

CIRCA-PREDICT: epigenetic insights into Chronotype-Integrated Radiotherapy for prostate CAncer - PREDICTing the impact on efficacy and toxicity of treatment timing with circadian rhythms

Rúben Diogo Oliveira Sousa

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Radiotherapy for prostate CAncer - PREDICTing the impact on
efficacy and toxicity of treatment timing with circadian rhythms**

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“Todas as casas onde há livros e quadros e discos são bonitas. E são feias
todas as casas, por mais luxuosas, onde faltem essas coisas.”

Eugénio de Andrade

“Sempre chegamos ao sítio aonde nos esperam.”

José Saramago



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Resumo

Introdução: O cancro da próstata (CaP) é o segundo cancro mais prevalente nos homens a nível mundial. Cerca de um terço dos doentes com CaP são tratados com radioterapia externa (RTE) como terapia de primeira linha. No entanto, apesar das elevadas taxas de sucesso, um número considerável de doentes experiencia progressão ou recorrência da doença, assim como efeitos secundários debilitantes. Estes dados realçam a necessidade de otimizar as estratégias de tratamento. O ritmo circadiano (RC) é um processo biológico que governa ciclos fisiológicos diários. Tem também um impacto relevante na reparação do DNA, no metabolismo e na resposta imunitária, aspetos biológicos fundamentais para o sucesso da radioterapia (RT). As evidências clínicas sugerem que alinhar a hora de tratamento com o RC individual pode melhorar os resultados do tratamento e minimizar as toxicidades associadas.

Objetivos: Este estudo teve como objetivo investigar o impacto do RC nos resultados da RT em doentes com CaP. Além disso, procurámos investigar os principais genes do RC e dissecar os mecanismos epigenéticos subjacentes à regulação do RC para identificar potenciais alvos terapêuticos e novas abordagens de radiosensibilização.

Materiais e Métodos: Conduzimos um estudo de coorte retrospectivo que incluiu 224 doentes com CaP localizado tratados com RTE no Instituto Português de Oncologia do Porto entre 2012 e 2021; um estudo de coorte prospetivo a decorrer no mesmo instituto, atualmente com 8 doentes com CaP. Em três linhas celulares de CaP (PC3, 22Rv1-P e 22Rv1-RR), foram realizadas experiências *in vitro*, incluindo sincronização do CR, ensaios clonogénicos e silenciamento, pelo método siRNA, dos genes *BMAL1* e *PER2* para investigar o papel destes reguladores do RC na radiosensibilidade. Também conduzimos análises de sequenciação de RNA e metilação do DNA (EPIC array).

Resultados: Na nossa análise retrospectiva, a RTE de manhã foi associada a uma melhoria significativa da sobrevivência livre de metástases (SLM) em comparação com o tratamento de tarde (log-rank $p < 0,05$). A análise de risco proporcional de Cox confirmou o tratamento de tarde como um preditor significativo de pior SLM ($p < 0,05$), e o tratamento de manhã reduziu o risco de toxicidades gastrointestinais e geniturinárias tardias graves ($p < 0,05$). Estes resultados sugerem uma influência do RC na resposta à RTE, o que motivou a investigação dos mecanismos moleculares subjacentes. No nosso estudo molecular, o silenciamento de *BMAL1* aumentou significativamente a radiosensibilidade nas células 22Rv1-RR ($p < 0,01$), enquanto o silenciamento de *PER2* levou a um aumento da

radorresistência nas células 22Rv1-P ($p < 0,01$). A análise de metilação do ADN indicou que o alinhamento circadiano é uma das principais vias desreguladas nas células radorresistentes, contribuindo, possivelmente, para a diminuição da expressão de genes circadianos importantes, como o *PER2* e o *RORB*.

Conclusão: Esta dissertação destacou o potencial da cronorradioterapia na otimização do tratamento de CaP. Alinhar a RT com o RC pode melhorar os resultados do tratamento. Além disso, marcadores como *BMAL1* e *PER2* – que mostraram ser potencialmente regulados por mecanismos epigenéticos - podem oferecer novas possibilidades para minimizar o fenómeno de radorresistência. Estes resultados contribuem para reforçar a crescente evidência de que a integração da RC na prática clínica pode conduzir a estratégias terapêuticas mais personalizadas e menos tóxicas para os doentes com CaP.

Abstract

Introduction: Prostate cancer (PCa) is the second most prevalent cancer in men globally. Nearly a third of PCa patients are treated with external beam radiotherapy (EBRT) as first-line therapy. However, despite high success rates, a considerable number of patients experience disease progression or recurrence, along with debilitating side effects. This emphasises the need to optimise treatment strategies and schedules. Circadian rhythm (CR) is a biological process that governs daily physiological cycles. It influences DNA repair, cellular metabolism, and immune response, all of which are important biological processes for radiotherapy (RT) efficacy. Clinical evidence suggests that aligning treatment timing with the individual CR may enhance treatment outcomes and minimise associated toxicities.

Aims: This study aimed to investigate the impact of CR on RT outcomes in PCa patients. Furthermore, we aimed to dissect epigenetic mechanisms underlying CR regulation to identify new potential targets and novel radiosensitising approaches.

Materials and Methods: We conducted a retrospective cohort study comprising 224 patients with localised PCa treated with EBRT at the Portuguese Oncology Institute of Porto between 2012 and 2021; an ongoing prospective cohort study involving 8 PCa patients; and *in vitro* experiments, including CR synchronisation, clonogenic assays, and siRNA transfections were performed on three PCa cell lines (PC3, 22Rv1-P, and 22Rv1-RR) to investigate the role of circadian regulators BMAL1 and PER2 in radiosensitivity. We also conducted RNA sequencing and DNA methylation analyses (EPIC array).

Results: Our retrospective analysis revealed that morning EBRT was associated with significantly improved metastasis-free survival (MFS) compared to afternoon treatment (log-rank $p < 0.05$). Cox proportional hazard analysis confirmed that afternoon treatment was a significant predictor of worse MFS ($p < 0.05$), and morning treatment reduced the risk of severe late gastrointestinal and genitourinary toxicities ($p < 0.05$). These results suggest an influence of CRs on the response to EBRT, which motivated the investigation of the underlying molecular mechanisms. In our molecular study, *BMAL1* knockdown significantly increased radiosensitivity in 22Rv1-RR cells ($p < 0.01$), while *PER2* knockdown led to increased radioresistance in 22Rv1-P cells ($p < 0.01$). DNA methylation analysis indicated circadian entrainment as a top dysregulated pathway in radioresistant cells, possibly contributing to key circadian gene downregulation such as *PER2* and *RORB*.

Conclusion: This dissertation highlights the potential of chronoradiotherapy in optimising PCa management. Aligning RT with CRs might enhance treatment outcomes. Furthermore,

targeting BMAL1 and PER2 - along with epigenetic modulation insights – may provide new possibilities to minimise radioresistance. These findings contribute to the growing evidence that integrating CR into clinical practice can lead to more personalised and less toxic therapeutic strategies for PCa patients.

Table of Contents

Chapter 1 General introduction.....	3
General Introduction	3
PROSTATE CANCER	3
Epidemiology and risk stratification	3
Conventional treatment	5
Radiotherapy treatment for localised disease	6
Radiobiology of prostate cancer	7
CIRCADIAN RHYTHM	9
EPIGENETICS.....	11
CHRONORADIO THERAPY IN PROSTATE CANCER	12
REFERENCES.....	14
Chapter 2 Aims	23
Aims	25
Chapter 3 Materials and methods.....	27
Material and Methods	29
Chapter 4 Retrospective cohort study	31
Impact of Radiotherapy Timing On Survival and Radiotoxicity Outcomes In Localized Prostate Cancer	33
ABSTRACT	34
INTRODUCTION	35
MATERIALS AND METHODS	36
Patient selection criteria	36
Radiotherapy treatment procedures	36
Toxicity evaluation and follow-up protocols	36
Data collection and endpoints definition	36
Statistical analysis.....	37
RESULTS.....	38
Patient characteristics.....	38
Morning EBRT is associated with improved metastasis-free survival	39
Morning treatment is linked to lower grades of late GI and GU toxicities	45
DISCUSSION	47
CONCLUSION	49
REFERENCES.....	49
Chapter 5 Molecular study	53

Circadian Rhythm's Impact on Prostate Cancer Radiotherapy Response: Exploring the Role of <i>PER2</i> and <i>BMAL1</i> Genes	55
ABSTRACT	56
INTRODUCTION	57
MATERIAL AND METHODS	58
Cell lines and cell culture	58
Ionising radiation (IR)	58
RNA extraction, cDNA synthesis, and reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)	59
Protein extraction & quantification and SDS-Page western blot.....	59
Clonogenicity assay.....	60
siRNA transfection for <i>PER2</i> and <i>BMAL1</i> gene silencing	60
Infinium Methylation EPIC Array	61
RNA sequencing	61
Statistical analysis.....	61
RESULTS.....	62
Opposing circadian dynamics of <i>BMAL1</i> and <i>PER2</i> in prostate cancer cell lines	62
<i>PER2</i> and <i>BMAL1</i> expression modulates radiosensitivity in PC3 cells: insights from simulating 22Rv1-RR dynamics	62
Silencing <i>BMAL1</i> enhances radiosensitivity in 22Rv1-RR cells.....	65
Phosphorylation of H2AX (γ -H2AX) increased upon irradiation in <i>BMAL1</i> knocked down cells	65
Silencing <i>BMAL1</i> and <i>PER2</i> disrupts DNA repair pathways: p53, KAT2B, and SIRT1 as key players	67
Epigenetic regulation of circadian rhythm dynamics in 22Rv1-P and -RR cell lines: Insights from DNA methylation analysis and RNA sequencing.....	68
DISCUSSION AND CONCLUSION	70
REFERENCES.....	73
Chapter 6 Prospective cohort study.....	79
Impact of circadian rhythm-based radiotherapy timing on toxicities in localised prostate cancer patients: a prospective cohort study protocol.....	81
ABSTRACT	82
RATIONALE OF THE STUDY	83
Prostate cancer epidemiology and treatment remarks	83
Advancements and challenges in RT for PCa.....	83
Understanding circadian rhythms and their potential influence on RT response	83

Timing of radiotherapy and treatment response	84
Research gaps.....	84
Study Importance	85
References.....	85
STUDY DESIGN	87
STUDY POPULATION - INCLUSION AND EXCLUSION CRITERIA.....	87
ENDPOINTS	87
ANALYSED CRITERIA	87
VISITS AND EXAMINATIONS.....	88
DATA COLLECTION	88
SAFETY AND ETHICAL CONSIDERATIONS	89
STATISTICAL ANALYSIS	90
EXPECTED RESULTS.....	90
PRELIMINARY DATA	90
Chapter 7 General discussion	95
General Discussion	97
Appendix.....	ci
APPENDIX I	ci
APPENDIX II	ciii
APPENDIX III	cxviii
APPENDIX IV	cxix

Index of Figures

Chapter 1

- Figure 1. Graphical representation of the global incidence and mortality rates of cancer in males, 2022.** Adapted from Bray, Laversanne [1]. Prostate cancer was the second most frequent neoplasia (14%) and the fifth leading cause of cancer-related deaths (7%). 3
- Figure 2. Projected prostate cancer incidence and mortality from 2022 to 2050.** Adapted from Bray, Laversanne [1]. By 2050, incidence is expected to rise 96.2%, while mortality is projected to increase 136.4%. 4
- Figure 3. Circadian rhythm mechanism.** The circadian rhythm is regulated by three molecular feedback loops involving key regulatory genes and proteins. Among them, the heterodimer of BMAL1 and CLOCK promotes the expression of PERs and CRYs genes which in turn inhibit BMAL1 and CLOCK activity. CCGs, Clock-controlled genes; RRE, retinoic acid-related orphan receptor response elements. Oliveira-Sousa, R. unpublished. 10
- Figure 4. Epigenetic mechanisms of regulation of gene expression.** The most common epigenetic mechanisms involve histone post-translational modifications, chromatin remodelling complexes, histone variants, non-coding RNAs and DNA methylation. Created with BioRender.com. Oliveira-Sousa, R. unpublished. 11

Chapter 4

- Figure 1.** (upper) Kaplan-Meier curves of metastasis-free survival (MFS) in morning and afternoon external beam radiotherapy groups for the unmatched first approach (n = 224) (A) and for the matched first approach (n = 202) (B). The analysis demonstrates a statistically significant difference in MFS favoring the morning treatment group (Log-rank test: (A) p = 0.014; (B) p = 0.016). (lower) Number of patients at risk within the afternoon and morning groups, along the time. 43

Chapter 5

- Figure 1. Distinct circadian rhythm patterns in PC3, 22Rv1-P and 22Rv1-RR cell lines.** Relative gene expression levels of BMAL1 (A) and PER2 (B) after a 48h-period in PC3, 22Rv1-P and 22Rv1-RR cells. Relative BMAL1 and PER2 gene expression in PC3 (C), 22Rv1-P (D) and 22Rv1-RR (E) PCa cells over the 48 hour-period. Data are presented as

mean±SEM from at least three independent biological replicates of 22Rv1-RR vs 22Rv1-P or PC3 cell extracts. *p < 0.05, **p < 0.01, ****p < 0.0001. RR, radioresistant; P, parental.

.....63

Figure 2. Timing radiotherapy for maximum impact based on PER2 and BMAL1 expression in PC3 cells.

(A) Heatmap of the relative gene expression of PER2 and BMAL1 in PC3 cells over a 48-hour period. GUS-β was used as the housekeeping gene. Transcript (B) and protein (C) levels of PER2 (140kDa) and BMAL1 (75-86kDa) at 4- and 44h-time points. (D) Cell survival fraction of PC3 cells irradiated at 4 hours (High PER2) and at 44 hours (Low PER2), represented through a linear-quadratic model ($LQ = (S = e - (xD + BD^2))$). Data are presented as mean±SEM from at least three independent biological replicates. *p < 0.05, **p < 0.01, ****p < 0.0001. DeF, dose enhancement factor; SF, survival fraction; RR, radioresistant. 64

Figure 3. Silencing BMAL1 led to a substantial radiosensitising effect in 22Rv1-RR.

