calmette-Guérin immunotherapy. *Urologic oncology*;36(12):531.e9-.e17.

21. Dowell AC, Cobby E, Wen K, Devall AJ, During V, Anderson J, et al. 2017 Interleukin-17-positive mast cells influence outcomes from BCG for patients with CIS: Data from a comprehensive characterisation of the immune microenvironment of urothelial bladder cancer. *PLoS ONE*;12(9).
22. Cormio L, Sanguedolce F, Cormio A, Massenio P, Pedicillo M, Cagiano S, et al. 2017 Human epidermal growth factor receptor 2 expression is more important than Bacillus Calmette Guérin treatment in predicting the outcome of T1G3 bladder cancer. *Oncotarget*;8(15):25433-41.
23. Miyake M, Tatsumi Y, Gotoh D, Ohnishi S, Owari T, Iida K, et al. 2017 Regulatory T Cells and Tumor-Associated Macrophages in the Tumor Microenvironment in Non-Muscle Invasive Bladder Cancer Treated with Intravesical Bacille Calmette-Guérin: A Long-Term Follow-Up Study of a Japanese Cohort. *International journal of molecular sciences*;18(10).
24. Pichler R, Fritz J, Zavadil C, Schäfer G, Culig Z, Brunner A. 2016 Tumor-infiltrating immune cell subpopulations influence the oncologic outcome after intravesical Bacillus Calmette-Guérin therapy in bladder cancer. *Oncotarget*;7(26):39916-30.
25. Gaya J, López-Martínez J, Karni-Schmidt O, Bonal D, Algaba F, Palou J, et al. 2015 ΔNp63 expression is a protective factor of progression in clinical high grade T1 bladder cancer. *United States*2015-4. 1144-50 p.
26. Bertz S, Otto W, Denzinger S, Wieland W, Burger M, Stöhr R, et al. 2014 Combination of CK20 and Ki-67 immunostaining analysis predicts recurrence, progression, and cancer-specific survival in pT1 urothelial bladder cancer. *European urology*;65(1):218-26.
27. Lima L, Oliveira D, Tavares A, Amaro T, Cruz R, Oliveira M, et al. 2014 The predominance of M2-polarised macrophages in the stroma of low-hypoxic bladder tumors is associated with BCG immunotherapy failure. *Urologic oncology*;32(4):449-57.
28. Oderda M, Ricceri F, Pisano F, Fiorito C, Gurioli A, Casetta G, et al. 2013 Prognostic factors including Ki-67 and p53 in Bacillus Calmette-Guérin-treated non-muscle-invasive bladder cancer: a prospective study. *Urol Int*;90(2):184-90.
29. Ajili F, Kourda N, Darouiche A, Chebil M, Boubaker S. 2013 Prognostic value of tumor-associated macrophages count in human non-muscle-invasive bladder cancer treated by BCG immunotherapy. *Ultrastructural pathology*;37(1):56-61.
30. Ajili F, Kaabi B, Darouiche A, Tounsi H, Kourda N, Chebil M, et al. 2012 Prognostic value of Bcl-2 and Bax tumor cell expression in patients with non muscle-invasive bladder

cancer receiving bacillus Calmette-Guerin immunotherapy. *Ultrastructural pathology*;36(1):31-9.

31. Ajili F, Kacem M, Tounsi H, Darouiche A, Enayfer E, Chebi M, et al. 2012 Prognostic impact of angiogenesis in nonmuscle invasive bladder cancer as defined by Microvessel density after immunohistochemical staining for CD34. *Ultrastructural Pathology*;36(5):336-42.
32. Otto W, Rubenwolf P, Burger M, Fritsche H, Rößler W, May M, et al. 2012 Loss of aquaporin 3 protein expression constitutes an independent prognostic factor for progression-free survival: an immunohistochemical study on stage pT1 urothelial bladder cancer. *BMC cancer*;12:459.
33. Alkhateeb SS, Neill M, Bar-Moshe S, Rhijn BV, Kakiashvili DM, Fleshner N, et al. 2011 Long-term prognostic value of the combination of EORTC risk group calculator and molecular markers in non-muscle-invasive bladder cancer patients treated with intravesical Bacille Calmette-Guérin. *Urol Ann*;3(3):119-26.
34. Cormio L, Tolve I, Annese P, Saracino A, Zamparese R, Sanguedolce F, et al. 2010 Retinoblastoma protein expression predicts response to bacillus Calmette-Guérin immunotherapy in patients with T1G3 bladder cancer. *Urol Oncol*;28(3):285-9.
35. Cai T, Nesi G, Dal Canto M, Tinacci G, Mondaini N, Piazzini M, et al. 2010 Loss of Heterozygosis on IFN- α Locus is a Prognostic Indicator of Bacillus Calmette-Guerin Response for Nonmuscle Invasive Bladder Cancer. *Journal of Urology*;183(5):1738-43.
36. Cormio L, Tolve I, Annese P, Saracino A, Zamparese R, Sanguedolce F, et al. 2009 Altered p53 and pRb expression is predictive of response to BCG treatment in T1G3 bladder cancer. *Anticancer Res*;29(10):4201-4.
37. Palou J, Algaba F, Vera I, Rodriguez O, Villavicencio H, Sanchez-Carbayo M. 2009 Protein expression patterns of ezrin are predictors of progression in T1G3 bladder tumours treated with nonmaintenance bacillus Calmette-Guérin. *European urology*;56(5):829-36.
38. Ayari C, LaRue H, Hovington H, Decobert M, Harel F, Bergeron A, et al. 2009 Bladder tumor infiltrating mature dendritic cells and macrophages as predictors of response to bacillus Calmette-Guérin immunotherapy. *European urology*;55(6):1386-95.
39. Queipo-Zaragoza JA, Ruiz-Cerda JL, Vera-Donoso CD, Vera-Sempere F, Budia-Alba A, Jimenez-Cruz JF. 2007 Prognostic value of p53, Ki-67, microstaging and microvessel density in pT1G3 bladder tumors: Creation of risk groups for progression. *Scandinavian Journal of Urology and Nephrology*;41(4):283-9.

40. Saint F, Le Frere Belda MA, Quintela R, Hoznek A, Patard JJ, Bellot J, et al. 2004 Pretreatment p53 nuclear overexpression as a prognostic marker in superficial bladder cancer treated with Bacillus Calmette-Guérin (BCG). *Eur Urol*;45(4):475-82.
41. Asakura T, Takano Y, Iki M, Suwa Y, Noguchi S, Kubota Y, Masuda M. 1997 Prognostic Value of Ki-67 for Recurrence and Progression of Superficial Bladder Cancer. *The Journal of Urology*;158(2):385-8.
42. Lee E, Park I, Lee C. 1997 Prognostic markers of intravesical bacillus Calmette-Guérin therapy for multiple, high-grade, stage T1 bladder cancers. *Int J Urol*;4(6):552-6.
43. Lacombe L, Dalbagni G, Zhang ZF, Cordon-Cardo C, Fair WR, Herr HW, Reuter VE. 1996 Overexpression of p53 protein in a high-risk population of patients with superficial bladder cancer before and after bacillus Calmette-Guérin therapy: correlation to clinical outcome. *J Clin Oncol*;14(10):2646-52.
44. Sanders J, Frasier C, Matulay J, Steuerwald N, Zhu J, Grigg C, et al. 2021 Genomic analysis of response to bacillus Calmette-Guérin (BCG) treatment in high-grade stage 1 bladder cancer patients. *Translational andrology and urology*;10(7):2998-3009.
45. Bastos DA, Mattedi RL, Barreiro R, Dos Santos FF, Buzatto V, Masotti C, et al. 2020 Genomic Biomarkers and Underlying Mechanism of Benefit from BCG Immunotherapy in Non-Muscle Invasive Bladder Cancer. *Bladder Cancer*;6(2):171-86.
46. Batista R, Lima L, Vinagre J, Pinto V, Lyra J, Máximo V, et al. 2020 TERT promoter mutation as a potential predictive biomarker in BCG-treated bladder cancer patients. *International Journal of Molecular Sciences*;21(3).
47. Agundez M, Grau L, Palou J, Algaba F, Villavicencio H, Sanchez-Carbayo M. 2011 Evaluation of the methylation status of tumour suppressor genes for predicting bacillus Calmette-Guérin response in patients with T1G3 high-risk bladder tumours. *European Urology*;60(1):131-40.
48. Alvarez-Múgica M, Cebrian V, Fernández-Gómez JM, Fresno F, Escaf S, Sánchez-Carbayo M. 2010 Myopodin methylation is associated with clinical outcome in patients with T1G3 bladder cancer. *Journal of Urology*;184(4):1507-13.
49. Lopez-Beltran A, Ordóñez J, Otero A, Blanca A, Sevillano V, Sanchez-Carbayo M, et al. 2010 Cyclin D3 gene amplification in bladder carcinoma in situ. *Virchows Archiv : an international journal of pathology*;457(5):555-61.

50. Piao X, Kim Y, Byun Y, Zheng C, Moon S, Kim K, et al. 2022 Expression of RPL9 predicts the recurrence of non-muscle invasive bladder cancer with BCG therapy. *Urologic oncology*;40(5):197.e1-.e9.
51. Awadalla A, Zahran M, Abol-Enein H, Zekri A, Elbaset M, Ahmed A, et al. 2022 Identification of Different miRNAs and Their Relevant miRNA Targeted Genes Involved in Sister Chromatid Cohesion and Segregation (SCCS)/chromatin Remodeling Pathway on T1G3 Urothelial Carcinoma (UC) Response to BCG Immunotherapy. *Clinical genitourinary cancer*;20(3):e181-e9.
52. Kim YJ, Ha YS, Kim SK, Yoon HY, Lym MS, Kim MJ, et al. 2010 Gene signatures for the prediction of response to Bacillus Calmette-Guerin immunotherapy in primary pT1 bladder cancers. *Clin Cancer Res*;16(7):2131-7.
53. Lima L, Severino P, Silva M, Miranda A, Tavares A, Pereira S, et al. 2013 Response of high-risk of recurrence/progression bladder tumours expressing sialyl-Tn and sialyl-6-T to BCG immunotherapy. *British journal of cancer*;109(8):2106-14.
54. Lim CJ, Nguyen PHD, Wasser M, Kumar P, Lee YH, Nasir NJM, et al. 2020 Immunological Hallmarks for Clinical Response to BCG in Bladder Cancer. *Front Immunol*;11:615091.
55. Lima L, Ferreira JA, Tavares A, Oliveira D, Morais A, Videira PA, et al. 2014 FASL polymorphism is associated with response to bacillus Calmette-Guérin immunotherapy in bladder cancer. *Urol Oncol*;32(1):44.e1-7.
56. Nishikawa R, Miyake M, Morizane S, Shimizu R, Teraoka S, Honda M, et al. 2023 C-reactive protein as a prognostic predictor for non-muscle invasive bladder cancer after intravesical bacillus Calmette-Guérin therapy: A Japan Urological Oncology Group study analysis. *International Journal of Urology*;30(3):299-307.
57. Zhong W, Wang B, Yu H, Lin J, Xia K, Hou W, et al. 2020 Serum CCL27 predicts the response to Bacillus Calmette-Guerin immunotherapy in non-muscle-invasive bladder cancer. *Oncoimmunology*;9(1):1776060.
58. Mbeutcha A, Shariat S, Rieken M, Rink M, Xylinas E, Seitz C, et al. 2016 Prognostic significance of markers of systemic inflammatory response in patients with non-muscle-invasive bladder cancer. *Urologic oncology*;34(11):483.e17-.e24.
59. Kobayashi M, Fujiyama N, Tanegashima T, Narita S, Yamamoto Y, Fujimoto N, et al. 2022 Effect of HLA genotype on intravesical recurrence after bacillus Calmette-Guérin therapy for non-muscle-invasive bladder cancer. *Cancer immunology, immunotherapy* : CII;71(3):727-36.

60. Ryk C, Koskela LR, Thiel T, Wiklund NP, Steineck G, Schumacher MC, de Verdier PJ. 2015 Outcome after BCG treatment for urinary bladder cancer may be influenced by polymorphisms in the NOS2 and NOS3 genes. *Redox Biology*;6:272-7.
61. Lima L, Oliveira D, Ferreira JA, Tavares A, Cruz R, Medeiros R, Santos L. 2015 The role of functional polymorphisms in immune response genes as biomarkers of bacille Calmette-Guérin (BCG) immunotherapy outcome in bladder cancer: Establishment of a predictive profile in a Southern Europe population. *BJU International*;116(5):753-63.
62. Jaiswal PK, Goel A, Mandhani A, Mittal RD. 2012 Functional polymorphisms in promoter survivin gene and its association with susceptibility to bladder cancer in north indian cohort. *Molecular Biology Reports*;39(5):5615-21.
63. Chiong E, Kesavan A, Mahendran R, Chan Y, Sng J, Lim Y, et al. 2011 NRAMP1 and hGPX1 gene polymorphism and response to bacillus Calmette-Guérin therapy for bladder cancer. *European urology*;59(3):430-7.
64. Srivastava P, Jaiswal PK, Singh V, Mittal RD. 2010 Role of p53 gene polymorphism and bladder cancer predisposition in northern India. *Cancer Biomarkers*;8(1):21-8.
65. Qu K, Gu J, Ye Y, Williams SB, Dinney CP, Wu X, Kamat A. 2017 High baseline levels of interleukin-8 in leukocytes and urine predict tumor recurrence in non-muscle invasive bladder cancer patients receiving bacillus Calmette-Guerin therapy: A long-term survival analysis. *Oncoimmunology*;6(2):e1265719.
66. Elsayy AA, Abol-Enein H, Laymon M, Ahmed AE, Essam A, Hamam ET, et al. 2022 Predictive value of immunological markers after bacille Calmette-Guérin induction in bladder cancer. *BJU Int*;130(4):444-53.
67. Murakami K, Kamat AM, Dai Y, Pagano I, Chen R, Sun Y, et al. 2022 Application of a multiplex urinalysis test for the prediction of intravesical BCG treatment response: A pilot study. *Cancer Biomarkers*;33(1):151-7.
68. Cai T, Nesi G, Mazzoli S, Meacci F, Tinacci G, Luciani LG, et al. 2012 Prediction of response to bacillus Calmette-Guérin treatment in non-muscle invasive bladder cancer patients through interleukin-6 and interleukin-10 ratio. *Exp Ther Med*;4(3):459-64.
69. Watanabe E, Matsuyama H, Matsuda K, Ohmi C, Tei Y, Yoshihiro S, et al. 2003 Urinary interleukin-2 may predict clinical outcome of intravesical bacillus Calmette-Guérin immunotherapy for carcinoma in situ of the bladder. *Germany*2003-8. 481-6 p.
70. Muto S, Lu Y, Ide H, Yamaguchi R, Saito K, Kitamura K, et al. 2021 The Use of Urine Mycobacterium tuberculosis Complex Polymerase Chain Reaction as a Predictive Factor

for Recurrence and Progression After Intravesical Bacillus Calmette-Guérin Therapy in Patients with Non-muscle-invasive Bladder Cancer. *Eur Urol Open Sci*;27:10-8.