(A) BMAL1 and PER2 transcript levels across the 22Rv1-RR-negative control (NC) and BMAL1-KD clones 13.1 to 13.3 at 72 hours. (B) Corresponding protein levels of BMAL1 and PER2 across the 22Rv1-RR-negative control (NC) and BMAL1-KD clones 13.1 to 13.3 at 72 hours. (C) Cell survival fraction of 22Rv1-RR-negative control (BMAL1 C-) and BMAL1-KD clones #1 to #3 transfected cells represented through linear-quadratic model ($LQ = (S = e - (xD + BD^2))$). (D) BMAL1 and PER2 transcript levels across the 22Rv1-P-negative control (NC) and PER2-KD clones 13.1 to 13.3 at 72 hours. (E) Corresponding protein levels of BMAL1 and PER2 across the 22Rv1-P-negative control (NC) and PER2-KD clones 13.1 to 13.3 at 72 hours. (F) Cell survival fraction of 22Rv1-P-negative control (PER2 C-) and PER2-KD clones #1 to #3 transfected cells represented through linear-quadratic model ($LQ = (S = e - (xD + BD^2))$). Data are presented as mean±SEM from at least three independent biological replicates. *p < 0.05, **p < 0.01, ****p < 0.0001, ns, not significant. DeF, dose enhancement factor; SF, survival fraction; KD, knockdown; RR, radioresistant; P, parental. 66

Figure 4. Phosphorylation of H2AX (γ-H2AX) in BMAL1 and PER2 knocked-down irradiated cells.

(A) γ-H2AX protein levels across 22Rv1-RR-negative control (NC) and BMAL1-KD clones 13.1 to 13.3, normalise to NC and 0 Gy baseline. (B) γ-H2AX protein levels across 22Rv1-P-negative control (NC) and PER2-KD clones 13.1 to 13.3, normalise to NC and 0 Gy baseline. Data are presented as mean±SEM from at least three independent biological replicates. KD, knockdown; RR, radioresistant; P, parental. 67

Figure 5. Silencing BMAL1 and PER2 disrupts DNA repair pathways: p53, KAT2B, and SIRT1 as key players.

Transcript levels of TP53, KAT2B and SIRT1 across the 22Rv1-RR-negative control (NC) and BMAL1-KD clones 13.1 to 13.3 at 72 hours (A) and across the 22Rv1-P-negative control (PER2 C-) and PER2-KD clones #1 to #3 at 72 hours (B).

Protein levels of KAT2B, SIRT1, and p53 across the 22Rv1-RR-negative control (NC) and BMAL1-KD clones 13.1 to 13.3 at 72 hours (C) and across the 22Rv1-P-P-negative control (PER2 NC) and PER2-KD clones #1 to #3 at 72 hours (D). Data are presented as mean±SEM from at least three independent biological replicates. *p < 0.05. KD, knockdown; RR, radioresistant; P, parental. 69

Figure 6. Epigenetic regulation of circadian rhythm dynamics in 22Rv1-P and 22Rv1-RR cell lines: Insights from DNA methylation analysis and RNA sequencing. (Ai)

KEGG pathway analysis for the top ten dysregulated pathways in 22Rv1-RR cells compared to 22Rv1-P on DNA methylation data. (Aii) Diagram of the circadian rhythm pathway, with red-marked genes representing those deregulated in 22Rv1-RR compared to 22Rv1-P. (B) Heatmap showing significantly hypermethylated CpG sites (p < 0.05) within the promoter regions of circadian-related genes in 22Rv1-RR versus 22Rv1-P cells. (C) Heatmap of RNA-sequencing data displaying significant (p < 0.05) differential expression of circadian-related genes between 22Rv1-P and 22Rv1-RR cells. (D) Volcano plot illustrating differentially expressed genes in 22Rv1-RR versus 22Rv1-P cells. Genes such as PER1, CRY1, CRY2, RORC, MYC, and NR1D2 were downregulated (blue dots), while TP53, SIRT1, BMAL1, RORA, CSNK1E, and KITLG were upregulated (red dots). 71

Chapter 6

Figure 1. Mean melatonin levels for the three collection moments (before, middle and after treatment). 93

Figure 2. Representative MLT curves for a morning-type and evening-type chronotypes. 93

Appendix II

Supplementary Figure 1. ROC curve analysis for determining the optimal cut-off point of hour of treatment in predicting metastasis-free survival. The optimal cut-off was determined using Youden's Index (sensitivity + specificity - 1). AUC, area under the curve; CI, confidence interval.....ciii

Supplementary Figure 2. Covariate balance before and after matching for the first cohort. Standardized mean differences (SMD) for all covariates used in the propensity score matching process. The red dots represent the unadjusted SMDs before matching, while the blue dots depict the adjusted SMDs after matching. The dashed vertical lines indicate the thresholds for acceptable covariate balance (SMD range of -0.2 to 0.2). Covariate balance was achieved when the blue dots fell within these limits, indicating

successful adjustment and minimal residual confounding between treatment groups. ECOG, Eastern Cooperative Oncology Group; ADT, androgen deprivation therapy. ciii

Supplementary Figure 3. Covariate balance before and after matching for the second cohort. Standardized mean differences (SMD) for all covariates used in the propensity score matching process. The red dots represent the unadjusted SMDs before matching, while the blue dots depict the adjusted SMDs after matching. The dashed vertical lines indicate the thresholds for acceptable covariate balance (SMD range of -0.2 to 0.2). Covariate balance was achieved when the blue dots fell within these limits, indicating successful adjustment and minimal residual confounding between treatment groups. ECOG, Eastern Cooperative Oncology Group; ADT, androgen deprivation therapy. civ

Supplementary Figure 4. (upper) Kaplan-Meier curves of biochemical-free survival (BFS) (A), relapse-free survival (RFS) (B), progression-free survival (C), and overall-survival (D) in morning (n = 112) and afternoon (n = 112) external beam radiotherapy groups for the unmatched first approach. The analysis demonstrates no statistically significant difference in survival rates for any of the outcomes (Log-rank test: $p > 0.05$). (lower) Number of patients at risk within the afternoon and morning groups, along the time. cv

Supplementary Figure 5. (upper) Kaplan-Meier curves of metastasis-free survival (MFS) in morning (n = 86) and afternoon (n = 86) external beam radiotherapy groups for the unmatched first approach. The analysis demonstrates a statistically significant difference in MFS favoring the morning treatment group (Log-rank test $p = 0.026$). (lower) Number of patients at risk within the afternoon and morning groups, along the time. cvi

Supplementary Figure 6. (upper) Kaplan-Meier curves of biochemical-free survival (BFS) (A), relapse-free survival (RFS) (B), progression-free survival (C), and overall-survival (D) in morning (n = 86) and afternoon (n = 86) external beam radiotherapy groups for the matched second cohort. The analysis demonstrates no statistically significant difference in survival rates for any of the outcomes (Log-rank test: $p > 0.05$). (lower) Number of patients at risk within the afternoon and morning groups, along the time. cvii

Supplementary Figure 7. (upper) Kaplan-Meier curves of biochemical-free survival (BFS) (A), relapse-free survival (RFS) (B), progression-free survival (C), and overall-survival (D) in morning (n = 86) and afternoon (n = 86) external beam radiotherapy groups for the matched second cohort. The analysis demonstrates no statistically significant difference in survival rates for any of the outcomes (Log-rank test: $p > 0.05$). (lower) Number of patients at risk within the afternoon and morning groups, along the time. cviii

Appendix IV

Supplementary Figure 1. Chronogram of the collection process. cxix

Supplementary Figure 2. Instructions flyer (front and back) in Portuguese.	cxx
Supplementary Figure 3. Time sheet to record the collection times in Portuguese.	cxxi

Index of Tables

Chapter 1

Table 1. Prostate cancer risk-group stratification. 5

Table 2. NCCN treatment guidelines for prostate cancer. 7

Chapter 4

Table 1. Patient clinicopathological characteristics and survival endpoints in the first unmatched and matched approaches. 40

Table 2. Patient clinicopathological characteristics of the matched first and second cohorts
.....44

Table 3. Comparison of acute and late gastrointestinal, genitourinary and radiation dermatitis toxicities by grade (<2 vs. ≥2) between treatment groups in the first matched and unmatched approach. 46

Chapter 6

Table 1. Patient clinicopathological characteristics. 90

Table 2. Incidence of acute gastrointestinal and genitourinary toxicities, radiation dermatitis and erectile dysfunction. 92

Table 3. Fatigue, quality of life and chronotype scores before and after treatment. 92

Appendix I

Supplementary Table 1. Histological International Society of Urological Pathology (ISUP) Grade Groups for prostate cancer. ci

Supplementary Table 2. TNM classification of prostate cancer according to the 8th edition of the American Joint Committee on Cancer (AJCC) guidelines. cii

Appendix II

Supplementary Table 1. Patient clinicopathological characteristics and survival endpoints in the second matched approach. cix

Supplementary Table 2. Multivariable Cox regression analysis of predictors of survival endpoints, in the first unmatched approach. cxi

Supplementary Table 3. Univariable Cox regression analysis of predictors of survival endpoints, in the matched approaches. cxiii

Supplementary Table 4. Incidence of all grades of acute and late gastrointestinal, genitourinary and radiation dermatitis toxicities, in the first unmatched approach.	cxiv
Supplementary Table 5. Incidence of all grades of acute and late gastrointestinal, genitourinary and radiation dermatitis toxicities, in the first unmatched approach.	cxv
Supplementary Table 6. Incidence of all grades of acute and late gastrointestinal, genitourinary and radiation dermatitis toxicities, in the second matched approach.	cxvi
Supplementary Table 7. Comparison of acute and late gastrointestinal, genitourinary and radiation dermatitis toxicities by grade (<2 vs. ≥2) between treatment groups in the second matched approach.	cxvii
Supplementary File 1. Logistic regression results for toxicities: Available here	cxvii

Appendix III

Supplementary Table 1. RT-qPCR (Fw and Rv) primers details.....	cxviii
Supplementary Table 2. Antibodies details for western blot (WB).	cxviii

Appendix IV

Supplementary Table 1. RTOG/EORTC acute and late urinary and lower gastrointestinal radiation morbidity scoring schema.	cxxii
---	-------

List of Common Abbreviations

3

3D-CRT - Three-Dimensional
Conformal Radiation Therapy

A

ADT - Androgen Deprivation Therapy

AJCC - American Joint Committee on
Cancer

AM - *Ante Meridiem*

AR - Androgen Receptor

B

BCR - Biochemical Recurrence

BFS - Biochemical-Free Survival

BMAL1 - Brain and Muscle ARNT-Like
1

BMI - Body Mass Index

C

Castration-resistant PCa (CRPC)

CBP - CREB-Binding Protein

CC - Circadian Clock

CHK1 – Checkpoint Kinase 1

ChIP - Chromatin immunoprecipitation

CI - Confidence Interval

CLOCK - Circadian Locomotor Output
Cycles Kaput

CR - Circadian Rhythm

CRY – Cryptochrome Circadian
Regulator

D

DBP - D-box Binding Protein

DeF - Dose enhancement Factor

DFS - Disease-Free Survival

DGE - Differential Gene Expression

DLMO - Dim Light Melatonin Onset

DNA - Deoxyribonucleic Acid

DRE - Digital Rectal Examination

DSB - Double-Strand Break

E

EBRT - External Beam Radiotherapy

ECOG - Eastern Cooperative
Oncology Group

EMT - Epithelial-Mesenchymal
Transition

EORTC QLQ C30 - European
Organization for Research and
Treatment of Cancer Quality of Life
Questionnaire Core-30

F

FACIT-F - Functional Assessment of
Chronic Illness Therapy – Fatigue

FBS - Fetal Bovine Serum

G

GI - Gastrointestinal

GU – Genitourinary

H

HAT - Histone Acetyltransferase

HDAC - Histone Deacetylase
HDI - Human Development Index
HR - Homologous Recombination

I

IARC - Agency for Research on Cancer
IGRT - Image-Guided Radiotherapy
IMRT - Intensity-Modulated Radiotherapy
IPO Porto - Portuguese Oncology Institute of Porto
IR - Ionising Radiation
ISUP - International Society of Urological Pathology

K

KD - Knockdown
KM - Kaplan-Meier

L

LQ - Linear-Quadratic
LUTS - Lower Urinary Tract Symptoms

M

MCTQ - Munich ChronoType Questionnaire
MFS - Metastasis-Free Survival
MLT - Melatonin
mpMRI - Multiparametric Magnetic Resonance Imaging
MR - Magnetic Resonance

N

NCCN - National Comprehensive Cancer Network
NEPC - Neuroendocrine Prostate Cancer
NFIL3 - Nuclear Factor, Interleukin 3 Regulated
NHEJ - Non-Homologous End Joining
NPAS2 - Neuronal PAS Domain Protein 2
NPC - Nasopharyngeal Carcinoma
NR1D - Nuclear Subfamily 1 Receptor Group D
ns - Not Significant
NTCP - Normal Tissue Complication Probability

O

OAR - Organs at Risk
OS - Overall Survival

P

P – Parental
p53 – Tumour Protein p53
p-p53 - p53
PCa - Prostate Cancer
pCAF - p300/CBP-Associated Factor
PER - Period Circadian Regulator
PFS - Progression-Free Survival
PIC – Protease Inhibitor Cocktail
PIN – Prostatic Intraepithelial Neoplasia
PM - *Post Meridiem*
PS - Propensity Scores
PSA - Prostate-Specific Antigen

Q

QoL - Quality of Life

R

RFS - Recurrence-Free Survival

RNA - Ribonucleic Acid

ROC - Receiver Operating
Characteristic

ROR - Retinoic acid-related Orphan
Receptors

ROS - Reactive Oxygen Species

RR – Radioresistant or Radioresistance

RRE - Retinoic Acid-Related Orphan
Receptor Response Elements

RT - Radiotherapy

RTOG - Radiation Therapy Oncology
Group

RT-qPCR - reverse transcriptase
quantitative polymerase chain reaction

S

S.E. - Standard Error

SBRT - Stereotactic Body
Radiotherapy

SD - Standard Deviation

SF - Survival Fraction

siRNA - small interfering RNA

Sirtuin 1 - SIRT1

SMD - Standardised Mean Difference

SSD - Skin-Source Distance

SV - Seminal Vesicles

T

TCP - Tumour Control Probability

TNM - Tumour, node, metastasis

TRUS - Transrectal Ultrasound

V

VMAT - Volumetric-Modulated Arc
Therapy

Y

γ -H2AX - H2AX phosphorylated

CHAPTER 1

General introduction

General Introduction

PROSTATE CANCER

Epidemiology and risk stratification

According to the International Agency for Research on Cancer (IARC), prostate cancer (PCa) is the second most frequent neoplasia and the fifth leading cause of cancer-related deaths worldwide in men (Figure 1) [1]. The incidence of PCa is greater in countries with higher Human Development Index (HDI) scores, where the diagnosis rate is approximately three times greater than in nations with low to medium HDI scores [1]. Disparities in male life expectancy, diagnostic approaches (Prostate-Specific Antigen [PSA] testing), ethnicity, and geography primarily account for these differences [2]. However, the gap is less significant for mortality rates between these countries, indicating discrepancies in early detection and treatment [1]. Moreover, the epidemiological burden of PCa is projected to rise significantly by the year 2050 globally, with a 96.2% increase in incidence and a 136.4% increase in mortality (Figure 2). Despite these trends, the aetiology of the disease remains not fully understood. Advancing age, African ancestry, family history of the disease, and specific genetic mutations (particularly in genes responsible for deoxyribonucleic acid [DNA] damage repair) are PCa-established risk factors. Furthermore, other factors such as smoking, obesity, and dietary habits have been suggested to influence the risk of developing PCa, though their impact is less well-defined [1].

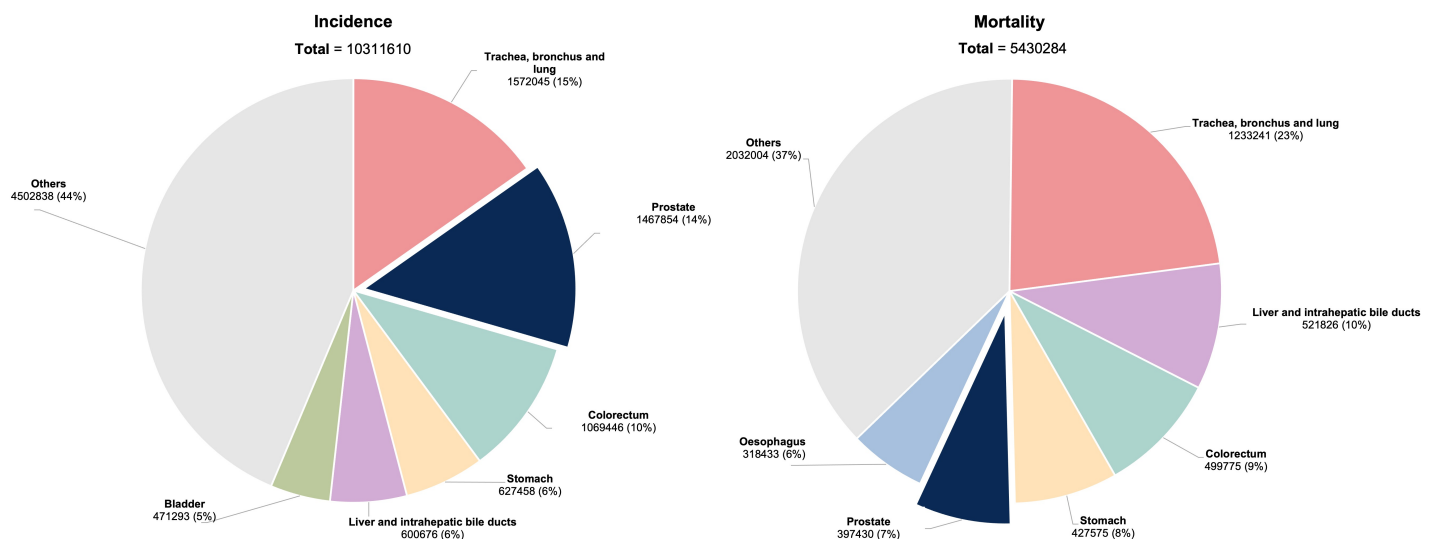


Figure 1. Graphical representation of the global incidence and mortality rates of cancer in males, 2022. Adapted from Bray, Laversanne [1]. Prostate cancer was the second most frequent neoplasia (14%) and the fifth leading cause of cancer-related deaths (7%).

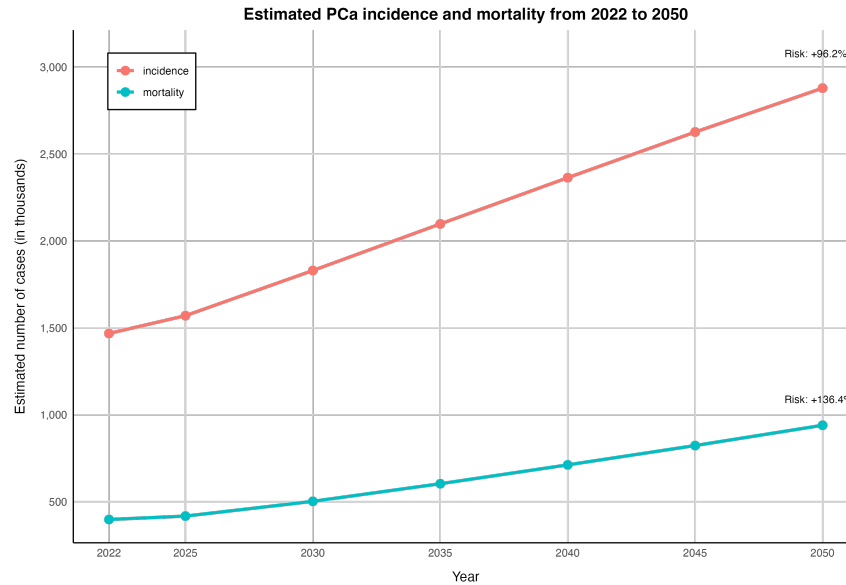


Figure 2. Projected prostate cancer incidence and mortality from 2022 to 2050. Adapted from Bray, Laversanne [1]. By 2050, incidence is expected to rise 96.2%, while mortality is projected to increase 136.4%.

Digital rectal examination (DRE) and serum PSA are the gold standard tools for PCa screening [3]. However, results from the DRE are subjective to the clinicians' expertise and possess low compliance and sensitivity [4]. PSA screening has a high rate of false positives, resulting in overdiagnosis and overtreatment of non-life-threatening PCa [5]. A transrectal ultrasound (TRUS) guided biopsy is the gold standard for a definitive diagnosis [6]. Nevertheless, less than a third of the TRUS biopsies have a positive result in patients with abnormal DRE or elevated PSA levels [7]. Additionally, the systematic biopsy is associated with a high rate of false negatives and tumour under-grading [8]. Hence, the diagnosis can be complemented with imaging techniques such as multiparametric magnetic resonance imaging (mpMRI) [9].

PCa exhibits considerable heterogeneity, ranging from small, clinically indolent, and low-grade tumours to large, highly aggressive, and potentially lethal forms [10]. To guide treatment decisions and support patient and clinician decision-making, PCa is currently stratified into very low-, low-, favourable intermediate-, unfavourable intermediate-, high-, and very high-risk categories (Table 1) [11]. This risk classification system is primarily based on PSA serum levels, the histological International Society of Urological Pathology (ISUP) grade group, and the tumour, node, metastasis (TNM) classification (Appendix I: Supplementary Tables 1-2) [12, 13]. Risk stratification in PCa is fundamental for predicting disease outcomes, including morbidity and mortality, and assisting treatment decision-making [14]. It also helps prevent overtreatment, particularly in cases of indolent or low-risk cancer.

Table 1. Prostate cancer risk-group stratification.

	Very low risk	Low risk	Intermediate risk [†]		High risk [†]	Very high risk
			Favourable	Unfavourable		
Clinical stage	T1c	T1-T2a	T2b-T2c	T2b-T2c	T3a	T3b-T4 or N+
ISUP grade	1	1	2	3	4 or 5	Any
PSA serum levels	<10 ng/mL	<10 ng/mL	10-20 ng/mL	10-20 ng/mL	>20 ng/mL	Any

[†]At least one of the listed criteria is needed for risk classification.

Adapted from NCCN guidelines [41].

Abbreviations: ISUP, International Society of Urological Pathology; PSA, Prostate-Specific Antigen; NCCN, National Comprehensive Cancer Network.

Histologically, 90% of PCa cases are acinar adenocarcinomas [15, 16]. It is characterised by the absence of the basal cell layer and the presence of abnormal mitosis with nuclear atypia [17]. On the other hand, PCa is highly heterogenous morphologically and molecularly with distinct clonal areas, making targeted treatment challenging. PCa typically starts as prostatic intraepithelial neoplasia (PIN) and progresses to localised and then locally advanced adenocarcinomas [15]. One common form of treatment and radiation resistance in PCa is neuroendocrine prostate cancers (NEPC) which are particularly aggressive. Although primary NEPCs are rare (< 1% of PCa cases), approximately one-third of prostate adenocarcinomas may progress to NEPC following androgen deprivation therapy (ADT) or radiotherapy (RT), being characterised by the loss of androgen receptor (AR) and PSA expression [18]. The emergence of castration-resistant PCa (CRPC), many of which are therapy-induced NEPC, often results from ADT, leading to disease progression and poor prognosis [19, 20].

Conventional treatment

Most cases of PCa are asymptomatic and remain confined to the prostate gland. This characteristic makes them suitable for curative treatment options such as radical prostatectomy or radiotherapy - external beam radiotherapy (EBRT) and brachytherapy [21]. Alternatively, conservative management strategies comprise active surveillance and watchful waiting. The latter is reserved for patients unsuitable for curative treatment, where the focus is palliative treatment, i.e., symptom management and maintaining quality of life. Active surveillance is used to delay or avoid unnecessary treatments in patients with a life expectancy of at least ten years. These patients are closely monitored through regular PSA testing, clinical exams,

magnetic resonance (MR) imaging, and biopsies [2, 22]. Treatment selection is typically guided by risk stratification, with low-risk patients often managed conservatively, while in high-risk and locally advanced cases, neoadjuvant and concomitant ADT is commonly prescribed [21, 22]. However, within ten years of completing initial curative treatment, 20–50% of patients may experience biochemical recurrence (BCR) [23, 24].

Radiotherapy treatment for localised disease

Surgery and RT are the principal treatment modalities available for localised PCa. Assuming adequate administered radiation doses, both have comparable outcomes in equivalent risk groups [25, 26]. Approximately 30% of newly diagnosed PCa patients undergo EBRT as the first line of treatment, mainly as adherence to radical prostatectomy declines [27]. Its objective is to administer high doses of radiation to eradicate the tumour cells with the least possible damage to normal tissues. The gold standard of EBRT for PCa is dose-escalated intensity-modulated radiotherapy (IMRT) or volumetric-modulated arc therapy (VMAT) [13]. In combination with image-guided radiotherapy (IGRT), they are associated with less toxicity than the three-dimensional conformal radiation therapy technique [13]. VMAT presents the main advantage of shorter treatment times [2]. MR-guided adaptive RT is under investigation [28], with studies showing reduced doses to organs at risk (OAR) culminating in low rates of acute gastrointestinal and genitourinary toxicity [29]. However, its implementation presents drawbacks, such as the long time required for each fraction (reaching almost 45 minutes) or the heavy MR workflow [28]. EBRT treatment is tailored to the patient's risk group, prognosis and preference. The local tumour burden and risk of recurrence guide the choice of irradiation volume, irradiation doses, and ADT while respecting patient choices [30]. Table 2 outlines the NCCN recommendations for RT treatment in localised PCa. The combination of EBRT with ADT has proven more effective than RT alone. In intermediate-risk patients, a short duration of 4-6 months is optimal, while high-risk patients benefit from long-term ADT of 2-3 years. For low-risk patients, neoadjuvant or concomitant treatment with ADT is not recommended [2]. Evidence suggests that escalation of radiation doses of 74 to 80 Gy is associated with significantly higher 10-year biochemical-free survival (BFS), 10-20% improvement, and improved PCa-specific mortality [31-34]. In contrast, these doses are associated with an increased risk of higher degrees of late gastrointestinal toxicity [35]. EBRT, especially when conventional fractionation is prescribed, demands multiple hospital visits, resulting in increased patient transportation costs and prolonged linear accelerator occupancy [36]. As a result, clinical trials have showcased the viability of

escalating daily radiation doses to 2.4–3.4 Gy, effectively shortening the overall treatment duration [37]. Notably, short-term moderately hypofractionated EBRT is well tolerated and feasible, mirroring similar toxicity profiles with conventional high-dose EBRT while retaining its oncological efficacy [38-40].

Table 2. NCCN treatment guidelines for prostate cancer.