71. Orihuela E, Varadachay S, Herr H, Melamed M, Whitmore WJ. 1987 The practical use of tumor marker determination in bladder washing specimens. Assessing the urothelium of patients with superficial bladder cancer. *Cancer*;60(5):1009-16.

72. Villoldo G, Pombo M, Aris M, Chemi J, Mandó P, Nagaraju S, et al. 2023 A Th2-score in the tumor microenvironment as a predictive biomarker of response to Bacillus Calmette Guérin in patients with non-muscle invasive bladder carcinoma: A retrospective study. *Oncology research*;31(2):207-20.

73. El-Babouly IM, Desoky EAE, El Sayed D, Ali MM, Harb OA, Ragab A, et al. 2018 The role of neural precursor cell-expressed developmentally down-regulated protein 9 in predicting bacillus Calmette-Guerin response in nonmuscle invasive bladder cancer. *Urologic Oncology: Seminars and Original Investigations*;36(5):242.e9-.e14.

74. Nunez-Nateras R, Castle EP, Protheroe CA, Stanton ML, Ocal TI, Ferrigni EN, et al. 2014 Predicting response to bacillus Calmette-Guérin (BCG) in patients with carcinoma in situ of the bladder. *Urologic Oncology: Seminars and Original Investigations*;32(1):45.e23-45.e30.

75. Ilijazi D, Pulverer W, Ertl I, Lemberger U, Kimura S, Abufaraj M, et al. 2020 Discovery of Molecular DNA Methylation-Based Biomarkers through Genome-Wide Analysis of Response Patterns to BCG for Bladder Cancer. *Cells*;9(8).

76. Kamat AM, Briggman J, Urbauer DL, Svatek R, Noguera González GM, Anderson R, et al. 2016 Cytokine Panel for Response to Intravesical Therapy (CyPRIT): Nomogram of Changes in Urinary Cytokine Levels Predicts Patient Response to Bacillus Calmette-Guérin. *Eur Urol*;69(2):197-200.

77. Koyama A. 2008 Re: History of bacillus Calmette-Guerin and bladder cancer: an immunotherapy success story: H. W. Herr and A. Morales. *J Urol* 2008; 179: 53-56. *J Urol*;180(5):2255; author reply

78. Mollier S, Descotes JL. 1997 Prognostic value of KI67 immunostaining in transitional cell carcinoma of the bladder stage pT1. *British Journal of Urology*;80:29.

79. Adhit KK, Wanjari A, Menon S, K S. 2023 Liquid Biopsy: An Evolving Paradigm for Non-invasive Disease Diagnosis and Monitoring in Medicine. *Cureus*;15(12):e50176.

80. Lodewijk I, Duenas M, Rubio C, Munera-Maravilla E, Segovia C, Bernardini A, et al. 2018 Liquid Biopsy Biomarkers in Bladder Cancer: A Current Need for Patient Diagnosis and Monitoring. *Int J Mol Sci*;19(9).
81. Jiang S, Redelman-Sidi G. 2022 BCG in Bladder Cancer Immunotherapy. *Cancers (Basel)*;14(13).
82. Taniguchi K, Koga S, Nishikido M, Yamashita S, Sakuragi T, Kanetake H, Saito Y. 1999 Systemic immune response after intravesical instillation of bacille Calmette-Guérin (BCG) for superficial bladder cancer. *Clin Exp Immunol*;115(1):131-5.
83. Lobo N, Hensley PJ, Bree KK, Nogueras-Gonzalez GM, Navai N, Dinney CP, et al. 2022 Updated European Association of Urology (EAU) Prognostic Factor Risk Groups Overestimate the Risk of Progression in Patients with Non-muscle-invasive Bladder Cancer Treated with Bacillus Calmette-Guérin. *Eur Urol Oncol*;5(1):84-91.

PART II

Predicting BCG Failure in Bladder Cancer Patients: a microRNA-Driven Biomarker Approach

Rui Ribeiro-Pereira, Ana Teixeira-Marques, Cláudia Martins-Lima, Rui Freitas, João André Carvalho, Jorge Ferreira, Marta Dueñas, Sara Monteiro-Reis, Rui Henrique, Carmen Jerónimo

Manuscript Under Preparation

Abstract

Bladder cancer (BlCa) represents a significant global health burden. High-risk non-muscle-invasive bladder cancer (NMIBC) is commonly treated with *Bacillus Calmette-Guérin* (BCG) instillations. Despite its widespread use, this therapy has efficacy limitations due to suboptimal response rates and adverse effects presented by some patients. Moreover, the lack of a clinically validated biomarker to predict treatment outcomes poses a substantial challenge in optimising patient management. MicroRNAs have emerged as promising candidates for non-invasive liquid biopsy biomarkers in BlCa. Their involvement in tumorigenesis, progression, and treatment response underscores their potential utility in guiding therapeutic decisions. However, the integration of miRNA-based biomarkers into clinical guidelines for BCG therapy remains elusive.

This study sought to validate a previously identified four-miRNA signature using droplet digital PCR (ddPCR) analysis of urine sediments from BCG-treated patients. The signature, comprising hsa-miR-483-3p, hsa-miR-579-3p, hsa-miR-874-5p, and hsa-miR-4443, demonstrated significant discriminatory capability between true BCG responders and non-responders when combined in panel. Incorporation of this signature into a nomogram model further enhanced predictive accuracy, by retaining their significance in the multivariate model, achieving 58% sensitivity and 76% specificity values. Moreover, based on this model, high-risk patients showed increased risk in non-responding to BCG when compared to low-risk individuals.

These findings underscore the potential of miRNA-based biomarkers to improve patient selection for BCG therapy and reduce the burden of unnecessary treatment. By accurately identifying patients most likely to benefit from BCG, this approach can contribute to more personalised and effective care for individuals with BlCa.

Introduction

Bladder cancer (BlCa) is the ninth most common cancer globally, with 614,000 newly diagnosed cases in 2022, while being the thirteenth leading cause of death, claiming nearly 220,000 lives in the same year (1). Moreover, BlCa is among the most costly cancers to treat per patient, imposing a significant socio-economic burden (2). Most BlCa cases originate from the urothelium and are often referred to as urothelial cell carcinomas, accounting for 90% of all cases. BlCa is classified into non-muscle invasive BlCa (NMIBC, 75-80% of cases) and muscle-invasive BlCa (MIBC, 20-25% of cases), based on muscle invasion. These two types often arise from distinct molecular pathways and have different prognoses and treatments (3, 4).

Based on the histopathologic characteristics of BICa and other clinical factors, patients with NMIBC are usually categorised according to prognostic groups, which help foresee the course of treatment (5, 6). Of those, high-risk patients often receive BCG treatment, which acts by delaying disease recurrence and progression (5, 6). BCG, an immunotherapy agent, is administered via intravesical instillation, targeting the bladder mucosa. This intervention elicits a robust immune response within the bladder lining, resulting in the selective destruction of adjacent tumour cells (7). However, this approach overlooks BICa molecular heterogeneity and complexity, leading to a 30-40% relapse rate (8, 9). Currently, there are no FDA/CE-approved biomarkers to identify patients who might benefit from BCG therapy prospectively. Furthermore, discovering predictive and prognostic biomarkers for BCG immunotherapy response would significantly enhance patient prognosis and prevent ineffective treatment for non-responding patients, leading to better patients' life quality and diminished healthcare costs (10).

Epigenetic mechanisms play a significant role in the development and progression of cancer, being now considered an important hallmark of cancer (11). In recent years, many studies have been implicating microRNAs (miRNAs) deregulation in the tumorigenesis, and tumour treatment response. MicroRNAs are essentially constituted by sequences of 17-25 nucleotides that interact with protein-coding mRNAs, mostly leading to target degradation or translation inhibition (12, 13). MiRNAs' role in key cell functions, abundance in body fluids and easy/low-cost detecting methods make them promising disease biomarkers (14).

Indeed, the use of miRNAs as biomarkers has already been reported in a few studies, although none of them have been implemented in clinical practice (15, 16). This may be due to inconsistent outcomes across various publications and the absence of testing this preliminary data on separate robust independent validation cohorts (17, 18). Additionally, there is limited knowledge about the changes in miRNA expression patterns during treatment. Consequently, new insights about previously identified miRNAs are needed to advance patients' response to treatment prediction tools. The current work, developed in the scope of the UCIPredict project Consortium (TRANSCAN3/0001/2021), aimed at validating the prognostic power of a four-microRNA signature, previously identified as being differentially expressed in tumours of BCG responders versus non-responders, and in urines of an independent cohort of patients.

Methods

Patient Selection

This study involved 79 NMIBC patients treated with BCG instillations between 2015 and 2024 at the Portuguese Institute of Oncology of Porto (IPO Porto). After undergoing

transurethral resection of bladder tumours (TURBT), patients typically received six weekly intravesical BCG instillations as induction therapy. In responders, maintenance therapy, comprising BCG instillations every three months for one to three years, was initiated to optimise treatment outcomes (19). Detailed patients' clinicopathological information is displayed in Table 1. All urine samples were collected prior to the initiation of the BCG treatment and at the last treatment moment available. Additionally, time of follow-up was considered from the first TURBT performed until the latest BCG treatment, except in cases of recurrence and/or progression. This study was approved by the ethics committee of IPO Porto (CES_62/023).

Table 1: Demographic and clinicopathological features of the NMIBC patients in the retrospective cohort.

Demographic and Clinicopathological Features	NMIBC Cases
Patients, <i>n</i>	79
Mean Age, yrs (range)	68.1 (46-89)
Mean Follow-up, mos (range)	19.2 (1-62)
Gender, <i>n</i>	
Males	66
Females	13
Histological Grade, <i>n</i>	
LG	15
HG	64
Pathological Stage, <i>n</i>	
pTis	2
pTa	22
pT1	55
Smoker Status, <i>n</i>	
Non-Smoker	25
Active Smoker	20
Former Smoker	34
Treatment Response, <i>n</i>	
BCG Responders	42
BCG Failures	37
Disease Recurrence, <i>n</i>	
No	67
Yes	12
Disease Progression, <i>n</i>	

No	73
Yes	6

NMIBC (Non-muscle Invasive Bladder Cancer); LG (Low-grade Tumour); HG (High-grade Tumour); yrs (years); mos (months); BCG (*Bacillus Calmette-Guérin*).

Sample Processing

All urine samples were processed within four hours after collection by double centrifugation at a minimum of 229 g and a double wash with phosphate-buffered saline (PBS). After pelleting the exfoliated cells, the urine pellets were then stored at -80°C until further use.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from the urine pellet samples by the conventional tryzol isolation method. As a technical control, a non-human synthetic spike-in, ath-miR-159a, was added to all sample mixtures with the tryzol reagent. Afterwards, the conversion of RNA to cDNA was carried out using the TaqMan™ MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's protocol. Five microliters of previously isolated RNA were reversely transcribed in a Veriti (Applied Biosystems™, Waltham, MA, USA) or GeneAmp PCR System 2700 (ThermoFisher Scientific, Waltham, MA, USA) thermal cyclers for the miRNAs of interest and the respective spike-in (ath-miR-159a, hsa-miR-483-3p, hsa-miR-579-3p, hsa-miR-874-5p, hsa-miR-4443).

Droplet Digital PCR (ddPCR)

microRNAs levels were evaluated by droplet digital PCR (ddPCR) (Bio-Rad Laboratories, Hercules, CA, USA) in 96-well plates and according to previous optimisations for cDNA input and annealing temperature performed by our research team (20). Briefly, each well contained a specific amount of cDNA input (2 µL of cDNA for ath-miR-159a; 3 µL of cDNA for hsa-miR-483-3p and hsa-miR-4443; 6 µL of cDNA for hsa-miR-579-3p and hsa-miR-874-5p), 1 µL of TaqMan hsa-miRNA Assay (5X) (hsa-miR-483-3p: 002339 - FAM; hsa-miR-579-3p: 002398 - FAM; hsa-miR-874-5p: 467234_mat - FAM; hsa-miR-4443: 463010_mat - FAM), 11 µL of 2X ddPCR Supermix for probes (no dUTP) (Bio-Rad Laboratories, Hercules, CA, USA) and a specific volume of bi-distilled water (8 µL for ath-miR-159a; 7 µL for hsa-miR-483-3p and hsa-miR-4443; 4 µL of cDNA for hsa-miR-579-3p and hsa-miR-874-5p) to ensure a final volume of 22 µL per well. For droplet generation, 20 µL of the reaction mix and 70 µL of Droplet Generation Oil for Probes (Bio-Rad Laboratories, Hercules, CA, USA) were pipetted into cartridges and then placed on the QX200 Droplet

Generator (Bio-Rad Laboratories, Hercules, CA, USA). Subsequently, 96-well plates were placed on the C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA) and the PCR run was set as described: 95°C for 10 min, 50 cycles of 94°C for 30 s, optimised Annealing Temperature (56°C for the ath-miR-159a and the hsa-miR-4443; 54°C for hsa-miR-483-3p, hsa-miR-579-3p and hsa-miR-874-5p) for 1 min; ramp rate 2°C/s and 98°C for 10 min. Ultimately, droplets were read on the QX200 Droplet Reader (Bio-Rad Laboratories, Hercules, CA, USA).

The limit of the blank (LOB) and the limit of detection (LOD) were calculated for each target microRNA according to (21, 22). A target was considered positive when the value of positive droplets was equal to or higher than LOD.

Statistical Analysis

Non-parametric tests were performed to compare and assess the levels of the microRNAs to differentiate BCG responders from failures. Mann-Whitney was used for two group comparisons. For each microRNA, the respective cases were categorised into “high” or “low expression levels” based on cutoff values determined by the Youden J index. This index combines the highest sensitivity and specificity obtained from the Receiver-Operating Characteristic (ROC) curve analysis. The ROC curves were generated through the true positive rates (sensitivity) against the false positive rates (1 - specificity). Subsequently, the respective areas under the curve (AUC) were computed (23).

The performance of the predictive biomarker was evaluated by determining sensitivity and specificity. Different combinations of panels were investigated to enhance the predictive capability of the chosen microRNAs. A positive outcome was recorded if at least one target microRNA was identified as positive in the analysis. Survival analysis was conducted using the Kaplan–Meier curves method, with the log-rank test used to compare treatment responses between groups considering clinicopathological variables and miRNA levels status.