Group risk	Radiotherapy	ADT
Very low risk	Active surveillance is preferable	Not recommended
Low risk	Active surveillance is preferable Active treatment: EBRT* or brachytherapy ^a	Not recommended
Favourable intermediate risk[†]	EBRT*, brachytherapy or brachytherapy boost combined with EBRT	Not recommended
Unfavourable intermediate risk[†]	EBRT* or brachytherapy ^b	Neoadjuvant and/or concomitant Short-term (4-6 months) ^c
High and very high risk^{† ‡}	EBRT* or brachytherapy boost combined with EBRT	Adjuvant and Concomitant Long-term (2-3 years)

*IMRT or VMAT with IGRT

^aFor patients with a life expectancy greater than 10 years.

^bFor patients with a life expectancy of 5 to 10 years.

^cIf comorbidities prevent the prescription of short-term ADT to patients, EBRT is performed alone.

[†]Includes irradiation of seminal vesicles.

[‡]Prophylactic nodal radiation should be considered.

Adapted from NCCN guidelines [41].

Abbreviations: NCCN, National Comprehensive Cancer Network; ADT, androgen deprivation therapy; EBRT, external beam radiotherapy; IMRT, intensity-modulated radiotherapy; VMAT, volumetric-modulated arc therapy; IGRT, image-guided radiotherapy.

Radiobiology of prostate cancer

Radiobiology is the branch of science that studies the effects of electromagnetic radiation on living organisms and biological tissues, combining radiation physics and biology [42]. It allowed the scientific development of RT as a therapeutic weapon. When ionising radiation penetrates biological material, it interacts with atoms and molecules, depositing the photon's energy through a series of events. In the direct effect, radiation directly ionises DNA and other vital cellular components, known as the physical stage. The indirect effect occurs when radiation interacts with water molecules, forming reactive oxygen species (ROS) during the physico-chemical stage. The subsequent biochemical stage involves reactions between the free radicals and intact molecules, leading to structural and functional abnormalities [42, 43]. Finally, the biological stage encompasses the development of biological damage and

reparative processes. Thus, following irradiation, cells may experience four possible outcomes: (1) no effect; (2) division delay; (3) apoptosis; or (4) reproductive failure [43].

In theory, any malignant tumour could be eradicated by administering a sufficiently high radiation dose, but the associated complication rates due to the irradiation of normal surrounding tissues prevent the use of such high doses [42]. Therefore, it is imperative to understand the six Rs of radiobiology - repair, redistribution, repopulation, reoxygenation, radiosensitivity, and reactivation of immune response - to optimise the therapeutic ratio, i.e., maximise tumour control probability (TCP) while minimising normal tissue complication probability (NTCP) [43, 44]. In practice, the typical RT treatment aims for a $TCP \geq 0.5$ and an $NTCP \leq 0.05$ [43].

The Linear-Quadratic (LQ) model adequately describes the relationship between radiation dose, cell survival, and energy deposition [45]. In this model, the alpha (α) parameter represents the initial slope of the dose-response curve, indicating the rate of cell kill via single-hit process, which is a non-repairable form of damage. The beta (β) parameter reflects the curve's curvature, corresponding to cell kill by two-hit mechanism, which is repairable [46, 47]. The α/β ratio is a critical parameter in radiobiology, as it indicates the sensitivity of tissues to fractionated radiation [45].

Proliferative tissues generally have a high α/β ratio and respond acutely to radiation. However, PCa exhibits a slow proliferative pattern, as evidenced by low Ki-67 expression and a median pretreatment potential doubling time of 42 days [48]. This resembles late-reacting normal tissues, having a low α/β ratio, translating into a hypersensitivity of PCa cells to increased dose per fraction [46]. As a result, hypofractionation, which delivers larger doses per fraction over fewer sessions, aiming to reduce the overall treatment duration, profits on the tumour's heightened sensitivity of these higher doses [47].

Moreover, many PCa cells may be in a quiescent state having higher repair and misrepair capability [49]. The quiescent cells usually have the cell arrest in G0 phase, spending more time in radioresistant phases of the cell cycle, through an increase in p-p53 (p53 phosphorylated) and p21 by CHK1/2 activation [50, 51]. DNA double-strand breaks (DSBs) induced by radiation are repaired primarily through two pathways - the non-homologous end joining (NHEJ), a faster but more error-prone process, more active during the G0/G1 phases, and by accurate homologous recombination (HR), which predominantly occurs during the S and G2 phases. The efficiency of these repair mechanisms can influence a tumour's radioresistance [52-54]. PCa cells that efficiently

repair DSBs through these pathways may exhibit increased resistance to RT, requiring higher doses or combination therapies to achieve optimal outcomes.

CIRCADIAN RHYTHM

The circadian rhythm (CR) has been extensively researched due to its essential role in regulating and synchronising daily biological functions with day-night cycles. The central clock in the hypothalamic suprachiasmatic nucleus synchronises with external stimuli and communicates with the peripheral clocks of different tissues [55]. In reaction to environmental cues, known as "zeitgebers", these peripheral clocks independently govern molecular, biochemical, and physiological processes that affect metabolism, cell cycle, hormone production, sleep patterns and body temperature [56]. For example, the acrophase of body temperature is reached between 06:00-08:00 PM, and the batiphase between 06:00-08:00 AM [57]. Additionally, cortisol secretion peaks around 07:00-08:00 AM and reaches nadir around 02:00-04:00 AM [58, 59]. In contrast, melatonin secretion typically starts rising 2-3 hours before the usual bedtime, with an acrophase around 02:00-04:00 AM [60, 61].

The molecular core circadian clock (CC) is controlled by autoregulatory transcriptional-translational feedback loops (Figure 3) [55]. The positive loop involves the circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like 1 (BMAL1) proteins. These proteins form a heterodimer that drives the expression of clock-controlled genes, leading to the expression of cryptochrome circadian regulator (CRY) and the period circadian regulator (PER). PER and CRY interact and repress the transcriptional effect of the CLOCK/BMAL1 complex, representing the negative loop [62]. Furthermore, CLOCK and BMAL1 activate the nuclear receptors REV-ERB α and REV-ERB β (encoded by the nuclear subfamily 1 receptor group D member 1 and 2 [NR1D1 and NR1D2]), competing with the retinoic acid-related orphan receptors (ROR α and ROR β) to bind retinoic acid-related orphan receptor response elements (RREs) in the *BMAL1* promoter. BMAL1 is activated by the RORs and repressed by the REV-ERBs. Nuclear factor, interleukin 3 regulated (NFIL3), D-box binding protein (DBP), and the CLOCK/BMAL1 heterodimer regulate the rhythmic expression of REV-ERB α/β [63, 64].

Circadian disruption has been increasingly linked to increased cancer risk and progression [63]. Studies have revealed CR interaction with cancer-related pathways, such as cell proliferation, apoptosis, inflammation, metabolic reprogramming, DNA repair, and metastasis [65].

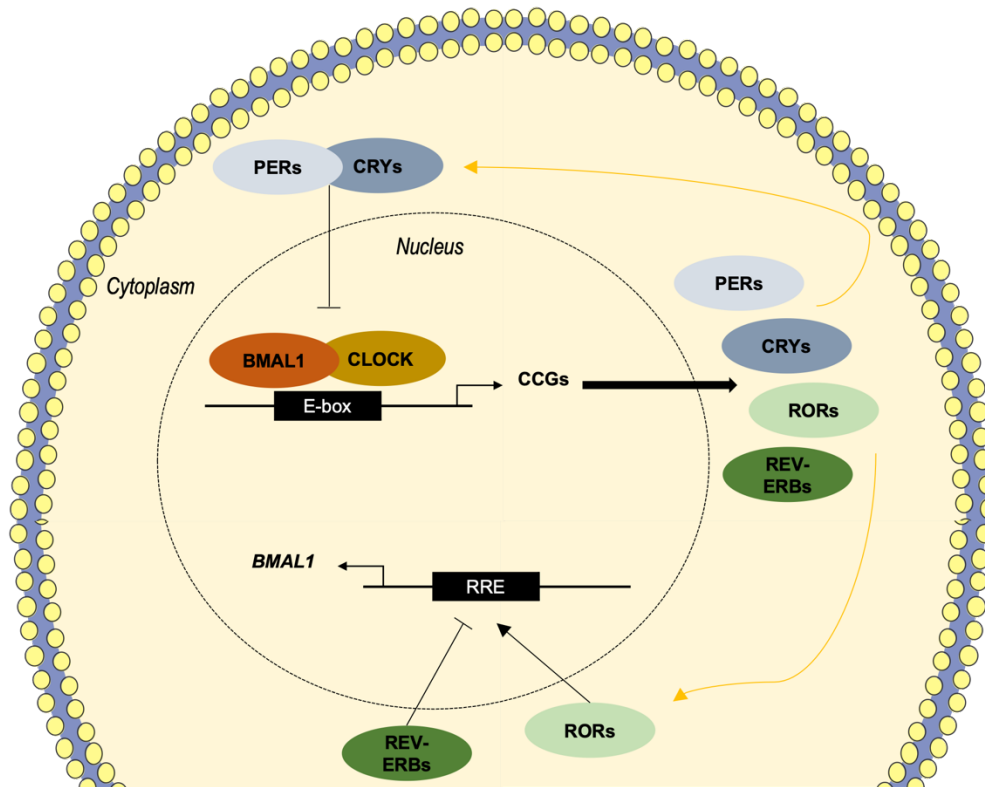


Figure 3. Circadian rhythm mechanism. The circadian rhythm is regulated by three molecular feedback loops involving key regulatory genes and proteins. Among them, the heterodimer of BMAL1 and CLOCK promotes the expression of *PERs* and *CRYs* genes which in turn inhibit BMAL1 and CLOCK activity. CCGs, Clock-controlled genes; RRE, retinoic acid-related orphan receptor response elements. Oliveira-Sousa, R. unpublished.

For example, the G2/M cell cycle checkpoint kinase inhibitor, WEE1, is regulated by the CLOCK/BMAL1 complex; and the ROR and REV-ERB proteins control G1/S cell cycle inhibitor p21 [66]. Regarding DNA damage response, the PER proteins interact with ATM and Checkpoint kinase 1 (CHK1) to modulate the DNA damage response, facilitating the repair of DNA double-strand breaks [65]. Furthermore, Tumour protein p53 (p53), along with c-MYC and RAS, have complex links with the CC [65]. p53 downregulates PER2 expression, and in turn stabilises p53 by binding to it [67, 68]. The heterodimer formed by BMAL1 and neuronal PAS domain protein 2 (NPAS2; paralogue of CLOCK) regulates MYC, which inversely influences cell proliferation [69]. Additional studies have identified an association between CC dysregulation and epithelial-mesenchymal transition (EMT), immune cell exhaustion, and cancer cell dissemination [70-72]. Moreover, it appears that the role of the circadian genes may vary depending on context. Certain clock genes were identified as tumour suppressors, while some appear to have oncogenic properties [73].

EPIGENETICS

Epigenetics refers to the mechanisms that regulate gene expression without altering the DNA sequence [74]. The major epigenetic mechanisms include DNA methylation, histone modifications, histone variants, chromatin remodelling complexes, and various families of non-coding ribonucleic acid (RNA) (Figure 4) [75]. These processes, crucial to maintaining normal cell development and differentiation, are heritable during cell division and can be reversible [76]. However, when disrupted, they can lead to uncontrolled cell growth and differentiation defects [77]. As such, they are extensively studied as alternative drivers of carcinogenesis and are recognised as an important hallmark of cancer [78].

DNA methylation involves the covalent addition of methyl groups to cytosines, predominantly in cytosine residues present in CpG dinucleotides. These CpG sites cluster in regions called CpG islands often located in the 5' end promoter region of gene [79]. In normal cells, CpG islands of tumour suppressor genes are usually unmethylated, but aberrant methylation can silence these genes expression. In cancer this process is associated with the inactivation of tumour suppressor genes, DNA repair genes, and other key regulators of cellular homeostasis [80].

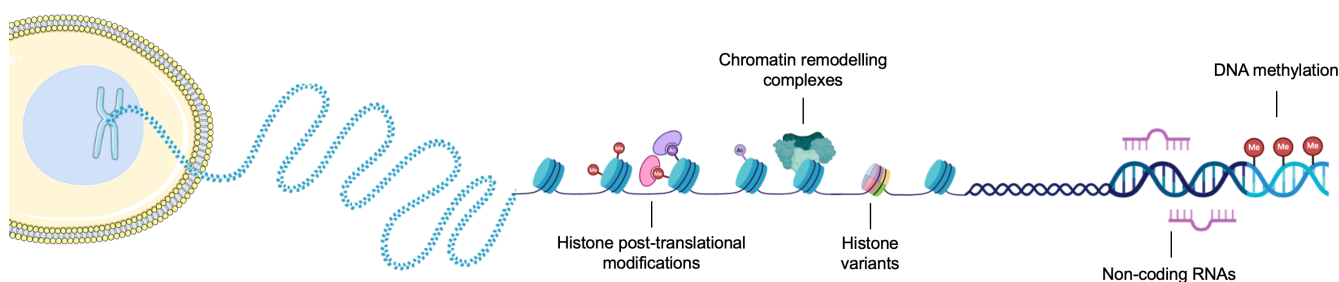


Figure 4. Epigenetic mechanisms of regulation of gene expression. The most common epigenetic mechanisms involve histone post-translational modifications, chromatin remodelling complexes, histone variants, non-coding RNAs and DNA methylation. Created with BioRender.com. Oliveira-Sousa, R. *unpublished*.

Histone modifications regulate chromatin structure and accessibility, thereby influencing gene transcription [74], and include but not only acetylation, methylation, and phosphorylation of specific residues on histone proteins [81]. Histone acetylation is generally associated with transcriptional activation, as it leads to a more open chromatin structure, facilitating access to the transcriptional machinery [81]. The dynamic balance between acetylation and deacetylation reactions regulates the levels of acetylation [82]. Histone acetyltransferases (HATs) enzymes, known as "writers," catalyse the addition of acetyl groups, promoting gene activation. On the other hand, histone deacetylases (HDACs) enzymes, referred as "erasers", remove acetyl groups, leading to chromatin condensation and transcriptional repression [81].

In addition to the genetic transcriptional-translational feedback loops and posttranslational regulation of the core CC, there is an interplay between the CC and epigenetics, where dynamic transitions in the chromatin state prepare the genome for rhythmic gene expression [83]. The core heterodimer CLOCK/BMAL1 engages with the transcriptional co-activators histone acetyltransferases p300, CREB-binding protein (CBP), and p300/CBP-associated factor (pCAF [KAT2B]) to acetylate histones and create an open chromatin state conducive to transcription at the onset of the transcription cycle [84, 85]. CLOCK was identified as an intrinsic histone acetyltransferase (KAT13D) that acetylates histone H3 on lysine 9 and 14 residues [84, 86]. Additionally, the circadian levels of NAD⁺, regulated by CLOCK-BMAL1, drive a rhythm in the activity of sirtuin 1 (SIRT1), in turn, feedback inhibiting the CLOCK-BMAL1 heterodimer [87]. SIRT1 also deacetylates PER2, promoting its degradation, and BMAL1, reducing its ability to interact with CRY1 [88, 89].

CHRONORADIOTHERAPY IN PROSTATE CANCER

Chronotherapy refers to the practice of optimising treatment timing to coordinate with the CR, aiming to maximise treatment efficacy and minimise side effects [65, 90]. The goal is to chronomodulate treatment delivery based on biological rhythms. Multiple clinical trials of phase I to III have validated the importance of timing treatments according to CR, confirming its potential to enhance outcomes [60]. Three approaches to chronotherapy have been proposed. First, “training the clock” involves actions that help to maintain or improve an optimal CR. Second, “drugging the clock” refers to the use of small molecules or drugs that modulate core clock functions, such as reinforcing the clock’s amplitude or altering its period phase. Finally, “clocking the drug” focuses on optimising the timing of drug or radiation administration to enhance efficacy and reduce toxicity [65, 91]. While chronomodulation has been extensively studied in chemotherapy, the principles of chronotherapy have also been applied to RT [92].

Studies on chronoradiotherapy, which primarily fall under the “clocking the drug” approach, have demonstrated that the timing of RT can significantly impact both treatment response and side effects. A systematic review of nine studies examined the impact of RT timing on tumour control and normal tissue response [93]. Most of the studies reviewed were retrospective, lacking prospective randomised trials comparing morning and afternoon treatments. Of the five studies evaluating overall survival, three reported significantly better outcomes with morning RT [94-96]. Regarding tumour control, three out of nine studies observed better results with morning treatment, while

one study found midday treatment to be more effective for women, and another reported better outcomes with afternoon RT [94-98]. When examining the quality of life and treatment-related symptoms, five studies found significant differences between morning and afternoon treatment groups, with morning RT associated with fewer side effects [94, 99-102]. In PCa, a retrospective cohort study in Taiwan found that patients who received RT in the evening experienced worse freedom from grade ≥ 2 late gastrointestinal (GI) toxicities. Additionally, in patients with T2b-T3, the 5-year BFS rate was significantly worse in the evening group [94]. Another study on localised PCa, not addressed in the review, observed that morning RT led to significantly improved quality of life and reduced worsening of lower urinary tract symptoms (LUTS) [103]. Although with inconsistent results, timing impacts were more prominently concerning side effects rather than oncological outcomes. This highlights the need for further prospective trials to fully assess the influence of chronoradiotherapy on both tumour control and toxicities.

Response to RT appears to be related to the expression of circadian genes. A study of RT in rectal cancer patients with complete tumour regression had *CLOCK*, *CRY2* and *PER2* genes and the downstream target gene *c-MYC* with significant higher expression [104]. In PCa, a risk score based on the expression of nine circadian clock genes (*CSNK1D*, *BTRC*, *CLOCK*, *CSNK1E*, *FBXL3*, *PRKAA2*, *DBP*, *NR1D2*, and *RORB*) was developed, demonstrating strong predictive value, particularly for disease-free survival (DFS). Higher risk scores were significantly associated with shorter DFS, early disease progression, and higher mortality rates [105]. In another study, a ten-gene signature, including key clock genes, was established to predict progression risk in PCa. *CLOCK*, *PER* (1, 2, 3), *CRY2*, *NPAS2*, *RORA*, and *BMAL1* showed greater correlation with anti-oncogenes and *CSNK1D* and *CSNK1E* with oncogenes. Patients with higher risk scores had lower gene expression of *PER1*, *PER2*, and *CRY2* and higher expression of *CSNK1E*, with a greater likelihood of disease progression [106].

One of the key challenges in implementing chronotherapy is the variation in individual chronotypes [107]. Each person has unique characteristics that influence the timing of their circadian phases. Dim light melatonin onset (DLMO) is a common non-invasive method to assess an individual's circadian phase, using saliva samples [108]. Also, other emerging techniques, such as blood tests, aim to determine circadian rhythms by analysing gene expression patterns. A promising tool called BodyTime can estimate a person's internal circadian time by measuring specific clock-related biomarkers, including *NR1D1*, *PER1*, *CRY1*, and several others, from a single blood sample [109].

In summary, understanding of the connection between CRs, epigenetic regulation, and RT in PCa, could lead to more personalised and effective treatments.

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CHAPTER 2

Aims

Aims

Prostate cancer (PCa) remains a significant health burden in men worldwide. While radiotherapy (RT) is a common curative treatment for localised disease, its efficacy and side effects can differ widely among patients. Research points to the role of circadian rhythms (CRs) in modulating cellular processes, such as DNA repair and cell cycle regulation, which are key to RT success. Despite this, the impact of CRs on RT outcomes remains a largely unexplored field in PCa management. Studies have indicated that RT timing may influence therapeutic efficacy and toxicity, suggesting that aligning treatment with patients' biological clock could enhance results. Addressing these gaps holds the potential to significantly improve patient care by enabling more personalised RT strategies.

Accordingly, the main goal of this project is to investigate the impact of CR on RT outcomes in PCa patients. Additionally, we aim to dissect epigenetic-based mechanisms behind CRs regulation to find new potential targets and novel radiosensitising approaches.

Accordingly, the specific aims of this project are:

- **Task 1:** Characterise the impact of RT treatment timing in PCa in a retrospective study cohort.
- **Task 2:** Mechanism dissection for CR controlling, by an epigenetic approach
 - Task 2.1.: Investigate the impact of circadian clock regulators, PER2 and BMAL1, on PCa RT response
 - Task 2.2.: Understand the epigenetic-based mechanism of circadian cycle regulation and its impact on the response to RT.
- **Task 3:** Evaluate the RT-related acute and late toxicities in a prospective study of PCa patients, based on the individual chronotype.

CHAPTER 3

Materials and methods

Material and Methods

This chapter outlines briefly the tasks, procedures, and techniques learned and applied throughout the MSc program, being specified in the respective chapters:

Chapter 4

- Retrospective cohort study:
 - o Patient selection;
 - o Clinical data management: collection and interpretation of the clinical data. Assessment to the treatment details, early and late toxicities, and follow-up information;
 - o Statistical analysis: survival analysis, univariate and multivariate Cox regression, multivariate binary logistic regression models, and propensity score matching.

Chapter 5

- Nucleic acid and protein extraction:
 - o RNA extraction from prostate cancer cell lines, using Trizol reagent;
 - o Total protein extraction from cell lines and quantification using BCA protein assay kit;
 - o Histone extraction and quantification.
- mRNA expression analyses:
 - o cDNA synthesis using Revert Aid RT kit;
 - o Real-time quantitative PCR, using SYBR green and specific primers.
- Protein analyses:
 - o Western blot.
- DNA methylation analyses:
 - o Genome-wide methylation analysis using the Infinium Methylation EPIC (Epic) BeadChip array.
- RNA sequencing
- *In vitro* experiments:
 - o Cell culture;
 - o Circadian rhythm synchronisation;
 - o Clonogenic assay;
 - o siRNA transfection.

Chapter 6

- Prospective cohort study:

- Protocol establishment;
- Patient recruitment;
- Application of standardised questionnaires: Munich ChronoType Questionnaire (MCTQ), European Organization for Research and Treatment of Cancer Quality of Life Questionnaire Core-30 (EORTC QLQ C30), and Functional Assessment of Chronic Illness Therapy – Fatigue (FACIT-F);
- Clinical data management: collection and interpretation of clinical data, including treatment details (e.g., technique, fractionation scheme, treated volume, treatment time), acute and late toxicities, and follow-up information;
- Radioimmunoassay for salivary melatonin quantification.

CHAPTER 4

Retrospective cohort study

Impact of Radiotherapy Timing On Survival and Radiotoxicity Outcomes In Localized Prostate Cancer

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ABSTRACT

Importance: Prostate cancer is the second most common cancer in men. Nearly 30% of patients diagnosed with prostate cancer are treated with external beam radiotherapy as first-line therapy. However, 27 to 53% of patients with localized prostate cancer experience disease progression or recurrence. The circadian rhythm regulates 24-hour biological cycles, influencing cell growth, DNA repair, metabolism and the immune system. Clinical evidence suggests that circadian rhythm and the time of external beam radiotherapy administration influence the response to treatment and its toxicities. A study in a Taiwanese population showed a significant increase in gastrointestinal complications and biochemical recurrences in patients treated in the evening. **Objective:** This study sought to evaluate the potential effect of external beam radiotherapy time of day on therapeutic effectiveness in patients with localized prostate cancer. **Design, Setting, and Participants:** This retrospective study included 224 patients with localized prostate cancer treated at the Portuguese Oncology Institute of Porto between 2012 and 2021. Patients were divided into two groups based on the ROC curve for metastasis-free survival using treatment time (before and after 12:45 PM). For further analysis, a second approach was established by separating the groups with 3.5 hours (08:30-11:30 AM and 03:00-08:00 PM). **Main Outcomes and Measures:** Clinical outcomes and toxicity levels were evaluated using Kaplan-Meier survival curves, Cox proportional hazards models, and binary logistic regression analyses. Matched-pair analysis using propensity scores was conducted to balance confounding variables between groups. **Results:** Morning external beam radiotherapy was consistently and significantly associated with improved for metastasis-free survival compared to afternoon treatment (log-rank $p < 0.05$). Cox proportional hazard analysis confirmed that afternoon treatment was a significant predictor of worse for metastasis-free survival ($p < 0.05$). Furthermore, morning treatment was significantly associated with lower grades of late gastrointestinal and genitourinary toxicities in a multivariable analysis ($p < 0.05$). **Conclusions and Relevance:** Consistent with Taiwan's study, our results demonstrate that the timing of external beam radiotherapy administration influences efficacy and toxicity in prostate cancer patients. Although more robust studies are needed for validation, external beam radiotherapy timing optimization could improve clinical outcomes and the quality of life of prostate cancer patients.

Keywords: Circadian rhythm, chronobiology, prostate cancer, oncological outcomes, treatment time of day

INTRODUCTION

Prostate cancer (PCa) remains a significant global health concern, ranking as the second most frequent male malignancy in 2022, with an expected 96.2% increase in incidence by 2050 [1]. Nearly 30% of newly diagnosed PCa patients undergo external beam radiotherapy (EBRT) as a first-line treatment, especially as adherence to radical prostatectomy tends to decline [2]. Despite the improvements in EBRT, including advanced techniques, improved fractionation schemes, and enhanced precision, 27 to 53% of localized PCa patients experience biochemical recurrence [3, 4]. Moreover, the therapeutic benefits of EBRT are often accompanied by potential debilitating side effects that can impair patients' functional capacity and quality of life [5].

Circadian rhythm (CR) is an approximately 24-hour biological cycle that governs numerous day-night variations in living organisms [6]. Importantly, clocks in peripheral tissues, including the prostate, operate independently but are influenced by the central clock, the suprachiasmatic nucleus, in the brain [7]. A close interplay between CRs and the cell cycle exists [8]. Indeed, circadian clock genes control tissue-specific cell cycle checkpoint proteins, resulting in circadian variations in cell growth and renewal [9]. Additionally, the CR regulates rhythmic events in DNA repair, oxidative defence mechanisms, metabolism, apoptosis, and the immune system [10].

Ionizing radiation (IR) disrupts DNA structure, impairs DNA repair mechanisms, and induces double-strand breaks [11]. These cytotoxic effects trigger cell cycle arrest, ultimately leading to cell death [11]. Cell radiosensitivity varies with the cell cycle phase, with G2/M being the most radiosensitive [12]. Therefore, this variation underscores the time-dependent resistance of cells to cytotoxic exposures, such as IR [13].

Previous clinical evidence suggests that CR and treatment timing influence RT response and associated toxicities. A study conducted by investigators at the National Taiwan University Hospital found that evening EBRT was associated with increased gastrointestinal (GI) complications, particularly in older patients, and worse biochemical-free survival (BFS) in PCa patients with T2b-T3 disease [9]. Similarly, another study observed that morning EBRT for localized PCa improved quality of life and reduced worsening of lower urinary tract symptoms [14].

This study aims to comprehensively characterize the impact of EBRT treatment timing in PCa patients through a retrospective analysis. Specifically, it seeks to validate previous findings observed in Taiwanese PCa patients within a Portuguese cohort.

MATERIALS AND METHODS

Patient selection criteria

This study enrolled 224 PCa patients treated at the Portuguese Oncology Institute of Porto (IPO Porto) from 2012 to 2021, according to specific inclusion and exclusion criteria. Eligible patients had a confirmed diagnosis of localized prostate adenocarcinoma and received definitive EBRT with or without androgen deprivation therapy (ADT). Exclusion criteria encompassed metastatic disease, history of cancer within five years of the PCa diagnosis, prior prostate surgery, treatments with three-dimensional conformal radiation therapy (3D-CRT) or stereotactic body radiotherapy (SBRT), and follow-up conducted outside of IPO Porto. Patients were excluded when at least two-thirds of their treatment fractions were not administered in the morning or the afternoon.