To calculate the risk of non-response, nomograms were developed using multivariable logistic regression analysis in RStudio. The performance of the models was evaluated using ROC curves and area under the curve (AUC) calculations, with the assistance of Rms, caTools, epicalc, and pROC packages. Clinical and pathological data such as age at diagnosis (age_cat), grade, previous treatment, and smoking status when constructing the nomograms. Response to treatment was used as a dependent variable. The Youden J Index was used to establish the ideal cutoff based on the linear predictor. The equivalent risk value was determined using the formula: $1 / (1 + e^{-\text{linear predictor}})$. A result was considered statistically significant when the p-value < 0.05. The GraphPad Prism (version 8.0) (GraphPad

Software, San Diego, CA, USA), IBM SPSS Statistics ver. 29.0 (IBM Co., Armonk, NY, USA) and RStudio (R software, version 4.3.1) were used for the analyses.

Results

Sample's Description

The BCG set consisted of samples from 79 patients with NMIBC who had received BCG therapy at two different timepoints: the first included urine samples collected before the initiation of BCG therapy, and the second included urine samples collected upon the last completed BCG instillation treatment. As a result, the initial cohort comprised 158 samples, from which two were excluded due to insufficient RNA, resulting in a final cohort of 156 urine samples.

MicroRNA Levels of the Individual Targets

Overall, the levels of the analysed miRNAs were very similar in the urines of BCG-responsive and non-responsive patients before and after BCG treatment. Additionally, no significant differences were observed for any microRNA levels before and after treatment in BCG responders and non-responders (Figure 1), even when analysing paired urine samples (Figure S1) (Mann Whitney, $p > 0.05$).

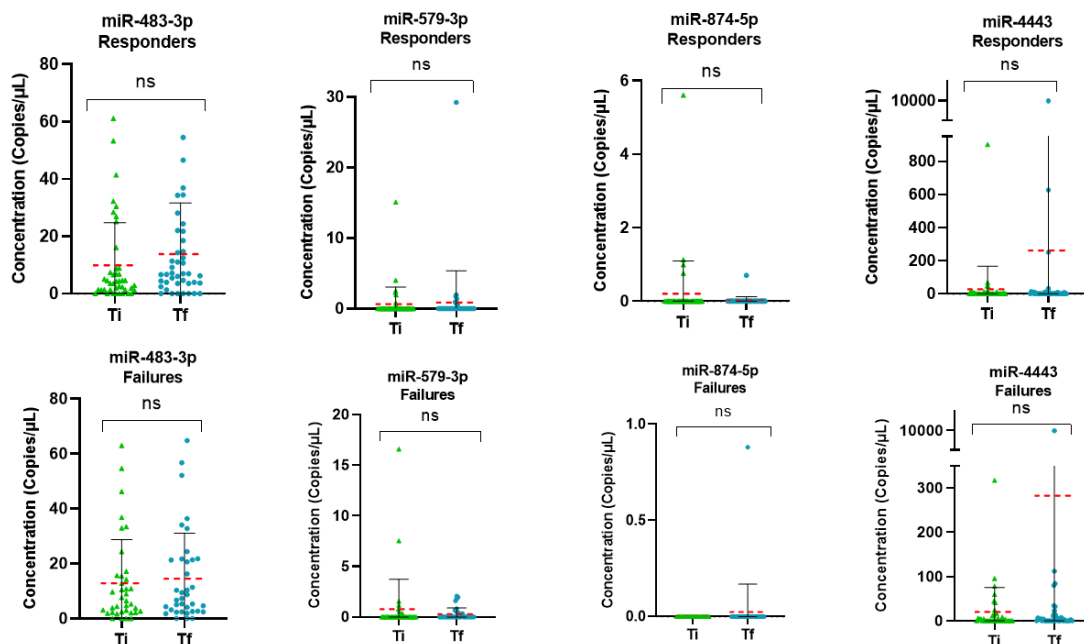


Figure 1: Variation of microRNA levels at the beginning and end of BCG treatment in both treatment response groups. ns: non-significant, Ti: initial timepoint, Tf: final timepoint.

All analysed microRNA levels (hsa-miR-483-3p, hsa-miR-579-3p, hsa-miR-874-5p and hsa-miR-4443) were not statistically different between the two treatment groups (Figure 2).

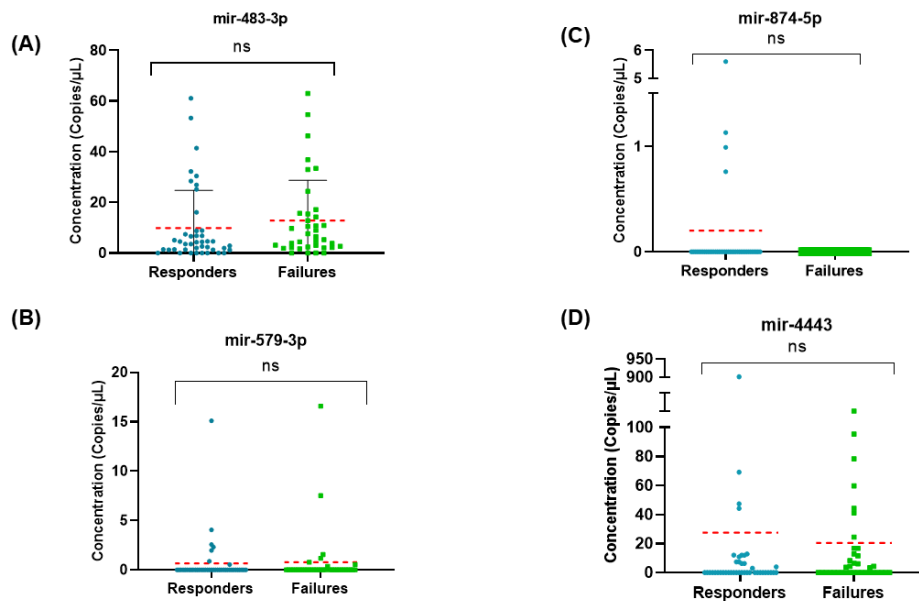


Figure 2: hsa-miR-483-3p, hsa-miR-579-3p, has-miR-874-5p and miR-4443 levels in BCG treatment patients. The microRNA levels in the urine samples prior to BCG from the 79 NMIBC patients were distributed according to their response status: Responders (n=42) and Failure (n=37). ns: non-significant

MicroRNA Levels, Performance and Survival Analysis of the microRNA Panel

Following the individual analysis of each target microRNA, all possible combinations of markers were studied to identify the most appropriate panels that could effectively distinguish between the BCG treatment groups, as no optimal performance was observed for each tested miR alone. Thus, several combinations of microRNA markers were assessed, considering whether at least 1, 2, 3, or all 4 markers were positive. These combinations were gradually evaluated, beginning with the inclusion of two markers and continuing until all four markers were included. All of the tested panels were able to significantly differentiate between the two treatment groups ($p < 0.05$) (Table S1). The cutoff value for each microRNA was determined based on the respective Responders' and Non-Responders' ROC Curve analysis (Figure S2).

The most promising panel identified included a combination of hsa-miR-483-3p, hsa-miR-579-3p, and hsa-miR-4443. The result was considered positive if at least one of these microRNAs was highly expressed (Figure 3A). This panel presented a 56.76% sensitivity and a 76.19% specificity, resulting in an AUC of 0.6647 (Table 2) and 67.09% accuracy (Table 2). Regarding survival analysis, patients with a positive result of the panel presented a 4.541-fold increased risk of not responding to the treatment than the patients with tumours with lower expression levels (Figure 3B).

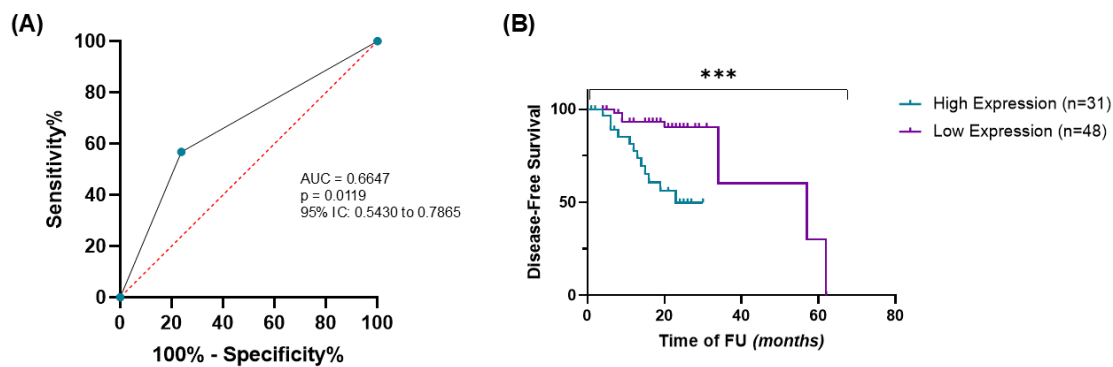


Figure 3: Performance and Survival Analysis of the Panel. (A) ROC Curve evaluating the performance of the Panel. **(B)** Kaplan-Meier Survival Curve for DFS based on the hsa-miR-483-3p/hsa-miR-579-3p/hsa-miR-4443 levels.

Table 2: Panel performance in distinguishing BCG response in BICa Patients.

Panel	TP	TN	FP	FN	Cutoff	SN	SP	PPV	NPV	Accuracy	AUC
483/579/4443	21	32	10	16	0.5	56.76%	76.19%	67.74%	66.67%	67.09%	0.6647

TP (True positive); TN (True Negative); FP (False Positive); FN (False Negative); SN (Sensitivity); SP (Specificity); PPV (Positive Predictive Value); NPV (Negative Predictive Value); AUC (Area Under the Curve).

Nomogram Model of the Targets in the microRNA Panel

To validate the previous results, several pipeline models were created and assessed to identify the model/panel that most accurately predicted BCG treatment response. In addition to the respective microRNA levels, each developed model included clinicopathological variables such as age at diagnosis, tumour grade, previous treatment history, smoking status, and response to treatment, as these are important risk factors for BICa patients. The gender variable was excluded from the pipelines due to the unequal distribution of cases, with 65 males and 13 females.

The nomogram of the model composed by hsa-miR-483-3p, hsa-miR-579-3p and hsa-miR-4443 is represented by the subsequent formula: Risk of non-response = $-3.95736 + 0.24026 \times \text{Smoke} - 0.01844 \times \text{Age_cat} + 0.46238 \times \text{Previous_Treatment} + 1.50385 \times \text{Grade} + 1.66483 \times \text{Panel}$, resulting in the generation of a risk model. Described information considering other parameters is presented in Table 3.

Table 3: Summary of the logistic model representative of the Risk Model.

Model	Coefficient	Odds Ratio	S.E.	Wald Z	p-value	95% Confidence Interval	
						Lower Bound	Upper Bound
Intercept	-3.95736	0.01911	1.62795	-2.431	0.01506	0.00079	0.46458
Smoke Status	0.24026	1.27158	0.33676	0.713	0.47558	0.65719	2.46032
Age_cat	-0.01844	0.98173	0.54101	-0.034	0.97282	0.34001	2.83465
Previous_treatment	0.46238	1.58785	0.39712	1.164	0.24429	0.72909	3.45807

Grade	1.50385	4.49898	0.73817	2.037	0.04162	1.05873	19.11799
Panel	1.66483	5.28477	0.54875	3.034	0.00241	1.80274	15.49241

S.E. (Standard Error).

By logistic multivariable model, 58.33% sensitivity and 78.57% specificity were attained (Table 4), augmenting the values obtained by the panel in univariable analysis. Furthermore, the nomogram showed a 0.7370 AUC (IC 95%: 0.628 to 0.847) in predicting BCG treatment response (Figure 4B).

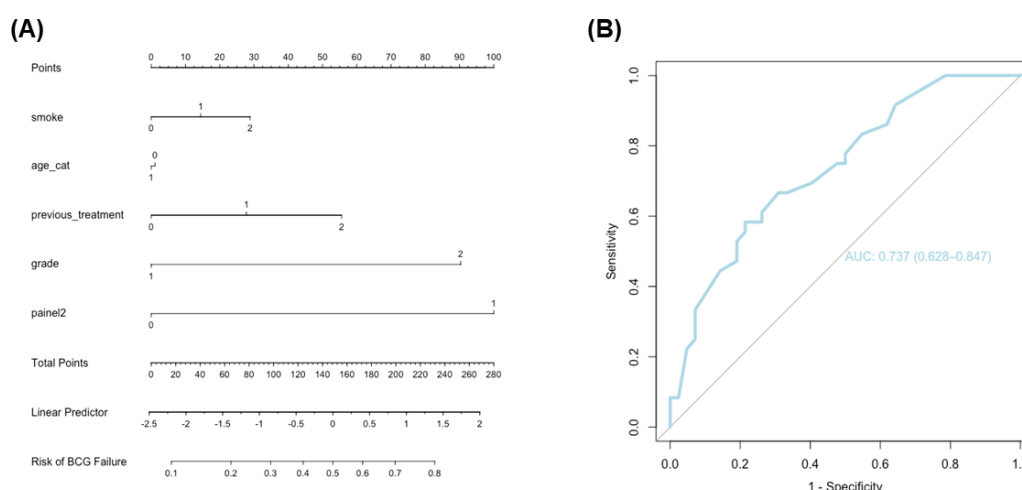


Figure 4: (A) Nomogram representative of the Risk of the Model, with the relevant clinicopathological variables: age at diagnosis (0: ≤68, 1: >68 years), smoke status (0: non-smoker, 1: former smoker, 2: active smoker), previous treatment (0: no previous treatment, 1: Mitomycin C, 2: BCG) and grade (1: low grade, 2: high grade). **(B) ROC curve to evaluate the performance of the nomogram model.** Panel categorisation- 0: low levels, 1: high levels.

Moreover, considering the disease-free survival analysis (Figure 5), high-risk patients presented a 4.217-fold increased risk of not responding to the treatment when compared to low-risk patients. Additionally, based on the linear predictor, patients with a ponderation higher than the 0.665 cutoff value were more likely not respond to the BCG treatment.

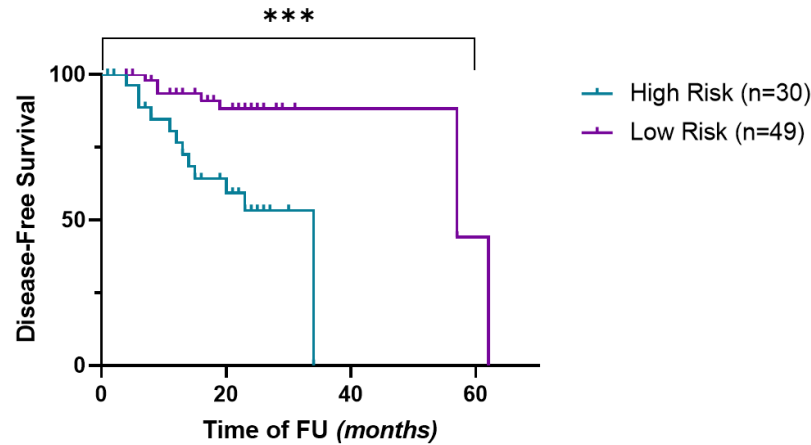


Figure 5: Kaplan-Meier Survival Curve for DFS based on patient stratification risk model. FU: follow-up.