Radiotherapy treatment procedures

EBRT was administered using the Novalis or the Trilogy linear accelerators from Varian Medical Systems with a 6 MV photon beam. Patients were immobilized in a supine position using individualized supports, with a comfortably full bladder and an empty rectum. Treatment techniques included intensity-modulated radiotherapy (IMRT) and volumetric modulated arc therapy (VMAT). Treatment schedules prioritized patient preference and appointment availability, disregarding individual clinical factors. All treatments occurred during the standard operating hours of the IPO Porto radiotherapy department (8:00 AM to 9:00 PM).

Toxicity evaluation and follow-up protocols

Genitourinary (GU), GI, and radiation dermatitis toxicities were graded using the Radiation Therapy Oncology Group (RTOG) acute and late morbidity scoring schema. Toxicities were defined as acute if they appeared until three months after the beginning of EBRT and as late if presented after this period. Follow-up was conducted at regular intervals according to the institute's protocol, with PSA measurements and toxicity assessments at specified intervals. Patients treated with ADT were monitored at six-month intervals for five years, while those who did not receive ADT had a follow-up every four months during the first year and every six months until the fifth year. After five years, all patients were followed annually.

Data collection and endpoints definition

Clinical and treatment data were obtained from the medical records and Varian's ARIA patient information system. The following endpoints (each measured from the time of

diagnosis until the occurrence of the respective event) were defined: BFS as the time without a prostate-specific antigen (PSA) increase exceeding 2.0 ng/ml above the nadir value (according to the Phoenix criteria); locoregional recurrence-free survival (LRFS) as the time without locoregional disease; metastasis-free survival (MFS) as the time without distant metastasis; progression-free survival (PFS) as the time without biochemical failure or locoregional or distant disease; and overall survival (OS) as the time until death from any cause.

Statistical analysis

Receiver operating characteristic (ROC) curves were generated for all endpoints using the R packages “rms”, “caTools”, “epicalc”, and “pROC”. The cohort was divided into two groups by the optimal cut-off selected with Youden's index from the most informative ROC curve: patients treated before 12:45 PM (n = 112) and after 12:45 PM (n = 112). For further analysis, a second approach was established by dividing patients into two groups separated by 3.5 hours: a morning group treated between 8:30 AM and 11:30 AM (n = 86) and an afternoon group treated between 3:00 PM and 8:00 PM (n = 101). Patients outside of these time frames were excluded.

Matched-pair analysis using propensity scores was conducted for group comparisons. Matching was performed with the “MatchIt” package (version 4.5.5) in R, employing the nearest-neighbour method. Propensity scores (PS) were calculated via logistic regression, adjusted by age, use of short- or long-term ADT, fractionation scheme, treated volume, Eastern Cooperative Oncology Group (ECOG) performance status, and risk group defined by the NCCN guidelines. Each morning EBRT case was matched to an afternoon EBRT case based on the closest PS without replacement, with a 1:1 ratio. We used standardized mean difference (SMD) with a target range of -0.2 to 0.2 for all covariates. A caliper of 0.4 standard deviations of the logit of the PS was applied if the SMD for any covariate exceeded this range, ensuring adequate balance, using the “cobalt” R package.

Normal distribution was checked using the Kolmogorov-Smirnov and Shapiro-Wilk tests, and the homogeneity of variances with Levene's test. Group differences in continuous clinical characteristics and survival outcomes were analysed using the Mann-Whitney U test.

Categorical clinical characteristics, survival outcomes, and grade distributions between groups were compared using the chi-squared test. For 2x2 contingency tables, a continuity correction was applied, and Fisher's exact test was employed when

over 20% of the expected cell counts were less than 5. Cramer's V was calculated to measure the effect size for Fisher's exact and chi-squared tests.

Kaplan-Meier (KM) curves were generated to estimate the endpoint rates, and the logrank test was used to evaluate survival differences between groups with the R packages "survival" and "survminer". Cox proportional hazards regression models, univariable and multivariable, were used to identify prognostic factors for oncological outcomes. Assumptions of the Cox models were verified using Schoenfeld residuals and proportional hazard tests. Multivariable binary logistic regression models were applied to explore the association between toxicities and covariates.

All descriptive and inferential analyses were conducted using IBM® SPSS® Statistics software (version 29 for macOS) (IBM Corp, Armonk, NY, USA) and RStudio (R software, version 4.3.1 for macOS) (PBC, Boston, MA, USA). The 2-tailed alpha level was set at 0.05, with a confidence interval of 95% for all statistical tests.

RESULTS

Patient characteristics

The median age of the 224 patients was 69 years (range 51-84). Most patients were classified as having favourable intermediate risk (72.3%), and only 14.3% received ADT. VMAT technique was used in 75% of cases, with 83% treated with moderate hypofractionation. In 49.1% of the patients, the treatment volume was confined to the prostate.

A cut-off time of 12:45 PM was derived as optimal for distinguishing between morning and afternoon treatments concerning MFS (Appendix II: Supplementary Figure 1). According to this division, 112 patients were treated between 08:30 AM and 12:45 PM and 112 between 12:45 PM and 8:00 PM. Table 1 presents a comparison of clinical and treatment characteristics between these groups. Patients treated after 12:45 PM only had a significantly lower proportion of clinical stage T2 (79.5% vs. 64.3%; $p = 0.031$; Cramer's $V = 0.175$) and ECOG 0 status (92.9% vs. 83.0%; $p = 0.045$; Cramer's $V = 0.162$) than the morning group. However, there were no significant differences in the risk group distribution.

We performed a matched-pair analysis to assess the impact of treatment time on MFS while balancing the groups for confounding variables (Table 1, Appendix II: Supplementary Figure 2). In the first matched approach, 11 patients from each group were excluded, leaving 101 patients in each treatment group ($n = 202$). The afternoon

treatment group remained with a significantly lower proportion of clinical stage T2 (80.2% vs. 63.4%; $p = 0.029$; Cramer's $V = 0.187$), but the distribution of risk groups remained balanced between the treatment groups (Table 1). Treatment groups were separated by a 3.5-hour gap in the second approach analysis. Following propensity score matching, this approach comprised 172 patients, equally distributed between the morning and afternoon treatment groups (Appendix II: Supplementary Figure 3 and Appendix II: Supplementary Table 1). All clinical characteristics were well-balanced between groups, with no significant variability observed (Appendix II: Supplementary Table 1).

Morning EBRT is associated with improved metastasis-free survival

For the entire cohort, the median follow-up was 66.3 months. KM estimates for the 5- and 10-year survival rates cohort-wide were: BFS at 93.3% and 60.2%, LRFS at 96.8% and 75.7%, MFS at 92.7% and 59.9%, PFS at 95.4% and 79.2%, and OS at 96.3% and 82.3%, respectively. The median survival rate for all outcomes showed no differences when comparing both groups (Table 1).

In the unadjusted KM analysis, the 10-year LRFS was significantly higher in patients treated after 12:45 PM than those treated earlier (81.1% versus 66.8%; $p = 0.0355$). In contrast, the 10-year MFS was significantly higher in patients treated before 12:45 PM (90.3% versus 68.2%; $p = 0.0002$). However, only the KM survival curves for MFS revealed a statistically significant difference with higher MFS in patients treated before 12:45 PM (logrank $p = 0.014$) (Figure 1A). There were no significant differences for other clinical endpoints between treatment groups (Appendix II: Supplementary Figure 4).

Multivariable analysis identified treatment after 12:45 PM as a significant predictor of worse MFS (HR = 0.2362; 95%CI - 0.0642, 0.8691; $p = 0.0299$; Table 2). Additionally, ECOG performance status significantly predicted poorer OS (HR = 4.9198; 95%CI - 1.7125; 14.1340; $p = 0.0031$). Detailed multivariable results for predictors of survival outcomes are summarized in Tables 2 and Appendix II: Supplementary Table 2.

Table 1. Patient clinicopathological characteristics and survival endpoints in the first unmatched and matched approaches.

Characteristics	1 st unmatched approach		<i>p</i> -value [†] (effect size) [‡]	1 st matched approach		<i>p</i> -value [†] (effect size) [‡]
	Before 12:45 PM	After 12:45 PM		Before 12:45 PM	After 12:45 PM	
	n (%)	n (%)		n (%)	n (%)	
Total	112	112		101	101	
Age			0.951			0.823
median (range)	69.0 (51-80)	69.0 (54-84)		69.0 (51-80)	69.0 (55-84)	
Clinical tumor stage			0.031 (0.175)			0.029 (0.187)
T1c	16 (14.3)	31 (27.7)		16 (15.8)	29 (28.7)	
T2	89 (79.5)	72 (64.3)		81 (80.2)	64 (63.4)	
T3	7 (6.3)	9 (8.0)		4 (4.0)	8 (7.9)	
ISUP grade			0.574			0.314
1	23 (20.5)	24 (21.4)		22 (21.8)	22 (21.8)	
2	69 (61.6)	67 (59.8)		65 (64.4)	61 (60.4)	
3	10 (8.9)	16 (14.3)		6 (5.9)	14 (13.9)	
4	8 (7.1)	4 (3.6)		6 (5.9)	3 (3.0)	
5	2 (1.8)	1 (0.9)		2 (2.0)	1 (1.0)	
Initial PSA (ng/mL)			0.639			0.201
<10	76 (67.9)	80 (71.4)		68 (67.3)	71 (70.3)	
10-20	30 (26.8)	29 (25.9)		27 (26.7)	29 (28.7)	
>20	6 (5.4)	3 (2.7)		6 (5.9)	1 (1.0)	
Risk group*			0.587			0.176
Low risk	4 (3.6)	6 (5.4)		4 (4.0)	6 (5.9)	
Favorable intermediate risk	83 (74.1)	79 (70.5)		81 (80.2)	72 (71.3)	
Unfavorable intermediate risk	8 (7.1)	13 (11.6)		4 (4.0)	12 (11.9)	

ADT	High risk	17 (15.2)	14 (12.5)	0.758	12 (11.9)	11 (10.9)	0.602
	None	94 (83.9)	98 (87.5)		85 (84.2)	90 (89.1)	
	Short-term	5 (4.5)	4 (3.6)		5 (5.0)	4 (4.0)	
	Long-term	13 (11.6)	10 (8.9)		11 (10.9)	7 (6.9)	
ECOG performance status				0.045 (0.162)			>0.999
	0	104 (92.9)	93 (83.0)		93 (92.1)	93 (92.1)	
	1	8 (7.1)	17 (15.2)		8 (7.9)	8 (7.9)	
	2	0	2 (1.8)		0	0	
Technique				>0.999			>0.999
	IMRT	28 (25.0)	28 (25.0)		26 (25.7)	26 (25.7)	
	VMAT	84 (75.0)	84 (75.0)		75 (74.3)	75 (74.3)	
Fractionation scheme				0.594			>0.999
	Conventional	21 (18.8)	17 (15.2)		17 (16.8)	16 (15.8)	
	Moderate hypofractionation	91 (81.3)	95 (84.8)		84 (83.2)	85 (84.2)	
Treated volume				0.337			>0.999
	Prostate	50 (44.6)	60 (53.6)		50 (49.5)	50 (49.5)	
	Prostate and SV	53 (47.3)	47 (42.0)		47 (46.5)	47 (46.5)	
	Prostate, SV and Pelvic lymph nodes	9 (8.0)	5 (4.5)		4 (4.0)	4 (4.0)	
Endpoints		n (%)	n (%)	p-value [†] (effect size) [‡]	n (%)	n (%)	p-value [†] (effect size) [‡]
Biochemical recurrence				0.544			0.514
	Yes	12 (10.7)	16 (14.3)		10 (9.9)	14 (13.9)	
	No	100 (89.3)	96 (85.7)		91 (90.1)	87 (86.1)	
Locoregional recurrence				>0.999			>0.999
	Yes	9 (8.0)	8 (7.1)		8 (7.9)	8 (7.9)	

	No	103 (92.0)	104 (92.9)		93 (92.1)	93 (92.1)	
Metastasis				0.032 (0.161)			0.037 (0.168)
	Yes	3 (2.7)	12 (10.7)		2 (2.0)	10 (9.9)	
	No	109 (97.3)	100 (89.3)		99 (98.0)	91 (90.1)	
Progression				0.426			0.393
	Yes	12 (10.7)	17 (15.2)		10 (9.9)	15 (14.9)	
	No	100 (89.3)	95 (84.8)		91 (90.1)	86 (85.1)	
Deaths				0.482			0.794
	Yes	8 (7.1)	12 (10.7)		7 (6.9)	9 (8.9)	
	No	104 (92.9)	12 (10.7)		94 (93.1)	92 (91.1)	
BFS (months)				0.329			0.206
	median (range)	65.7 (16.6-154.1)	63.6 (20.2-129.5)		65.8 (16.6-154.1)	63.0 (20.2-129.5)	
LRFS (months)				0.945			0.630
	median (range)	66.1 (16.6-154.1)	65.1 (26.9-143.9)		66.4 (16.6-154.1)	64.4 (26.9-143.9)	
MFS (months)				0.325			0.220
	median (range)	66.1 (16.6-154.1)	64.3 (24.7-139.6)		66.4 (16.6-154.1)	63.1 (24.7-139.6)	
PFS (months)				0.330			0.207
	median (range)	65.7 (16.6-154.1)	63.6 (20.2-129.5)		65.8 (16.6-154.1)	63.0 (20.2-129.5)	
OS (months)				0.971			0.103
	median (range)	66.7 (22.9-154.1)	65.9 (27.4-144.1)		67.0 (22.9-154.1)	64.7 (27.4-144.1)	

*Risk classification is based on clinical tumor stage, ISUP grade and initial PSA serum levels, according to the National Comprehensive Cancer Network (NCCN) guidelines.

†Statistical tests applied: Mann-Whitney U test for continuous variables, chi-squared test for categorical variables, Fisher's exact test when expected cell counts were less than 5, and chi-squared test with continuity correction for 2x2 contingency tables.