Table 4: Model Performance combining the different microRNAs and clinicopathological variables of interest in distinguishing BCG response in BICa Patients.

Multivariate Model												
Panel	TP	TN	FP	FN	Cutoff	LP	SN	SP	PPV	NPV	Accuracy	AUC
483/579/4443	21	33	9	15	0.513794	0.665	58.33%	78.57%	70.00%	68.75%	69.23%	0.7370

TP (True positive); TN (True Negative); FP (False Positive); FN (False Negative); LN (Linear Predictor) SN (Sensitivity); SP (Specificity); PPV (Positive Predictive Value); NPV (Negative Predictive Value); AUC (Area Under the Curve).

Discussion

High-risk NMIBC is one of the most commonly diagnosed forms of BICa and presents significant challenges, as patients experience high rates of relapse (8, 9). The standard treatment for these patients consists of periodical installations with BCG, the first immunotherapy drug, approved for the treatment of any cancer in 1976 (24). However, few efforts have been made to improve this treatment, and those attempted, like combining atezolizumab with BCG (25) have posed a significant financial burden on healthcare and led to high relapse rates with limited patient benefit, mainly because distinguishing true BCG therapy responders from non-responders remains challenging. Despite significant efforts to discover new biomarkers for predicting BCG treatment response, none have entered clinical practice largely due to the lack of validation in different clinical settings and the need for larger, robust independent cohort studies before clinical trials. Thus, this study aimed to validate a previously discovered four-microRNA signature (hsa-mir-483-3p, hsa-miR-579-3p, hsa-miR-874-5p and hsa-miR-4443) as a biomarker tool for predicting BCG treatment response.

The biological functions of the four microRNA targets were already previously assessed by others. For instance, hsa-miR-483-3p is known as a polycistronic microRNA that has a role in cell proliferation and apoptosis in hepatocellular carcinoma, neuroblastoma and breast cancer (26-28). hsa-miR-579-3p is an intronic microRNA known to act as a tumour suppressor or, less commonly, as an oncogene depending on the cancer model context (29). On the other hand, hsa-miR-874-5p is also a possible intronic microRNA that is acknowledged for inhibiting cancer cell growth and invasion in osteosarcoma and hepatocellular carcinoma (30, 31). Lastly, hsa-miR-4443 is an intergenic microRNA with tumor-suppressive and oncogenic roles, regulating, for example, cancer progression and drug resistance (32).

When analysing the oscillation levels of each microRNA before and after the BCG treatment in our study, no differences were observed regarding the levels between both treatment groups, indicating that microRNA levels' variation during the therapy does not associate with the patient's outcome. Moreover, the individual miRNAs were not able to discriminate the two analysed groups, possibly due to the limited number of cases, as these samples are very challenging to obtain. Nevertheless, a comprehensive panel analysis and BCG prediction response biomarkers validation are mandatory. Herein this issue was tackled in a small retrospective series of 79 NMIBC urine samples obtained from an IPO Porto BICa cohort of patients, though important and new results were found.

Following the selection of optimal cutoff values for each marker, combined performances of the different markers were attempted. Indeed, contrary to the individual analysis, the generated panels could significantly discriminate BCG responders from non-responders with rather good performance. For instance, the panel composed of hsa-miR-483-3p and hsa-miR-4443 was one of the most specific panels (76.19%) and the least sensitive one (54.05%) in predicting response to treatment, while the panel composed of the four microRNAs provided the opposite outcome, with higher sensitivity and lowest specificity, being 56.76% and 73.81%, respectively. Of note, increased costs may result if more markers are added to a panel, which may also impact in the reproducibility of the results. This approach relies on significant performance improvement, which is not evident in this case. Nonetheless, the panel composed of hsa-miR-483-3p, hsa-miR-579-3p and hsa-miR-4443 surfaced as the best performer in identifying non-responders with 56.76% sensitivity and 76.19% specificity. Moreover, higher microRNA levels were significantly associated with worse outcomes. Importantly, all the designed panels successfully predicted the BICa patients' response status, including recurrence, progression, or intolerance).

Ultimately, multivariate models were formulated including age, smoking status, previous treatment, and grade were used to create the most accurate model possible. Gender was also considered an important variable in BICa, as it is known that BICa is four times more incident in men than in women, and females usually own higher-grade disease associated with worse outcomes (33). Nevertheless, the reduced number of available samples from female patients, gender ended up not being included. Herein, grade and the miRs panel emerged as the sole significant input variables across all models. According with international guidelines, BCG therapy is given to intermediate and high-risk BICa patients' (34), and our cohort mirrors exactly that thus validating the observed findings. Remarkably, after the evaluation of the performance of all the multivariate models using ROC curves, the best model (hsa-mir-483-3p, hsa-miR-579-3p and hsa-miR-4443), improved both sensitivity and specificity by 2% with a new AUC of 0.7370. Additionally, DFS analysis linked to the multivariate model demonstrated that high-risk patients showed significantly decreased likelihood of response compared to low-risk counterparts. Importantly, this multivariate model identified the patients who truly will benefit from the therapy with higher accuracy, reducing the number of patients undergoing costly and aggressive treatments that would be ineffective, thereby preserving quality of life and lowering associated costs. Moreover, with no approved markers for predicting BCG response, this model offers a new and useful tool for patients to avoid invasive treatments like cystoscopy, the current standard of care for monitoring patients and detecting possible recurrences/progressions, and might also be more cost-effective.

Although one should acknowledge that different molecular mechanisms may contribute to treatment failure at different moments and that previous treatments to BCG instillations may also influence response, future steps include increasing the sample size and conducting analyses in larger, independent cohorts.

Conclusion

In this study, four microRNA signatures identified by collaborators of a consortium showed good performance in distinguishing BCG responders from non-responders. Moreover, a developed multivariable model was able to significantly increase the panel's accuracy in predicting BCG response, constituting an important step for future implementation in clinical practice. Larger-scale studies are now necessary to reliably validate the accuracy of these markers and pave the way for their application prior to BCG therapy in NMIBC patients.

References

1. Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I, Jemal A. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2024.
2. Svatek RS, Hollenbeck BK, Holmäng S, Lee R, Kim SP, Stenzl A, Lotan Y. The Economics of Bladder Cancer: Costs and Considerations of Caring for This Disease. *European Urology*. 2014;66(2):253-62.
3. Mar N, Dayyani F. Management of Urothelial Bladder Cancer in Clinical Practice: Real-World Answers to Difficult Questions. *J Oncol Pract*. 2019;15(8):421-8.
4. Minoli M, Kiener M, Thalmann GN, Kruithof-de Julio M, Seiler R. Evolution of Urothelial Bladder Cancer in the Context of Molecular Classifications. *Int J Mol Sci*. 2020;21(16).
5. Ritch CR, Velasquez MC, Kwon D, Becerra MF, Soodana-Prakash N, Atluri VS, et al. Use and Validation of the AUA/SUO Risk Grouping for Nonmuscle Invasive Bladder Cancer in a Contemporary Cohort. *J Urol*. 2020;203(3):505-11.
6. Lobo N, Hensley PJ, Bree KK, Nogueras-Gonzalez GM, Navai N, Dinney CP, et al. Updated European Association of Urology (EAU) Prognostic Factor Risk Groups Overestimate the Risk of Progression in Patients with Non-muscle-invasive Bladder Cancer Treated with Bacillus Calmette-Guérin. *Eur Urol Oncol*. 2022;5(1):84-91.
7. Redelman-Sidi G, Glickman MS, Bochner BH. The mechanism of action of BCG therapy for bladder cancer—a current perspective. *Nature Reviews Urology*. 2014;11(3):153-62.
8. Zlotta AR, Fleshner NE, Jewett MA. The management of BCG failure in non-muscle-invasive bladder cancer: an update. *Can Urol Assoc J*. 2009;3(6 Suppl 4):S199-205.
9. Kikuchi E, Hayakawa N, Fukumoto K, Shigeta K, Matsumoto K. Bacillus Calmette-Guérin-unresponsive non-muscle-invasive bladder cancer: Its definition and future therapeutic strategies. *Int J Urol*. 2020;27(2):108-16.
10. Aly A, Johnson C, Doleh Y, Chirikov V, Botteman M, Shenolikar R, Hussain A. The Real-World Lifetime Economic Burden of Urothelial Carcinoma by Stage at Diagnosis. *J Clin Pathw*. 2020;6(4):51-60.
11. Hanahan D. Hallmarks of Cancer: New Dimensions. *Cancer Discov*. 2022;12(1):31-46.
12. Zhang Y, Zhan L, Jiang X, Tang X. Comprehensive review for non-coding RNAs: From mechanisms to therapeutic applications. *Biochem Pharmacol*. 2024;224:116218.

13. Beermann J, Piccoli MT, Viereck J, Thum T. Non-coding RNAs in Development and Disease: Background, Mechanisms, and Therapeutic Approaches. *Physiol Rev.* 2016;96(4):1297-325.
14. Parizi PK, Yarahmadi F, Tabar HM, Hosseini Z, Sarli A, Kia N, et al. MicroRNAs and target molecules in bladder cancer. *Med Oncol.* 2020;37(12):118.
15. Suarez-Cabrera C, Estudillo L, Ramón-Gil E, Martínez-Fernández M, Peral J, Rubio C, et al. BlaDimiR: A Urine-based miRNA Score for Accurate Bladder Cancer Diagnosis and Follow-up. *Eur Urol.* 2022;82(6):663-7.
16. Padrão NA, Monteiro-Reis S, Torres-Ferreira J, Antunes L, Leça L, Montezuma D, et al. MicroRNA promoter methylation: a new tool for accurate detection of urothelial carcinoma. *Br J Cancer.* 2017;116(5):634-9.
17. Kutwin P, Konecki T, Borkowska EM, Traczyk-Borszynska M, Jablonowski Z. Urine miRNA as a potential biomarker for bladder cancer detection - a meta-analysis. *Cent European J Urol.* 2018;71(2):177-85.
18. Li Q, Wang H, Peng H, Huang Q, Huan T, Huang Q, et al. MicroRNAs: Key Players in Bladder Cancer. *Mol Diagn Ther.* 2019;23(5):579-601.
19. Guallar-Garrido S, Julián E. Bacillus Calmette-Guérin (BCG) Therapy for Bladder Cancer: An Update. *Immunotargets Ther.* 2020;9:1-11.
20. Sequeira JP, Lobo J, Constancio V, Brito-Rocha T, Carvalho-Maia C, Braga I, et al. DigiMir Test: Establishing a Novel Pipeline for MiR-371a Quantification Using Droplet Digital PCR in Liquid Biopsies From Testicular Germ Cell Tumor Patients. *Front Oncol.* 2022;12:876732.
21. Armbruster DA, Pry T. Limit of blank, limit of detection and limit of quantitation. *Clin Biochem Rev.* 2008;29 Suppl 1(Suppl 1):S49-52.
22. Milbury CA, Zhong Q, Lin J, Williams M, Olson J, Link DR, Hutchison B. Determining lower limits of detection of digital PCR assays for cancer-related gene mutations. *Biomol Detect Quantif.* 2014;1(1):8-22.
23. Youden WJ. Index for rating diagnostic tests. *Cancer.* 1950;3(1):32-5.
24. Lobo N, Brooks NA, Zlotta AR, Cirillo JD, Boorjian S, Black PC, et al. 100 years of Bacillus Calmette–Guérin immunotherapy: from cattle to COVID-19. *Nature Reviews Urology.* 2021;18(10):611-22.
25. Castellano D, Velasco Gd, Villarrubia JE, Dueñas M, Paramio J, Martinez VG, et al. BladderGATE: Atezolizumab + intravesical BCG (bacillus Calmette-Guerin) upfront combination in patients with high risk non-muscle invasive bladder cancer (NMIBC)—Phase I-II ONCOSUR study. *Journal of Clinical Oncology.* 2024;42(4_suppl):595-.

26. Lupini L, Pepe F, Ferracin M, Braconi C, Callegari E, Pagotto S, et al. Over-expression of the miR-483-3p overcomes the miR-145/TP53 pro-apoptotic loop in hepatocellular carcinoma. *Oncotarget*. 2016;7(21):31361-71.
27. Wu K, Wang J, He J, Chen Q, Yang L. miR-483-3p promotes proliferation and migration of neuroblastoma cells by targeting PUMA. *Int J Clin Exp Pathol*. 2018;11(2):490-501.
28. Cui K, Zhang H, Wang GZ. MiR-483 suppresses cell proliferation and promotes cell apoptosis by targeting SOX3 in breast cancer. *Eur Rev Med Pharmacol Sci*. 2019;23(5):2069-74.
29. Quintavalle C, Meyer-Schaller N, Roessler S, Calabrese D, Marone R, Riedl T, et al. miR-579-3p Controls Hepatocellular Carcinoma Formation by Regulating the Phosphoinositide 3-Kinase-Protein Kinase B Pathway in Chronically Inflamed Liver. *Hepatol Commun*. 2022;6(6):1467-81.
30. Tang W, Wang W, Zhao Y, Zhao Z. MicroRNA-874 inhibits cell proliferation and invasion by targeting cyclin-dependent kinase 9 in osteosarcoma. *Oncology letters*. 2018;15(5):7649-54.
31. Zhang Y, Wei Y, Li X, Liang X, Wang L, Song J, et al. microRNA-874 suppresses tumor proliferation and metastasis in hepatocellular carcinoma by targeting the DOR/EGFR/ERK pathway. *Cell Death Dis*. 2018;9(2):130.
32. Castaldo V, Minopoli M, Di Modugno F, Sacconi A, Liguoro D, Frigerio R, et al. Upregulated expression of miR-4443 and miR-4488 in drug resistant melanomas promotes migratory and invasive phenotypes through downregulation of intermediate filament nestin. *J Exp Clin Cancer Res*. 2023;42(1):317.
33. Doshi B, Athans SR, Woloszynska A. Biological differences underlying sex and gender disparities in bladder cancer: current synopsis and future directions. *Oncogenesis*. 2023;12(1):44.
34. P. Gontero (Chair) EMC, J.L. Dominguez Escrig, F. Liedberg, P. Mariappan, A. Masson-Lecomte, A.H. Mostafid, B.W.G. van Rhijn, M. Rouprêt, T. Seisen, S.F. Shariat, E.N. Xylinas, Patient Advocates: I. Gürses Andersson RWGAOC, B. Pradere, B.P. Rai, F. Soria, V. Soukup. EAU GUIDELINES ON NON-MUSCLE-INVASIVE (TaT1, CIS) BLADDER CANCER. EAU. 2023.

GENERAL DISCUSSION

General Discussion

Around the globe, BlCa is seen as a major health threat, being one of the most common incident cancers and causes of cancer-associated mortality (1). High-risk NMIBC cases are usually treated with BCG instillations from one up to three years, after the resection of the bladder tumour by a TURBT (2). However, even though BCG prevents progressions and recurrences, it is still associated with high relapse rates, with about 30-40% of BCG-treated patients not responding or tolerating the therapy within 5 years (3, 4). While prognostic indicators offer a means of patient stratification for treatment recommendations, they inadequately capture the molecular heterogeneity and complex nature of BlCa. To date, no FDA/CE-approved biomarkers exist for the pre-emptive identification of patients who may benefit from BCG therapy. The absence of such a biomarker contributes to higher mortality rates, increased comorbidities, and a notable economic strain on the healthcare systems. Identifying predictive biomarkers for BCG immunotherapy response could significantly enhance patients' prognosis and well-being while sparing them from ineffective, invasive, and costly treatment (5). The absence of validated BCG response biomarkers for guiding therapeutic decision has created a need to identify non-invasive biomarkers, particularly those based on urine, to predict the response to BCG. Consequently, this concept has been actively pursued among researchers and clinicians.

Epigenetics has become an established event related to deregulation in cancer, particularly in BlCa (6-8). Moreover, microRNAs have emerged as a prominent disease biomarker due to their key characteristics. Numerous studies have demonstrated their capacity to function as oncogenic or suppressor miRNAs (9), being upregulated or downregulated in several BlCa-associated signalling pathways (10).

Thus, in Part I of this Dissertation, by performing a systematic review, we comprehensively gathered and investigated existing published research that aimed to evaluate the promise of predictive and prognostic biomarkers for BCG failure in BlCa patients. Part II was then dedicated to carrying out an initial validation of a four-microRNA signature that had been previously discovered within the scope of the UCIPredict consortium.

Given the scarcity of recent literature on biomarkers related to BCG treatment response, with the last major reviews conducted over five years ago, the need for a comprehensive and updated exploration of this field has become apparent. In gathering the relevant data, we identified several distinct biomarkers, with a predominant focus on protein-based markers in tissue samples. However, recent studies have increasingly highlighted the potential of liquid biopsies, such as blood and urine samples, as valuable alternatives.

Particularly in the context of BICa, urinary samples may offer a unique advantage as biomarkers, due to their close interaction with tumour cells and the tumour microenvironment (11). Furthermore, immunology-based markers emerged as key candidates in this review, showing significant promise as biomarkers. This is especially pertinent given that the standard treatment for BICa involves immunotherapy. Consequently, stimulating the immune system is crucial, and the presence (or absence) of immune-related cells could help differentiate patients who are likely to respond to treatment from those who may not (12).

During our analysis of the retrieved publications' data, we encountered several difficulties, primarily due to the lack of detail and the elevated heterogeneity in the data reported by the records. For instance, many studies did not include significant BICa stages in their data, specifically excluding patients with *carcinoma in situ* (Tis) or concomitant Tis. Additionally, most of the research involved a limited number of patients, which could introduce bias and may not accurately reflect the disease's global burden. Furthermore, the majority of the analysed studies still require further validation in larger, more robust patient cohorts. Back in 2012, L. Lima et al (13) conducted a similar systematic review on predictive biomarkers of response to BCG. However, they only used one database for the literature search only addressing RFS and PFS. Nevertheless, the study presents a complete report of a huge variety of markers across different sources. As so, our systematic review came to importantly update, complement and comprehensively add newly discovered data within this decade, compiling all the essential information.

Regarding the utility of microRNAs as predictive biomarkers, the systematic review yielded a paucity of relevant literature, with only one study investigating their application. This sole investigation demonstrated that elevated miR-21 expression was independently associated with disease progression and non-responsiveness to BCG therapy, while decreased miR-31 levels served as an independent predictor of disease recurrence and progression (14). This statement, conjugated with the lack of published literature using this type of markers as predictors of BCG response, showcases the promise and novelty of using them in the current clinical scenario.

As was evident in Part I, there is a lack of extensive published research on the specific roles of these microRNAs in BICa development and response to BCG treatment. In response to this gap, we tested and developed various models using previously identified microRNAs in the context of the UCIPredict Project. UCIPredict is a European Commission-funded project under the ERA-NET TRANSCAN-3 program (TRANSCAN3/0001/2021), focused on developing molecular signatures to predict BCG response in BICa patients. The project aims to create a reliable and reproducible laboratory tool for this purpose. As part of

this initiative, one of the UCIPredict collaborators identified several microRNAs that were differentially expressed in tumour tissues from BICa patients who responded to BCG treatment compared to those who did not. Building on this discovery, we conducted an initial validation of a four-microRNA signature, consisting of hsa-miR-483-3p, hsa-miR-579-3p, hsa-miR-874-5p, and hsa-miR-4443.

The biological roles of the identified microRNAs were already explored by some authors. **hsa-miR-483-3p** is a polycistronic microRNA encoded within the intronic region of the *insulin-like growth factor 2* (IGF2) gene (15). Notably, hsa-miR-483-3p is involved in cancer-related pathways, including immune evasion, metabolism, chemoresistance, and resistance to programmed cell death, through its targeting of multiple genes (16). Furthermore, in certain cancers, it acts as an oncogene, promoting cell proliferation and inhibiting apoptosis, thereby contributing to tumour progression (17, 18).

Similarly, **hsa-miR-579-3p**, an intronic microRNA within its host gene *ZFR*, is also associated with cancer regulation, although its roles can vary significantly depending on the cancer type. (19-21). For instance, in melanoma, hsa-miR-579-3p functions as a tumour suppressor by targeting and downregulating oncogenes such as *MDM2*, which plays a crucial role in the *p53* tumour suppressor pathway (22). On the other hand, in glioblastoma, hsa-miR-579-3p has been shown to inhibit cell proliferation and invasion, further emphasising its potential as a therapeutic target (23). However, in hepatocellular carcinoma, it exhibits oncogenic behaviour by promoting colony formation, cell proliferation, growth, and migration through the *PIK3CA* pathway (21).

hsa-miR-874-5p is located within the intron region of a specific gene at 5q31.2, making it an intronic microRNA. It is frequently described as a tumour suppressor, often downregulated in a variety of cancers. By inhibiting certain targeting oncogenes, it plays a pivotal role in cell proliferation, apoptosis, invasion, migration, cell cycle, and epithelial-mesenchymal transition (EMT) (24-27).

Meanwhile, **hsa-miR-4443**, an intergenic microRNA, stands out due to its independent processing from Droscha and Dicer (28). Like hsa-miR-579-3p, a dual role has been reported in cancer, with its function being highly context-dependent. On the one hand, it has been linked to cancer progression and drug resistance, where it promotes cell proliferation, invasion, and migration by acting as an oncogene (29-32). On the other hand, in different contexts, hsa-miR-4443 also demonstrates tumour-suppressive functions, further highlighting the complexity of microRNA roles in cancer biology (29, 30).

As expected, combining multiple microRNAs consistently improved predictive performance. Nonetheless, careful consideration must guide panel construction, as

challenges such as reproducibility and associated costs may arise during implementation. Among the panels tested, the combination of three microRNAs (hsa-miR-483-3p/hsa-miR-579-3p/hsa-miR-4443) showed the best performance. Moreover, a notable association emerged between elevated expression of specific microRNAs of the panel and a decreased likelihood of treatment response. Patients exhibiting higher expression levels displayed a 7.041-fold increased risk of non-response compared to those with lower expression. Furthermore, the incorporation of the panel into a nomogram model resulted in a 2% improvement in predictive accuracy, while maintaining their significance in the multivariate model, in differentiating responders from non-responders to BCG treatment. Additionally, in this multivariable model, high-risk patients presented an increased risk of not responding to the treatment when compared to low-risk patients. This improvement suggests that incorporating such a model into clinical practice could enhance outcomes for high-risk NMIBC patients. By identifying those less likely to respond to BCG treatment early, clinicians could opt for more aggressive or alternative therapies, ultimately improving patient care.

Importantly, one should emphasize that the choice of microRNA, panel, or predictive model depends heavily on the specific research or clinical goal. For instance, when predicting treatment responses or monitoring for possible recurrences/progressions within a defined population, prioritising high specificity should take precedence over sensitivity. Conversely, in diagnostic settings where the primary goal is to detect malignant tumours, achieving high sensitivity becomes paramount. Thus, depending on the clinical context, the emphasis might shift between sensitivity and specificity (33).

Despite its significance, this study is subject to certain limitations. The retrospective cohort, derived from a single institution, was relatively small and heterogeneous in terms of the timepoint of BCG failure and disease status. Consequently, future investigations should aim to expand the sample size and incorporate larger, independent cohorts to enhance the robustness of our findings.

In conclusion, this work suggests that integrating a predictive model combining microRNA panels with clinicopathological variables might significantly enhance the accuracy of predicting BCG treatment response. Such an approach holds promise for improving patient care and quality of life by offering clinicians a novel tool for making informed decisions on the best therapeutic options for their patients.

References

1. Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I, Jemal A. 2024 Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*.
2. P. Gontero (Chair) EMC, J.L. Dominguez Escrig, F. Liedberg, P. Mariappan, A. Masson-Lecomte, A.H. Mostafid, B.W.G. van Rhijn, M. Rouprêt, T. Seisen, S.F. Shariat, E.N. Xylinas, Patient Advocates: I. Gürses Andersson RWGAOC, B. Pradere, B.P. Rai, F. Soria, V. Soukup. 2023 EAU GUIDELINES ON NON-MUSCLE-INVASIVE (TaT1, CIS) BLADDER CANCER. EAU.
3. Zlotta AR, Fleshner NE, Jewett MA. 2009 The management of BCG failure in non-muscle-invasive bladder cancer: an update. *Can Urol Assoc J*;3(6 Suppl 4):S199-205.
4. Kikuchi E, Hayakawa N, Fukumoto K, Shigeta K, Matsumoto K. 2020 Bacillus Calmette-Guérin-unresponsive non-muscle-invasive bladder cancer: Its definition and future therapeutic strategies. *Int J Urol*;27(2):108-16.
5. Witjes JA, Bruins HM, Cathomas R, Compérat EM, Cowan NC, Gakis G, et al. 2021 European Association of Urology Guidelines on Muscle-invasive and Metastatic Bladder Cancer: Summary of the 2020 Guidelines. *Eur Urol*;79(1):82-104.
6. Martinez VG, Munera-Maravilla E, Bernardini A, Rubio C, Suarez-Cabrera C, Segovia C, et al. 2019 Epigenetics of Bladder Cancer: Where Biomarkers and Therapeutic Targets Meet. *Front Genet*;10:1125.
7. Monteiro-Reis S, Miranda-Gonçalves V, Guimarães-Teixeira C, Martins-Lima C, Lobo J, Montezuma D, et al. 2023 Vimentin epigenetic deregulation in Bladder Cancer associates with acquisition of invasive and metastatic phenotype through epithelial-to-mesenchymal transition. *Int J Biol Sci*;19(1):1-12.
8. Padrao NA, Monteiro-Reis S, Torres-Ferreira J, Antunes L, Leca L, Montezuma D, et al. 2017 MicroRNA promoter methylation: a new tool for accurate detection of urothelial carcinoma. *Br J Cancer*;116(5):634-9.
9. Parizi PK, Yarahmadi F, Tabar HM, Hosseini Z, Sarli A, Kia N, et al. 2020 MicroRNAs and target molecules in bladder cancer. *Med Oncol*;37(12):118.
10. Taheri M, Shirvani-Farsani Z, Ghafouri-Fard S, Omrani MD. 2020 Expression profile of microRNAs in bladder cancer and their application as biomarkers. *Biomed Pharmacother*;131:110703.

11. Lodewijk I, Duenas M, Rubio C, Munera-Maravilla E, Segovia C, Bernardini A, et al. 2018 Liquid Biopsy Biomarkers in Bladder Cancer: A Current Need for Patient Diagnosis and Monitoring. *Int J Mol Sci*;19(9).
12. Jiang S, Redelman-Sidi G. 2022 BCG in Bladder Cancer Immunotherapy. *Cancers (Basel)*;14(13).
13. Lima L, Dinis-Ribeiro M, Longatto-Filho A, Santos L. 2012 Predictive biomarkers of bacillus calmette-guérin immunotherapy response in bladder cancer: where are we now? *Adv Urol*;2012:232609.
14. Awadalla A, Zahran M, Abol-Enein H, Zekri A, Elbaset M, Ahmed A, et al. 2022 Identification of Different miRNAs and Their Relevant miRNA Targeted Genes Involved in Sister Chromatid Cohesion and Segregation (SCCS)/chromatin Remodeling Pathway on T1G3 Urothelial Carcinoma (UC) Response to BCG Immunotherapy. *Clinical genitourinary cancer*;20(3):e181-e9.
15. Pepe F, Visone R, Veronese A. 2018 The Glucose-Regulated MiR-483-3p Influences Key Signaling Pathways in Cancer. *Cancers (Basel)*;10(6).
16. Pepe F, Visone R, Veronese A. 2018 The Glucose-Regulated MiR-483-3p Influences Key Signaling Pathways in Cancer. *Cancers*;10(6):181.
17. Wu K, Wang J, He J, Chen Q, Yang L. 2018 miR-483-3p promotes proliferation and migration of neuroblastoma cells by targeting PUMA. *Int J Clin Exp Pathol*;11(2):490-501.
18. Lupini L, Pepe F, Ferracin M, Braconi C, Callegari E, Pagotto S, et al. 2016 Over-expression of the miR-483-3p overcomes the miR-145/TP53 pro-apoptotic loop in hepatocellular carcinoma. *Oncotarget*;7(21):31361-71.
19. Boujemaa M, Mighri N, Chouchane L, Boubaker MS, Abdelhak S, Boussen H, Hamdi Y. 2022 Health influenced by genetics: A first comprehensive analysis of breast cancer high and moderate penetrance susceptibility genes in the Tunisian population. *PLoS One*;17(3):e0265638.
20. Hinske LC, Galante PA, Limbeck E, Möhnle P, Parmigiani RB, Ohno-Machado L, et al. 2015 Alternative polyadenylation allows differential negative feedback of human miRNA miR-579 on its host gene ZFR. *PLoS One*;10(3):e0121507.
21. Quintavalle C, Meyer-Schaller N, Roessler S, Calabrese D, Marone R, Riedl T, et al. 2022 miR-579-3p Controls Hepatocellular Carcinoma Formation by Regulating the Phosphoinositide 3-Kinase-Protein Kinase B Pathway in Chronically Inflamed Liver. *Hepatol Commun*;6(6):1467-81.