‡Effect size (Cramer's V) was computed for categorical variables when the *p*-value < 0.05.

Bold values indicate statistical significance (*p*-value < 0.05).

Abbreviations: PM, *post meridiem*; ISUP, International Society of Urological Pathology; PSA, prostate-specific antigen; AJCC, American Joint Committee on Cancer; ADT, androgen deprivation therapy; ECOG, Eastern Cooperative Oncology Group; SV, seminal vesicles; BFS, biochemical-free survival; LRFS, locoregional recurrence-free survival; MFS, metastasis-free survival; PFS, progression-free survival; OS, overall survival.

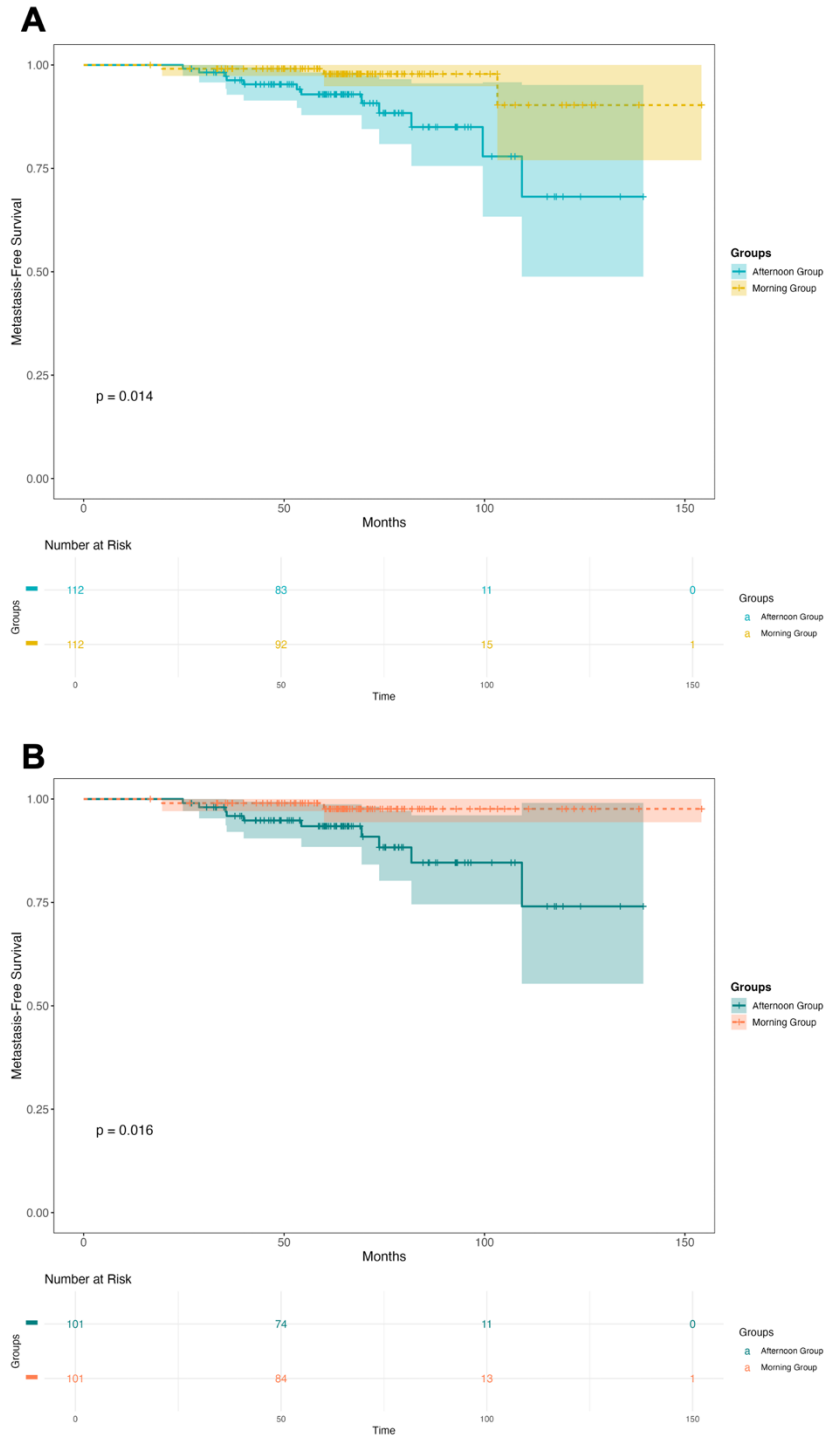


Figure 1. (upper) Kaplan-Meier curves of metastasis-free survival (MFS) in morning and afternoon external beam radiotherapy groups for the unmatched first approach ($n = 224$) (A) and for the matched first approach ($n = 202$) (B). The analysis demonstrates a statistically significant difference in MFS favoring the morning treatment group (Log-rank test: (A) $p = 0.014$; (B) $p = 0.016$). (lower) Number of patients at risk within the afternoon and morning groups, along the time.

Table 2. Patient clinicopathological characteristics of the matched first and second cohorts.

Approach	Variables	Coefficient (S.E.)	HR (95% CI)	Z	p-value
1st unmatched approach					
	Treatment group	-1.4430 (0.6647)	0.2362 (0.0642-0.8691)	-2.1710	0.0299
	ECOG performance status	0.0209 (0.7808)	1.0211 (0.2210-4.7174)	0.0270	0.9786
	Age	-0.0047 (0.0419)	0.9954 (0.9169-1.0805)	-0.1110	0.9114
	*Risk group	0.6966 (0.4490)	2.0070 (0.8324-4.8388)	1.5510	0.1208
	ADT	-0.2524 (0.4965)	0.7769 (0.2936-2.0559)	-0.5080	0.6112
	Fractionation scheme	-0.4892 (0.7967)	0.6131 (0.1286-2.9223)	-0.6140	0.5392
	Treated volume	-0.2622 (0.5646)	0.7694 (0.2544-2.3266)	-0.4640	0.6424
1st matched approach					
	Treatment group	-1.6643 (0.7748)	0.1893 (0.0415-0.8644)	-2.1480	0.0317

*Risk classification is based on clinical tumor stage, ISUP grade and initial PSA serum levels, according to the National Comprehensive Cancer Network (NCCN) guidelines.

Bold values indicate statistical significance (p -value < 0.05).

Abbreviations: ECOG, Eastern Cooperative Oncology Group; ADT, androgen deprivation therapy; HR, hazard ratio; S.E., standard error; CI, confidence interval.

The first matched approach analysis corroborated significant differences found in MFS between patients treated before and after 12:45 PM (logrank $p = 0.016$; HR = 0.1893; 95%CI - 0.0415, 0.8644; $p = 0.0317$; Figure 1B; Appendix II: Supplementary Table 3). In the second matched approach, significant differences in MFS were again observed between the morning and afternoon groups (logrank $p = 0.026$; HR = 0.2071; 95%CI - 0.04471, 0.9589; $p = 0.044$; Appendix II: Supplementary Figure 5; Appendix II: Supplementary Table 3). Other clinical endpoints showed no significant differences between the treatment groups in the matched approaches (Appendix II: Supplementary Figure 6-7).

Morning treatment is linked to lower grades of late GI and GU toxicities

Incidence of all grades of acute GI, GU, and radiation dermatitis between groups are shown in eTables 4-6 Supplement 1. In the first unmatched group, the morning group, compared to the afternoon group, had a significantly lower incidence of late GI grade 2 or higher (1.8% versus 10.7%; $p = 0.013$; Cramer's V = 0.184; Table 3) and late GU toxicities (12.5% versus 29.5%; $p = 0.003$; Cramer's V = 0.208; Table 3). Similar late GI and GU toxicities were observed in the matched approaches (Table 3 and Appendix II: Supplementary Table 7).

In the multivariable analysis of the matched (first and second) and unmatched first approach, morning treatment was significantly associated with a reduced risk of late GU toxicities across all approaches and GI toxicities in the first unmatched and matched. In the first unmatched approach, patients treated in the morning present an 86% lower risk of grade 2 or higher late GI toxicity (OR = 0.1383; 95% CI – 0.0209, 0.5332; $p = 0.012$; Appendix II: Supplementary File 1) and a 68% lower risk of grade 2 or higher late GU toxicity (OR = 0.3177; 95% CI – 0.1489, 0.6450; $p = 0.002$; Appendix II: Supplementary File 1). Similar findings were observed in the first and second matched approaches (Appendix II: Supplementary File 1). Across all approaches, patients treated with moderate hypofractionation had a significantly lower risk of grade 2 or higher acute GI toxicity (Appendix II: Supplementary File 1). Younger age was associated with a decreased risk of grade 2 or higher acute GU toxicity (Appendix II: Supplementary File 1), whereas older age and treatment with VMAT were associated with a higher incidence of erectile dysfunction (Appendix II: Supplementary File 1). Patients treated with VMAT had a significantly lower risk of developing anejaculation (Appendix II: Supplementary File 1).

Table 3. Comparison of acute and late gastrointestinal, genitourinary and radiation dermatitis toxicities by grade (<2 vs. ≥2) between treatment groups in the first matched and unmatched approach.

	1 st unmatched approach					1 st matched approach				
	Before 12:45 PM (n=112)		After 12:45 PM (n=112)		<i>p</i> -value [†] (effect size) [‡]	Before 12:45 PM (n=101)		After 12:45 PM (n=101)		<i>p</i> -value [†] (effect size) [‡]
	Grade <2	Grade ≥2	Grade <2	Grade ≥2		Grade <2	Grade ≥2	Grade <2	Grade ≥2	
Acute toxicities										
Gastrointestinal	105 (93.8)	7 (6.3)	98 (87.5)	14 (12.5)	0.169	95 (94.1)	6 (5.9)	88 (87.1)	13 (12.9)	0.148
Genitourinary	87 (77.7)	25 (22.3)	76 (67.9)	36 (32.1)	0.133	77 (76.2)	24 (23.8)	69 (68.3)	32 (31.7)	0.271
Late toxicities										
Gastrointestinal	110 (98.2)	2 (1.8)	100 (89.3)	12 (10.7)	0.013 (0.184)	99 (98.0)	2 (2.0)	90 (89.1)	11 (10.9)	0.022 (0.182)
Genitourinary	98 (87.5)	14 (12.5)	79 (70.5)	33 (29.5)	0.003 (0.208)	87 (86.1)	14 (13.9)	72 (71.3)	29 (28.7)	0.016 (0.181)
Radiation dermatitis										
	110 (98.2)	2 (1.8)	106 (94.6)	6 (5.4)	0.280	100 (99.0)	1 (1.0)	96 (95.0)	5 (5.0)	0.212

[†]Statistical tests applied: Fisher's exact test when expected cell counts were less than 5, and chi-squared test with continuity correction.

[‡]Effect size (Cramer's V) was computed for categorical variables when the *p*-value < 0.05.

Bold values indicate statistical significance (*p*-value < 0.05).

Abbreviations: PM, *post meridiem*.

DISCUSSION

This study aimed to evaluate the impact of EBRT treatment time on oncological outcomes and radiotoxicity in patients with localized PCa. ROC curve analyses across all oncological endpoints determined the ideal cut-off time to divide the groups. Setting the cut-off point at 12:45 PM allowed us to optimize the discrimination for MFS. In the second approach, we define two groups separated by a 3.5-hour window to test the findings in a narrower time range. While there is no consensus on the optimal method for defining treatment groups based on time of day, our dynamic approach enhanced the external validity of our results and ensured comparability with other studies. The results indicate that morning EBRT significantly impacts MFS and late radiotoxicity.

After the multivariable analysis, earlier treatment remained consistently associated with improved MFS and lower gradings of late GU and GI toxicities, minimizing the likelihood of observed differences between the groups being attributable to confounding variables. Furthermore, the matched-pair analysis reinforced these results, independently of potential clinical characteristics imbalances between groups, as illustrated in Figure 2. Specifically, morning treatment was associated with a 76.4% reduction in the risk of metastasis and an 86.4% and 68.4% reduction in the risk of grade 2 or higher late GI and GU toxicities, respectively. While other oncological endpoints showed no significant differences, it's important to note that MFS is the most relevant endpoint in PCa. These findings suggest that the response to EBRT varies throughout the day, aligning with previous research [15].

CR is crucial in the regulation of cell cycle checkpoint proteins [8, 16]. Research shows that the clock genes Period Circadian Regulator 1 (*Per1*), Period Circadian Regulator 2 (*Per2*), and Brain and Muscle ARNT-Like 1 (*Bmal1*) significantly influence cell growth [17-19], with their expression levels inversely correlated with the G2/M phase of the cell cycle and positively correlated with increased apoptosis [20]. Additionally, CR regulates the activity of DNA repair genes, which fluctuate throughout the day. Notably, studies have observed a tenfold increase in their activity at different times [21]. For instance, mouse splenocytes display higher expression levels of genes responsible for DNA double-strand break repair and base excision repair during the light phase [22]. These biological mechanisms may explain why patients treated in the afternoon exhibit higher metastasis rates.

DNA synthesis in human rectal mucosa is at its lowest during the evening and peaks in the morning [23, 24], as does the expression of topoisomerase II alpha, a critical enzyme for DNA repair and replication [25]. Since cells in the late S-phase of the cell

cycle are more resistant to IR, this could explain the lower incidence of GI toxicity observed in patients treated in the morning. Additionally, from clinical experience, the timing of a patient's bowel movements can impact GI toxicities during RT. Patients treated outside their usual bowel movement time often require laxatives to ensure an empty rectum, and daily use of laxatives over multiple treatment sessions may contribute to increased GI toxicity. Nevertheless, conflicting evidence comes from two randomized controlled trials with pelvic IR, which reported an increased incidence and severity of diarrhoea in patients undergoing morning EBRT [20, 26]. However, it is crucial to consider that these were younger cohorts than those typically treated for PCa, and it is well-established that circadian rhythms shift with age. Older adults are more likely to exhibit a morning chronotype, characterized by earlier melatonin secretion [27, 28]. Given that melatonin is thought to mitigate radiation-induced side effects [29], this circadian shift could help explain the varying effects of EBRT observed in PCa patients.