22. Fattore L, Mancini R, Acunzo M, Romano G, Laganà A, Pisanu ME, et al. 2016 miR-579-3p controls melanoma progression and resistance to target therapy. *Proc Natl Acad Sci U S A*;113(34):E5005-13.
23. Kalhori MR, Irani S, Soleimani M, Arefian E, Kouhkan F. 2019 The effect of miR-579 on the PI3K/AKT pathway in human glioblastoma PTEN mutant cell lines. *J Cell Biochem*;120(10):16760-74.
24. Zhang Q, Zhong C, Yan Q, Zeng LH, Gao W, Duan S. 2022 miR-874: An Important Regulator in Human Diseases. *Front Cell Dev Biol*;10:784968.
25. Tang W, Wang W, Zhao Y, Zhao Z. 2018 MicroRNA-874 inhibits cell proliferation and invasion by targeting cyclin-dependent kinase 9 in osteosarcoma. *Oncology letters*;15(5):7649-54.
26. Zhang Y, Wei Y, Li X, Liang X, Wang L, Song J, et al. 2018 microRNA-874 suppresses tumor proliferation and metastasis in hepatocellular carcinoma by targeting the DOR/EGFR/ERK pathway. *Cell Death Dis*;9(2):130.
27. Zhang Q, Zhong C, Yan Q, Zeng L-h, Gao W, Duan S. 2022 miR-874: An Important Regulator in Human Diseases. *Frontiers in Cell and Developmental Biology*;10.
28. Meerson A. 2020 Leptin-Responsive MiR-4443 Is a Small Regulatory RNA Independent of the Canonic MicroRNA Biogenesis Pathway. *Biomolecules*;10(2).
29. Castaldo V, Minopoli M, Di Modugno F, Sacconi A, Liguoro D, Frigerio R, et al. 2023 Upregulated expression of miR-4443 and miR-4488 in drug resistant melanomas promotes migratory and invasive phenotypes through downregulation of intermediate filament nestin. *J Exp Clin Cancer Res*;42(1):317.
30. Song Z, Jia G, Ma P, Cang S. 2021 Exosomal miR-4443 promotes cisplatin resistance in non-small cell lung carcinoma by regulating FSP1 m6A modification-mediated ferroptosis. *Life Sci*;276:119399.
31. Wang J, Wang J, Gu Q, Yang Y, Ma Y, Zhu J, Zhang Q. 2020 [MiR-4443 promotes migration and invasion of breast cancer cells by inhibiting PEBP1 expression]. *Nan Fang Yi Ke Da Xue Xue Bao*;40(12):1712-9.
32. Mao Y, Shen J, Wu Y, Wenjing R, Zhu F, Duan S. 2022 Aberrant expression of microRNA-4443 (miR-4443) in human diseases. *Bioengineered*;13(6):14770-9.
33. Soorojebally Y, Neuzillet Y, Roumiguié M, Lamy PJ, Allory Y, Descotes F, et al. 2023 Urinary biomarkers for bladder cancer diagnosis and NMIBC follow-up: a systematic review. *World J Urol*;41(2):345-59.

CONCLUSIONS AND FUTURE PERSPECTIVES

Conclusions and Future Perspectives

This dissertation provides a valuable update on biomarkers for predicting BCG treatment response in BICa patients. In Part I significant gaps, such as inconsistent data reporting and small sample sizes, limit deeper analysis and reproducibility. Despite these challenges, a promising array of molecular markers show great promise as predictive indicators of non-responsiveness to BCG therapy.

Building on this, in Part II we were able to validate the expression of a specific microRNA signature for discriminating BCG non-responders from responders. Specifically, a microRNA signature (hsa-miR-483-3p, hsa-miR-579-3p, and hsa-miR-4443) effectively distinguished BCG responders from non-responders, with the model showing 56.76% sensitivity, 76.19% specificity, and an AUC of 0.737. This model highlights a higher risk of treatment failure in high-risk patients and offers hope for improving patient care by guiding therapeutic decisions. This work introduces a valuable tool that may aid in decision-making for the best therapeutic options, ultimately enhancing patient outcomes and quality of life. While these preliminary results are promising, a well-structured prospective multicentre trial with a larger patient cohort is essential to validate these findings and assess their clinical applicability. This will require recruiting a larger, and more representative patient cohort for a comprehensive evaluation of the biomarkers' clinical applicability in real-world settings.

APPENDIX

Part I Supplementary Material:

Table S1: Quality Assessment of each included study through Quality Assessment of Prognosis Accuracy Studies 2 (QUAPAS-2).

Author, year, journal	Study ID	Risk of bias																				Concerns of applicability					
		Participants				Index test					Outcome				Flow and timing				Analysis					P	T	O	F
		P1	P2	P3	P	T1	T2	T3	T4	T	O1	O2	O3	O	F1	F2	F3	F	A1	A2	A3	A4	A				
L. Zucca et al, 2024, Trans Oncol	V1	Y	U	Y	Low	Y	Y	U	U	Low	Y	Y	U	Low	Y	Y	Y	Low	Y	U	U	N	Possible	Low	Possible	Low	Low
N. Wook et al, 2023, Frontiers in Immunology	U4	Y	U	Y	Low	Y	Y	N	U	Possible	Y	Y	U	Low	Y	U	Y	Low	Y	U	U	Y	Low	Low	Possible	Low	Possible
R. Nishikawa et al, 2023, Inter J Urol	U2	Y	U	Y	Low	Y	Y	N	Y	Low	Y	Y	U	Low	U	Y	U	Possible	Y	U	U	N	Possible	Low	Possible	Low	High
X. Tan et al, 2023, Eur Urol Oncol	U1	Y	Y	Y	Low	Y	N	U	Y	Possible	Y	Y	U	Low	N	Y	Y	Possible	N	U	U	Y	Possible	Low	Low	Low	Low
X. M. Piao et al, 2022, Urol Oncology	T5	Y	U	Y	Low	N	Y	N	U	High	Y	Y	U	Low	U	Y	U	High	Y	U	U	Y	Low	High	High	Possible	Low
K. Murakami et al, 2022, Cancer Biomarkers	T4	U	U	Y	Possible	Y	Y	Y	U	Low	Y	Y	U	Low	U	Y	Y	Low	Y	U	U	Y	Low	Low	Low	Possible	Possible
A. Awadalla et al, 2022, Clin Genito Cancer	T3	N	U	Y	High	Y	Y	U	Y	Low	Y	Y	U	Low	Y	Y	Y	Low	Y	U	U	Y	Low	Possible	Low	Possible	Low
AA. Elsaywy et al, 2022, BJU Inter	T2	Y	Y	Y	Low	Y	Y	U	U	Low	U	U	U	Possible	Y	Y	Y	Low	Y	U	U	Y	Low	Low	Possible	Low	Low
M. Kobayashi et al, 2022, Can Immuno,	T1	Y	U	Y	Low	Y	Y	U	U	Low	Y	Y	U	Low	U	Y	U	Possible	Y	U	U	Y	Low	Low	High	Possible	Low
S. Muto et al, 2021, Euro Urol Open Science	S2	Y	U	Y	Low	N	Y	N	N	High	U	Y	N	High	Y	U	Y	Possible	Y	U	U	U	Possible	Low	High	Possible	Low
J. Sanders et al, 2021, Transl Androl Urol	S1	Y	N	Y	High	Y	Y	U	N	Possible	U	U	U	Possible	Y	Y	Y	Low	Y	U	U	U	Possible	Low	Possible	Possible	Low
D. A. Bastos et al, 2020, IOS Press	R4	Y	Y	Y	Low	Y	Y	U	N	Possible	N	Y	N	High	Y	Y	Y	Low	Y	U	U	Y	Low	Low	High	Low	Low
R. Batista et al, 2020, Inter Journ Molec Sciences	R3	N	U	Y	High	N	U	U	N	High	Y	U	U	Possible	Y	U	U	Possible	N	U	U	Y	Possible	Low	High	High	Possible
W. Zhong et al, 2020, OncoImmunology	R2	N	Y	Y	High	Y	Y	Y	Y	Low	Y	Y	U	Low	Y	Y	Y	Low	Y	U	U	Y	Low	High	Low	Low	Low
CJ. Lim et al, 2020, Frontiers in Immunology	R1	N	U	Y	High	Y	N	N	U	High	Y	N	U	High	Y	Y	U	Low	U	U	U	N	Possible	Low	High	High	High
R. Martínez et al, 2019, OncoImmunology	Q1	N	U	Y	High	U	Y	U	Y	Low	Y	Y	N	High	Y	Y	Y	Low	Y	U	U	N	Possible	High	Possible	Low	Low
F. Sanguedolce et al, 2018, Jour Canc Rese Cli On	P3	U	Y	Y	Low	Y	Y	U	Y	Low	Y	Y	Y	Low	Y	Y	Y	Low	Y	U	U	Y	Low	Low	Low	Low	Possible
K. Fukumoto et al, 2018, Ann Surg Oncol	P2	Y	Y	N	High	Y	Y	U	Y	Low	Y	Y	U	Low	U	U	Y	Low	Y	U	U	Y	Low	Low	Low	Possible	Low
R. Mano et al, 2018, Urologic Oncology	P1	Y	Y	Y	Low	Y	Y	Y	Y	Low	Y	Y	Y	Low	Y	Y	Y	Low	Y	U	U	Y	Low	Possible	Low	Possible	Possible
A. C. Dowell et al, 2017, Plos One	O4	Y	N	Y	High	Y	Y	Y	U	Low	U	Y	U	Possible	U	Y	Y	Low	U	U	U	U	Possible	Possible	Possible	High	Possible
M. Miyake et al, 2017, Inter Journ Molec Sciences	O3	Y	Y	Y	Low	Y	Y	U	Y	Low	U	Y	U	Possible	Y	Y	Y	Low	Y	U	U	Y	Low	Low	Low	Possible	Possible
L. Cormio et al, 2017, Oncotarget	O2	Y	U	Y	Low	N	U	U	U	Possible	U	Y	U	Possible	Y	N	Y	Possible	N	U	U	Y	Possible	Low	Low	Low	Low
K. Qu et al, 2017, OncoImmunology	O1	Y	Y	Y	Low	U	Y	U	N	High	Y	Y	U	Low	Y	Y	Y	Low	Y	U	U	N	Possible	Low	Possible	Possible	Possible
A. Mbeutcha et al, 2016, Urol Oncol	N2	U	U	Y	Possible	N	Y	U	Y	Possible	Y	Y	U	Low	N	Y	U	Possible	N	U	U	Y	Possible	Low	High	Low	Low
R. Pichler, 2016 et al, Oncotarget	N1	U	U	Y	Possible	N	U	U	U	Possible	U	U	U	Possible	U	Y	U	Possible	U	U	U	N	Possible	Low	High	Possible	Low
L. Lima et al, 2015, BJU Int	M3	U	N	Y	High	N	Y	Y	U	Possible	Y	Y	U	Low	U	Y	Y	Possible	Y	U	U	Y	Low	Low	High	Low	Low

C. Ryk et al, 2015, Redox Biol	M2	U	Y	Y	Low	U	Y	U	U	Possible	U	U	U	Possible	U	U	U	Possible	U	U	U	N	Possible	Low	Possible	Possible	Possible
JM. Gaya et al, 2015, J Urol	M1	U	U	Y	Possible	N	Y	U	Y	Possible	Y	Y	U	Low	N	Y	U	Possible	N	U	U	Y	Possible	High	High	Low	Low
L. Lima et al, 2014, Urol Oncol	L3	U	U	Y	Possible	U	Y	U	Y	Low	N	Y	U	High	Y	Y	Y	Low	Y	U	U	Y	Low	Low	Possible	Low	Low
L. Lima et al, 2014, Urol Oncol	L2	U	N	Y	High	N	Y	Y	U	Possible	U	Y	U	Possible	N	Y	Y	High	N	U	U	Y	Possible	Low	High	Possible	Low
S. Bertz et al, 2014, Eur Urol	L1	U	U	Y	Possible	U	Y	U	Y	Low	U	Y	U	Possible	U	U	U	Possible	U	U	U	Y	Possible	High	Possible	Possible	Possible
F. Ajili et al, 2013, Ultrastruct Pathol	K4	Y	U	Y	Low	N	Y	U	U	Possible	U	Y	U	Possible	Y	Y	Y	Low	Y	U	U	Y	Low	Low	High	Possible	Low
L. Lima et al, 2013, Br J Cancer	K3	U	U	Y	Possible	U	Y	U	U	Possible	U	U	U	Possible	Y	U	Y	Possible	Y	U	U	Y	Low	Low	Possible	Possible	Low
M. Oderda et al, 2013, Urol Int	K2	U	Y	Y	Low	U	Y	U	Y	Low	Y	Y	U	Low	U	Y	U	Possible	U	U	U	Y	Possible	Low	Possible	Low	Low
F. Ajili et al, 2012, Ultrastruct Pathol	J4	Y	Y	N	High	Y	Y	U	Y	Low	U	Y	U	Possible	Y	Y	U	Possible	U	U	U	Y	Possible	Low	Low	Possible	Low
F. Ajili et al, 2012, Ultrastruct Pathol	J3	Y	Y	N	High	U	Y	U	Y	Low	N	Y	U	High	Y	Y	U	Possible	Y	U	U	Y	Low	High	Possible	High	Low
PK. Jaiswal et al, 2012, Mol Biol Rep	J2	U	N	N	High	N	Y	U	U	Possible	U	U	U	Possible	U	U	U	Possible	U	U	U	Y	Possible	High	High	Possible	Possible
T. Cai et al, 2012, Exp Ther Med	J1	Y	Y	N	High	Y	Y	U	Y	Low	N	Y	U	High	Y	Y	U	Possible	Y	U	U	Y	Low	High	Low	High	Low
S. Alkhateeb et al, 2011, Urol Ann	I4	Y	U	Y	Low	Y	Y	U	Y	Low	Y	Y	U	Low	U	U	U	Possible	U	U	U	N	Possible	Low	Low	Low	High
W. Otto et al, 2011, J Urol	I3	U	U	Y	Possible	U	Y	U	U	Possible	U	U	U	Possible	N	N	U	High	N	U	U	Y	Possible	High	Possible	Possible	Possible
M. Agundez et al, 2011, Eur Urol	I2	U	U	Y	Possible	U	Y	U	U	Possible	U	U	U	Possible	U	U	U	Possible	U	U	U	Y	Possible	High	Possible	Possible	Possible
E. Chiong et al, 2011, Eur Urol	I1	U	U	Y	Possible	N	Y	U	Y	Possible	Y	Y	U	Low	U	U	U	Possible	U	U	U	Y	Possible	Low	High	Low	Possible
P. Srivastava et al, 2010, Cancer Biomark	H6	U	N	Y	High	N	Y	U	U	Possible	U	U	U	Possible	Y	U	Y	Possible	Y	U	U	N	Possible	High	High	Possible	Low
A. Lopez-Beltran et al, 2010, Virchows Arch	H5	Y	U	Y	Low	Y	Y	U	Y	Low	U	U	U	Possible	Y	U	U	Possible	U	U	U	U	Possible	High	Low	Possible	Possible
M. Alvarez-Múgica et al, 2010, J Urol	H4	U	U	Y	Possible	Y	Y	U	U	Low	U	U	U	Possible	N	U	U	Possible	N	U	U	Y	Possible	High	Low	High	Possible
T. Cai et al, 2010, J Urol	H3	Y	U	N	High	N	Y	U	U	Possible	N	Y	U	High	U	U	U	Possible	U	U	U	Y	Possible	High	High	High	Possible
L. Cormio et al, 2010, Urol Oncol	H2	U	U	Y	Possible	Y	Y	Y	Y	Low	Y	Y	U	Low	U	U	U	Possible	U	U	U	Y	Possible	High	Low	Low	Possible
YJ. Kim et al, 2010, Clin Cancer Res	H1	U	U	Y	Possible	N	Y	U	U	Possible	Y	Y	U	Low	U	U	U	Possible	U	U	U	Y	Possible	High	High	Low	Possible
C. Ayari et al, 2009, Eur Urol	G3	U	U	Y	Possible	N	Y	U	Y	Possible	U	U	U	Possible	N	U	U	Possible	Y	U	U	Y	Low	Low	High	Possible	Possible
J. Palou et al, 2009, Eur Urol	G2	U	U	Y	Possible	Y	Y	U	Y	Low	U	N	U	High	Y	N	U	High	Y	U	U	Y	Low	High	Low	Possible	High
L. Cormio et al, 2009, Anticancer Res	G1	U	U	Y	Possible	Y	Y	U	Y	Low	Y	Y	U	Low	U	U	U	Possible	U	U	U	N	Possible	Low	Low	Low	Possible
JA. Queipo-Zaragoza et al, 2007, Scand J Urol Nephrol	F1	U	U	Y	Possible	Y	Y	Y	Y	Low	U	U	U	Possible	Y	Y	N	High	Y	Y	U	Y	Low	High	Low	Possible	Low
F. Saint et al, 2004, Eur Urol	E1	Y	U	Y	Low	N	Y	U	U	Possible	Y	Y	U	Low	Y	U	Y	Possible	U	U	U	Y	Possible	Low	High	Low	Low
E. Watanabe et al, 2003, Cancer Immunol Immunother	D1	U	U	Y	Possible	Y	Y	U	U	Low	Y	Y	U	Low	U	U	U	Possible	U	U	U	Y	Possible	High	Possible	Low	Possible
T. Asakura et al, 1997, Eur Urol	C2	U	U	N	High	N	Y	U	Y	Possible	Y	Y	U	Low	Y	N	Y	High	Y	U	U	Y	Low	High	Low	Low	High
E. Lee et al, 1997, Int J Urol	C1	U	U	Y	Possible	N	Y	U	Y	Possible	N	Y	U	High	U	Y	Y	Low	U	U	U	Y	Possible	High	High	Low	Low
L. Lacombe et al, 1996, J Clin Oncol	B1	U	U	Y	Possible	N	Y	N	Y	High	U	U	U	Possible	N	U	N	High	U	U	U	Y	Possible	Low	Low	Possible	Possible
E. Orihuela et al, 1987, Cancer	A1	U	N	Y	High	N	Y	Y	Y	Low	Y	Y	U	Low	N	N	U	High	N	U	U	Y	Possible	Low	Possible	Low	Possible

QUAPAS items: P1= Participant selection is fully described; P2= A consecutive or random sample of patients was selected; P3= A case-control design was avoided; P= Risk of Bias on Participants; T1= The index test was well described; T2= Method for performing the index test was the same for all the participants; T3= The index test results were interpreted without knowledge of the outcome; T4= The threshold used was pre-specified; T= Risk of Bias on Index Test; O1= The Method used to measure the outcome was valid and reliable; O2= The method for measuring the outcome was the same for all participants; O3= The outcome was measured without knowledge of the index test results; O= Risk of Bias on Outcome; F1= Description of patients lost-to-follow up; F2= Appropriate interval between index and outcome; F3= All the patients outcome is available; F= Risk of Bias on Flow and Timming; A1= All the participants were enrolled in the analysis; A2= Data missing, the correct methods were used; A3= Appropriate methods used accounting censoring; A4= Competing events appropriate methods were used to account them; A= Risk of Bias on Analysis; Y= Fulfilled; N= Not fulfilled; U= Unclear

Table S2: Quality Assessment of each included study through Prediction model Risk Of Bias Assessment (PROBAST).

Author, year, journal	Study ID	Risk of bias																				Concerns of applicability							
		Participants				Predictors					Outcome				Flow and Timing				Analysis							P	T	O	F
		P1	P2	P3	P	T1	T2	T3	T4	T	O1	O2	O3	O	F1	F2	F3	F	A1	A2	A3	A4	A5	A					
G. M. Villoldo et al, 2023, Tech Science Press	U3	Y	Y	Y	Low	Y	Y	U	Y	Low	Y	Y	U	Low	Y	Y	Y	Low	Y	U	U	N	U	Possible	Low	Low	Low	Low	
K. Murakami et al, 2022, Cancer Biomarkers	T4	U	U	Y	Possible	Y	Y	Y	U	Low	Y	Y	U	Low	U	Y	Y	Low	Y	U	U	U	Y	Low	Low	Low	Possible	Possible	
S. Muto et al, 2021, Euro Urol Open Science	S2	Y	U	Y	Low	N	Y	N	N	High	U	Y	N	High	Y	U	Y	Low	Y	U	U	N	U	Possible	Low	High	Possible	Low	
W. Zhong et al, 2020, Oncolmmunology	R2	N	Y	Y	High	Y	Y	Y	Y	Low	Y	Y	U	Low	Y	Y	Y	Low	Y	U	U	U	Y	Low	High	Low	Low	Low	
D. Ilijazi et al, 2020, Cells	R5	Y	Y	Y	Low	Y	U	U	U	Possible	Y	Y	U	Low	Y	Y	Y	Low	Y	U	U	Y	U	Low	Low	Possible	Possible	Low	
R. Martínez et al, 2019, Oncolmmunology	Q1	N	U	Y	High	U	Y	U	Y	Low	Y	Y	N	High	Y	Y	Y	Low	Y	U	U	N	U	Possible	High	Possible	Low	Low	
I. M. El-Babouly et al, 2018, Urologic Oncology	P4	Y	Y	Y	Low	Y	Y	Y	Y	Low	U	Y	Y	Low	Y	Y	Y	Low	Y	U	U	N	Y	Possible	Low	Low	Possible	Possible	
L. Cormio et al, 2017, Oncotarget	O2	Y	U	Y	Low	N	U	U	U	Possible	U	Y	U	Possible	Y	N	Y	Possible	N	U	U	Y	Y	Possible	Low	Low	Low	Low	
A. M. Kamat et al, 2016, European Urology	N3	Y	U	Y	Low	N	Y	U	U	Possible	U	U	U	Possible	U	U	Y	Possible	Y	U	U	N	Y	Possible	Low	Low	Possible	Possible	
R. Pichler et al, 2016, Oncotarget	N1	U	U	Y	Possible	N	U	U	U	Possible	U	U	U	Possible	U	Y	U	Possible	U	U	U	N	N	High	Low	High	Possible	Low	
L. Lima et al, 2015, BJU International	M3	U	N	Y	High	N	Y	Y	U	Possible	Y	Y	U	Low	U	Y	Y	Low	U	U	U	N	N	High	High	Low	Low	Possible	
R. Nunez-Nateras et al, 2014	L4	Y	U	N	High	N	Y	U	Y	Possible	Y	Y	U	Low	Y	N	Y	High	U	U	U	N	Y	Possible	High	Possible	Low	High	

PROBAST items: P1= Participant selection is fully described; P2= A consecutive or random sample of patients was selected; P3= A case-control design was avoided; P= Risk of Bias on Patient Selection; T1= The index test was well described; T2= Method for performing the index test was the same for all the participants; T3= The index test results were interpreted without knowledge of the outcome; T4= The threshold used was pre-specified; T= Risk of Bias on Index Test; O1= The Method used to measure the outcome was valid and reliable; O2= The method for measuring the outcome was the same for all participants; O3= The outcome was measured without knowledge of the index test results; O= Risk of Bias on Outcome; F1= Description of patients lost-to-follow up; F2= Appropriate interval between index and outcome; F3= All the patients outcome is available; F= Risk of Bias on Flow and Timming; A1= All the participants were enrolled in the analysis; A2= Data missing, the correct methods were used; A3= Appropriate methods used accounting censoring; A4= Complexities in the data were accounted for appropriately; A5= Relevant model performance measures were evaluated appropriately; A= Risk of Bias on Analysis; Y= Fulfilled; N= Not fulfilled; U= Unclear

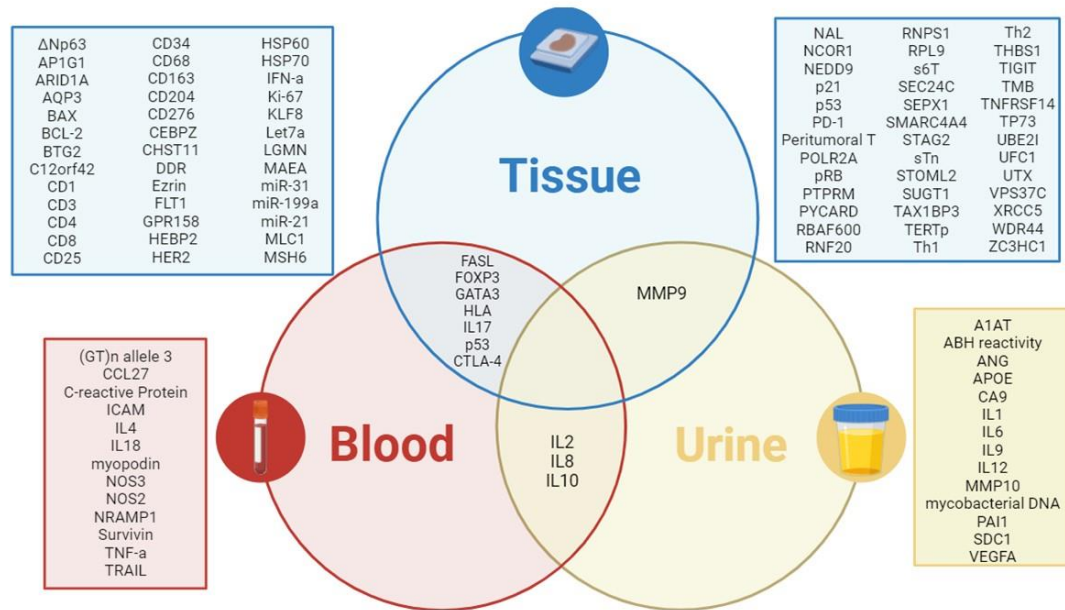


Figure S1: Venn Diagram of the studied biomarker molecules by source. Created with BioRender.com.

Part II Supplementary Material:

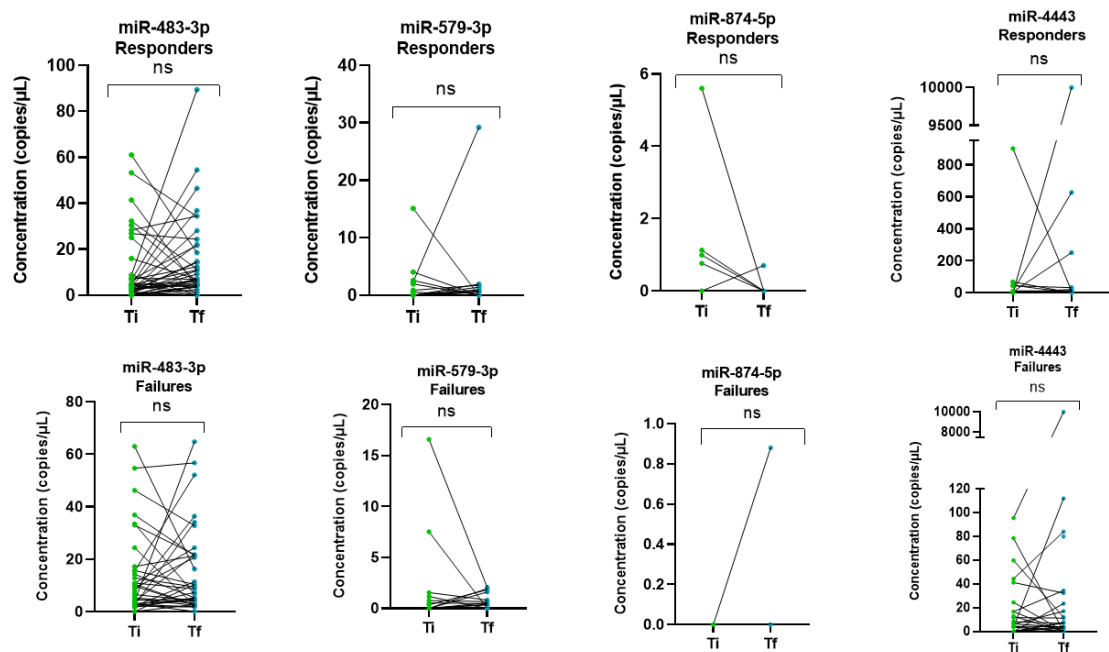


Figure S1: Variation of microRNA levels at the beginning and end of BCG treatment in both treatment response groups. ns: non-significant, Ti: initial timepoint, Tf: final timepoint.

Table S1: Summary of the relevant parameters of the tested Panels.

Panel	p-value	Standard Error	95% Confidence Interval
483/579/4443	0.0119	0.06212	0.5430 to 0.7865
483/579/874/4443	0.0196	0.06259	0.5302 to 0.7755
483/4443	0.0210	0.06272	0.5283 to 0.7741

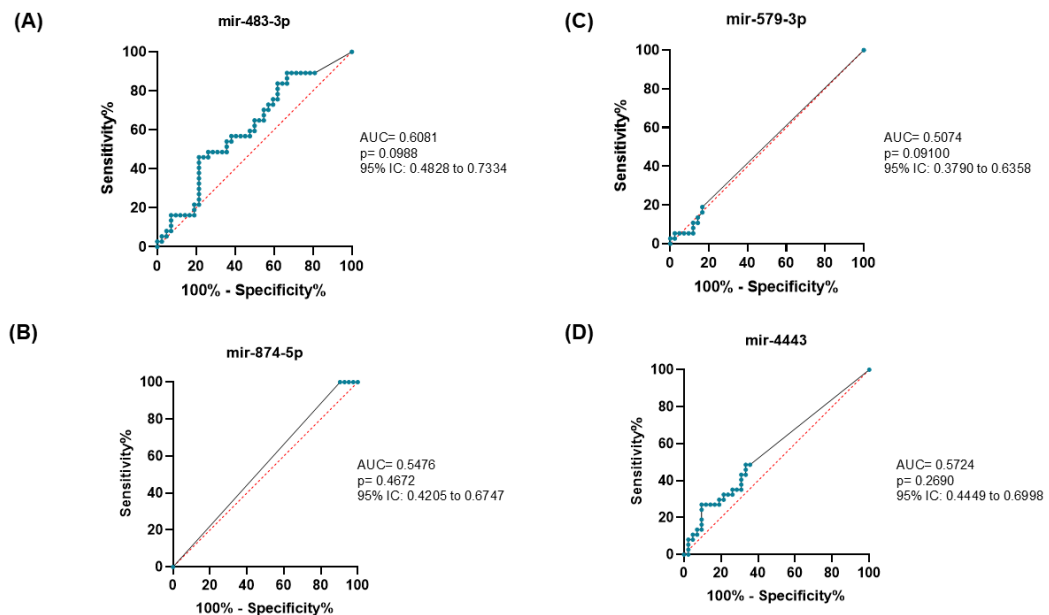


Figure S2: Respective Receiver Operator Curves (ROC) from each individual microRNA.

The construction of the ROC curves was essential for accurately classifying into "high" or "low expression levels" using cutoff values established by Youden's J index.

MicroRNA Levels, Performance and Survival Analysis of other Different microRNA Panels

All the tested panels' could significantly ($p < 0.05$) discriminate both treatment groups.

Panel composed by miR-483-3p, miR-579-3p, miR-874-5p and miR-4443

This Panel is composed of the four target microRNAs and the panel is considered positive if at least one microRNA is highly present (Figure S3A). This panel presented a sensitivity of 56.76% and a specificity of 73.81%, resulting in an AUC of 0.6528. Analysing the Kaplan-Meier curve, patients with a high expression of one of the microRNAs of the panel present a 6.271-fold increased risk of not responding to BCG compared with patients with a lower expression of a specific microRNA of the panel.

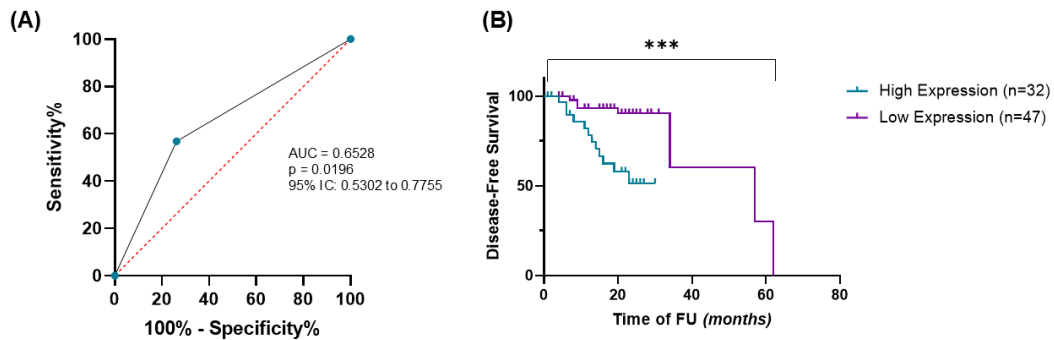


Figure S3: Performance and Survival Analysis of the Panel. (A) ROC Curve evaluating the performance of the Panel. **(B)** Kaplan-Meier Survival Curve for DFS based on the levels of the Panel microRNAs.

Panel composed by miR-483-3p and miR-4443

Combining two microRNAs in a Panel results in the panel being deemed positive if at least one microRNA is highly present (Figure S4A). This panel demonstrated a sensitivity of 54.05% and a specificity of 76.19%, yielding an AUC of 0.6512. For this specific panel, a high expression of a specific microRNA by a patient displays a 5.873-fold increased risk of not responding to the given therapy when compared to a patient with a lower expression level.

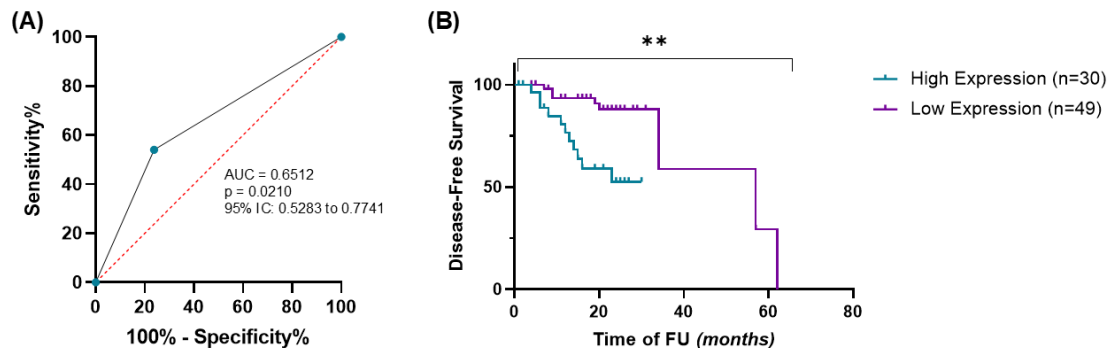


Figure S4: Performance and Survival Analysis of the Panel. (A) ROC Curve evaluating the performance of the Panel. **(B)** Kaplan-Meier Survival Curve for DFS based on the expression levels of the Panel microRNAs.

The accuracy values of the panels are demonstrated in Table S2. In general, all panels presented similar accuracies and AUCs.

Table S2: Performance of the panels combining the different microRNAs in distinguishing BCG response in BICa Patients.

Panel Markers											
Panel	TP	TN	FP	FN	Cutoff	SN	SP	PPV	NPV	Accuracy	AUC
483/579/874/4443	21	31	11	16	0.5	56.76%	73.81%	65.63%	65.96%	65.82%	0.6528
483/4443	20	32	10	17	0.5	54.05%	76.19%	66.67%	65.31%	65.82%	0.6512

TP (True positive); TN (True Negative); FP (False Positive); FN (False Negative); SN (Sensitivity); SP (Specificity); PPV (Positive Predictive Value); NPV (Negative Predictive Value); AUC (Area Under the Curve).

Nomogram Models of the Targets in other Different microRNA Panels

Model composed by miR-483-3p, miR-579-3p, miR-874-5p and miR-4443

A nomogram model was formulated for disease-free survival represented by the following formula: Risk of non-response = $-3.81448 + 0.26206 \times \text{Smoke} - 0.09943 \times \text{Age_cat} + 0.47432 \times \text{Previous_Treatment} + 1.45702 \times \text{Grade} + 1.51513 \times \text{Panel}$, resulting in the generation of risk model. More detailed information regarding other parameters is presented in Table S3.

Table S3: Summary of the logistic model representative of the Risk Model.

Model	Coefficient	Odds Ratio	S.E.	Wald Z	p-value	95% Confidence Interval	
						Lower Bound	Upper Bound
Intercept	-3.81448	0.02205	1.59966	-2.385	0.01710	0.00096	0.50702
Smoke Status	0.26206	1.29960	0.33469	0.783	0.43364	0.67440	2.50438
Age_cat	-0.09943	0.90535	0.52988	-0.188	0.85116	0.32047	2.55773
Previous_treatment	0.47432	1.60692	0.39571	1.199	0.23066	0.73989	3.48999
Grade	1.45702	4.29315	0.72707	2.004	0.04508	1.03250	17.85083
Panel	1.51513	4.55001	0.53420	2.836	0.00456	1.59697	12.96353

S.E. (Standard Error).

This model originated an AUC of 0.7240 (IC 95%: 0.612 to 0.836) (Figure S5B) with a sensitivity of 58.33% and a specificity of 76.19%, an improvement when combined with the clinicopathological variables of interest.

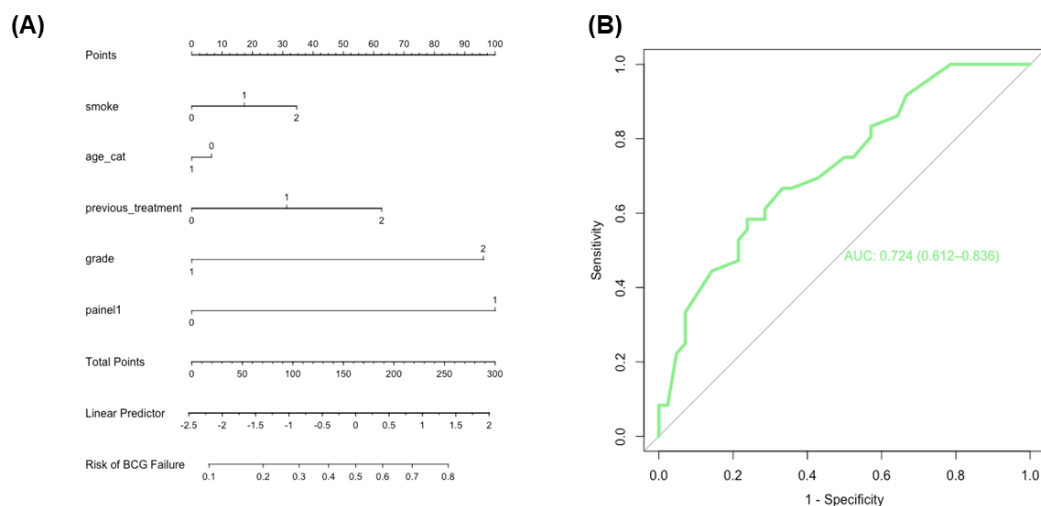


Figure S5: (A) Nomogram representative of the Risk Model, with the relevant clinicopathological variables: age at diagnosis (0: ≤ 68 , 1: > 68 years), smoke status (0: non-smoker, 1: former smoker, 2: active smoker), previous treatment (0: no previous treatment, 1: Mitomycin C, 2: BCG) and grade (1: low grade, 2: high grade). (B) ROC curve to evaluate the performance of the nomogram model. Panel categorisation - 0: low levels, 1: high levels.

Model composed by miR-483-3p and miR-4443

The nomogram is embodied by the formula: Risk of non-response = - 3.95736 + 0.24026*Smoke - 0.01844*Age_cat + 0.46238*Previous_Treatment + 1.50385*Grade + 1.66483*Panel, resulting in the generation of risk model. Other important parameters are described in Table S4.

Table S4: Summary of the logistic model representative of the Risk Model.

Model	Coefficient	Odds Ratio	S.E.	Wald Z	p-value	95% Confidence Interval	
						Lower Bound	Upper Bound
Intercept	-3.4949	0,03035	1.5586	-2.242	0.02494	0.0014	0,64400
Smoke Status	0.2226	1,24932	0.3298	0.675	0.49973	0.6545	2,38457
Age_cat	-0.0406	0,96021	0.5349	-0.076	0.93950	0.3366	2,73938
Previous_treatment	0.4111	1,50848	0.3900	1.054	0.29174	0.7025	3,23960
Grade	1.3347	3,79886	0.7214	1.850	0.06427	0.9239	15,62106
Panel	1.4430	4,23338	0.5364	2.690	0.00715	1.4793	12,11483

S.E. (Standard Error).

This model attained a sensitivity of 58.33% and a specificity of 78.57%, achieving an AUC of 0.7240 (IC 95%: 0,611 to 0.837) (Figure S6B), another improvement from the model without the clinicopathological variables included.

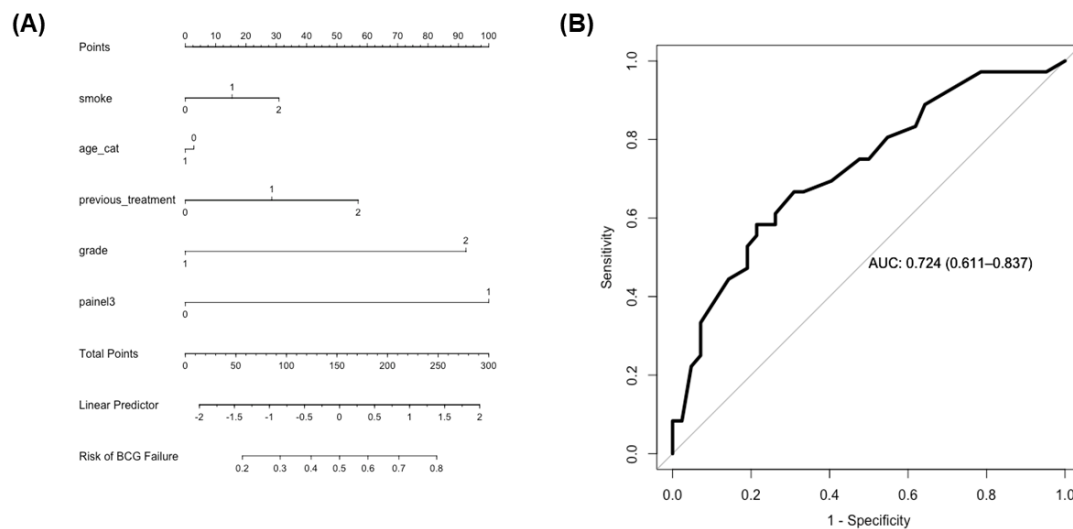


Figure S6: (A) Nomogram representative of the Risk of the Model, with the relevant clinicopathological variables: age at diagnosis (0: ≤68, 1: >68 years), smoke status (0: non-smoker, 1: former smoker, 2: active smoker), previous treatment (0: no previous treatment, 1: Mitomycin C, 2: BCG) and grade (1: low grade, 2: high grade). **(B) ROC curve to evaluate the performance of the nomogram model.** Panel categorisation - 0: low levels, 1: high levels.

The performance and accuracy values of the models are demonstrated in Table S5. All panels, when including the clinicopathological variables, still presented similar accuracies and AUCs.

Table S5: Performance of the models combining the different microRNAs and clinicopathological variables of interest in distinguishing BCG response in BICa Patients.

Multivariate Model												
Panel	TP	TN	FP	FN	Cutoff	LP	SN	SP	PPV	NPV	Accuracy	AUC
483/579/874/4443	21	32	10	15	0.5069034	0.665	58.33%	76.19%	67.74%	68.09%	67.95%	0.7240
483/4443	21	33	9	15	0.5110961	0.658	58.33%	78.57%	70.00%	68.75%	69.23%	0.7240

TP (True positive); TN (True Negative); FP (False Positive); FN (False Negative); LN (Linear Predictor) SN (Sensitivity); SP (Specificity); PPV (Positive Predictive Value); NPV (Negative Predictive Value); AUC (Area Under the Curve)