EBRT technique (IMRT versus VMAT) was not included as a confounding variable in the Cox multivariable analysis, as evidence suggests no significant differences in oncological outcomes between both modalities [30]. However, it was considered in the toxicities analysis [31]. Contrary to previous literature, our findings suggest that moderate hypofractionation is associated with lower risk of grade 2 or higher acute GI toxicity, presumably due to the lower proportion of patients receiving conventional fractionation in our cohort [32].

This study partially converges with the Taiwanese [9], which also identified a relationship between RT timing and its efficacy and toxicities. The observed differences between the studies may stem from the number of participants and methodological variations, namely the definition of treatment cut-off times, inclusion and exclusion criteria, and the confounding variables used in the multivariable models. We used the NCCN risk group classification integrating the clinical tumour stage, initial PSA, and ISUP grade instead of using them separately [33]. Our approach reflects current clinical practice, where risk groups guide treatment decision-making for patients and physicians [33, 34].

Our cohort aligns with trends observed in other PCa studies, with most patients falling into the intermediate-risk category [35, 36]. We noted a higher proportion of patients with ECOG 1 and 2 in the afternoon group but with a higher ratio of clinical T1c stage patients. However, more importantly, the risk group classification showed no significant differences between the treatment groups. Furthermore, risk group and ECOG were considered in the statistical analysis, mitigating their effects as much as

possible. It is worth highlighting that the clinical tumour stage is an established clinicopathological predictor of worse prognosis [37]. Therefore, if any bias were introduced by this variable, it would likely favour the afternoon treatment group. Despite this potential bias, the morning group still demonstrated significantly better outcomes in terms of MFS, though no significant difference was observed in LRFS.

This study is subject to inherent limitations typical of observational retrospective analyses, namely the potential for selection bias. Nonetheless, we employed multivariable analyses and propensity-matching methods incorporating a range of patient and treatment characteristics to minimize bias. Furthermore, the relatively small sample size (224 patients) constrains the generalization of our findings. Future research involving a broader database would enhance the robustness and external validity of the results. Another limitation is the absence of control over patients' biological and behavioural factors, such as individual circadian rhythms and lifestyle habits (which may act as confounding variables). It is also worth noting that during the pandemic, treatment strategies prioritized hypofractionation and confining the treatment volume to the prostate.

Given the high volume of PCa patients receiving EBRT, shifting all treatments to the morning would present significant logistical challenges. However, if treatment timing proves to be a determinant of patient outcomes, it would be worth consideration. Ultimately, we agree with previous research that the ideal scenario would be to select patients who could benefit from EBRT at specific times based on molecular markers related to DNA damage repair pathways or cell cycle regulation driven by circadian rhythms.

CONCLUSION

This study suggests that localized PCa benefits from EBRT in the morning. Although randomized studies are needed to confirm these findings, current evidence supports the consideration of treatment schedules as a potentially important factor in personalizing EBRT for these patients.

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CHAPTER 5

Molecular study

Circadian Rhythm's Impact on Prostate Cancer Radiotherapy Response: Exploring the Role of *PER2* and *BMAL1* Genes

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ABSTRACT

Introduction: Definitive radiotherapy (RT) is prescribed in approximately 30% of prostate cancer (PCa) patients, although disease progression or relapse represents a significant problem. Circadian rhythm (CR) is a regulator of key cellular processes relevant to RT response. Here, we sought to investigate the impact of key circadian clock regulators, *PER2* and *BMAL1*, on PCa RT response and identify putative epigenetic regulators on the circadian clock-RT response axis, exploring whether these could serve as potential biomarkers for treatment response monitoring or putative targets for epigenetic inhibitors aimed at enhancing radiosensitivity in PCa.

Methods: *In vitro* experiments were conducted using three prostate cancer cell lines (PC3, 22Rv1-P, and 22Rv1-RR), including CR synchronisation, clonogenic assays, and siRNA transfections, to investigate the role of circadian regulators *BMAL1* and *PER2* in radiosensitivity. Additionally, RNA sequencing and DNA methylation analyses (EPIC array) were conducted. **Results:** The expression profiles of *BMAL1* and *PER2* over 48 hours revealed an upregulation of *BMAL1* and a downregulation of *PER2* in 22Rv1 radioresistant cells compared to the parental cell line ($p < 0.0001$). Remarkably, the silencing of *BMAL1* in 22Rv1-RR cells led to a significant increase in radiosensitivity ($p < 0.001$), while *PER2* knockdown in 22Rv1-P enhanced radioresistance ($p < 0.05$). Interestingly, circadian entrainment was one of the top ten dysregulated KEGG pathways in 22Rv1-RR versus 22Rv1-P cells in the DNA methylation analysis ($p < 0.0001$). *RORB* and *PER2* genes were significantly downregulated and hypermethylated in radioresistant cells. **Conclusions:** Overall, our results suggested that *BMAL1* and *PER2* are radioresistance drivers of PCa cells. This study highlights the potential of targeting circadian regulators to enhance radiosensitivity in PCa. Furthermore, by dissecting crucial epigenetic mechanisms involved in CR regulation, we might be able to find new potential biomarkers and novel targeting complementary therapies.

Keywords: Circadian rhythm, Chronotherapy, Prostate Cancer, Radiobiology, *PER2*, *BMAL1*

INTRODUCTION

Prostate cancer (PCa) remains a global health concern, with the International Agency for Research on Cancer (IARC) projecting an increase of 96.2% and 136.4% in incidence and mortality by 2050, respectively [1]. Radiotherapy (RT) is a first-line treatment option in PCa therapy, with approximately 30% of patients undergoing RT with curative intent [2]. Despite advances in treatment, 20-40% of patients with advanced PCa may eventually progress or relapse [3]. Preliminary findings suggested that there is potential to improve treatment outcomes by timing RT when cancer cells are in a more radiosensitive stage, i.e. during the late S phase of the cell cycle, while normal radioresistant cells repopulate [4]. Indeed, normal and malignant cells may have distinct circadian cycles [5].

Circadian rhythm (CR) is an internal clock that regulates and synchronises daily biological functions with day-night cycles. Three autoregulatory transcriptional-translational feedback loops control the core circadian clock (CC) [6]. The positive loop involves the heterodimer circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like 1 (BMAL1) transcription factors. This complex drives the expression of genes in the negative loop – the cryptochrome circadian regulator 1 and 2 (CRY1 and CRY2) and the period circadian regulator 1-3 (PER1, PER2, and PER3). The protein products of these genes inhibit the activity of the CLOCK/BMAL1 complex, establishing a rhythmic cycle governing processes such as cell metabolism, cell cycle progression, hormone production, sleep patterns, and body temperature [7].

PER2 functions as a tumour suppressor, with low expression correlating with higher tumour grades and poorer prognosis in several cancers [8, 9]. PER2 also influences key DNA repair and apoptosis mechanisms, decreasing the expression of BCL2, p53, p27 and p21 while increasing BAX [10]. Elevated levels of PER2 significantly inhibited cell growth and viability of PCa cells [11]. Moreover, BMAL1 was associated with PCa progression, where its knockdown (KD) reduced the proportion of cells in the S-phase, thus impacting cell proliferation [12]. BMAL1 promotes prostate growth by modulating the cell cycle and repressing the p21 expression, effectively functioning as a cell cycle activator [13]. Furthermore, reduced levels of BMAL1 impaired DNA damage repair mechanisms, which can allow cancer cells survival advantages [14]. Both BMAL1 and PER2 regulate epithelial-mesenchymal transition (EMT) signalling pathways, contributing to tumour progression and migration. For instance, the downregulation of PER2 led to the activation of EMT genes in breast cancer, whereas the downregulation

of BMAL1 was associated with a decreased mesenchymal cell invasion in colorectal cancer [15].

Epigenetic modifications, a well-known cancer hallmark [16], are critical in regulating gene expression [17]. Within the framework of circadian regulation, DNA methylation and histone modifications seemed to be essential regulatory mechanisms that influence the expression of the core CC genes [18]. The epigenetic status of CC genes was correlated with CC gene expression, affecting the function of the CR [19]. Histone modifications also exhibit circadian patterns, helping to maintain peripheral clocks. Importantly, the acetyltransferase KAT2B enhanced the activity of the CLOCK-BMAL1 heterodimer [20]. Furthermore, the deacetylase SIRT1, which targets BMAL1 and PER2, has been shown to repress the transcription of various CC genes, further influencing the rhythmic expression of CC genes [19].

In this study, we sought to investigate the impact of key circadian clock regulators, PER2 and BMAL1, on PCa RT response and identify putative epigenetic regulators on the CC-RT response axis, exploring whether these could serve as potential biomarkers for treatment response or targets for epigenetic inhibitors aimed at enhancing radiosensitivity in prostate cancer.

MATERIAL AND METHODS

Cell lines and cell culture

Three human PCa cell lines, PC3, 22Rv1 parental (P) and 22Rv1 radioresistant (RR) (in-house generated as described elsewhere [21]), were cultured in RPMI 1640 (Biotechnómica, Porto, Portugal) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin and in a humidified incubator set at 37°C with 5% CO₂. Cells underwent periodic testing for mycoplasma contamination using MyTaq HS Red PCR Mycoplasma Detection Set (Bioline, Meridian Bioscience, London, UK).

For specific *in vitro* assays, PCa cells were synchronised by changing the culture medium. Timepoint 0h is defined as the timepoint of synchronisation.

Ionising radiation (IR)

IR was performed using a Varian Medical Systems DHX linear accelerator at room temperature with normal oxygen levels, with an isocentric technique, a 25×25 cm field

size at a depth of 3 cm (source-skin distance [SSD] = 97cm), a 6MV photon beam, and a dose rate of 600 MU/min.

RNA extraction, cDNA synthesis, and reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

PCa cells were seeded in 6-well plates at a density of 80×10^3 cells per well. RNA was subsequently extracted at designated time points using the Trizol reagent method. Then, 1000 ng of RNA extracts were used to convert it into cDNA using the Revert Aid RT Kit (Thermo Scientific Inc.), following the manufacturer's protocol. Subsequently, qRT-PCR was conducted on 384-well plates using a QuantStudio™ 12K Flex Real-Time PCR System (Applied Biosystems, USA). The reaction mixture for each well comprised 22.5 ng of cDNA, 5 μ L of Xpert Fast SYBR Green Mastermix with ROX (GRISP, Porto, Portugal), and specific primer concentrations (sequences, primers concentration and melting temperatures detailed in Appendix III: Supplementary Table 1) adjusted to a final volume of 10 μ L with sterile bi-distilled water. A homemade RNA pool derived from various human cell lines was used as a positive control. Transcript levels for target genes were normalised to the housekeeping gene β -GUS using the $\Delta\Delta$ Ct method and multiplied by 10^3 for easier tabulation. Results were derived from at least three independent biological replicates, and each sample run in technical triplicates.

Protein extraction & quantification and SDS-Page western blot

Following previously established protocols [22], total protein extraction was performed with RIPA lysis buffer supplemented with 1X protease inhibitor cocktail (PIC, Merck) from PCa cell line extracts under experimental conditions, including cell synchronisation and *PER2* or *BMAL1* gene silencing.

Histone proteins were extracted using a lysis buffer containing 0.5% Triton X-100 in 1x PBS and 1X PIC. The cell lysates were centrifuged at 2000 rpm for 10 minutes at 4°C, followed by 10 minutes in rotation at 4°C two times. The pellet was then resuspended in 0.2 M hydrochloric acid and incubated overnight in rotation at 4°C to allow histone precipitation. Subsequently, total and histone proteins were quantified using a commercially available colorimetric Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. Briefly, 5 ng of histone- and 50 ng of total- protein extracts were loaded. Western blotting was performed as previously described [22]. Beta-actin and H3 served as loading controls for total and histone protein extracts, respectively. Antibody concentrations and the respective host species are described on the Appendix III:

Supplementary Table 2. Chemiluminiscence signal was detected using a ChemiDoc system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) after adding Clarity Western ECL chemiluminescent substrate (Bio-Rad). Protein relative quantification was performed using Image Lab software (version 6.1, Bio-Rad Laboratories, Inc., Hercules, CA, USA). At least three independent biological replicates were performed.

Clonogenicity assay

PCa cells were subjected to cell synchronisation or gene silencing for *PER2* or *BMAL1*. Then, PCa cells were seeded into 6-well plates at an optimised density (100,000cells/mL for PC3 and 200,000cells/mL for 22Rv1-P and 22Rv1-RR) before being exposed to varying doses of ionising radiation (0 to 8 Gy). Following IR exposure, cells were cultured at low density (1000cells/well) for seven days to allow colonies development. Ultimately, colonies were fixed in methanol 10min (Merck) and then stained 20min with 1% Crystal Violet in 20% methanol solution (Sigma). Colonies comprising at least 50 cells were considered for analysis. Images for colony counting were captured using a Canon EOS 5D Mark III digital camera and quantified using ImageJ software tools, version 2.14 (National Institutes of Health). Each experiment was repeated with at least three independent biological replicates. Plating efficiency (PE) was calculated as the number of colonies formed divided by the number of cells plated, multiplied by 100. The survival fraction (SF) was determined by dividing the number of colonies formed by the product of the number of cells plated and the plating efficiency divided by 100. The dose enhancement factor (DeF) was defined by the ratio between the dose of radiation required to reduce survival to 50% (ID50) in the control with the ID50 of the target condition.

siRNA transfection for *PER2* and *BMAL1* gene silencing

Three distinct small interfering RNA (siRNA) oligos, for each gene, targeted the coding sequence (CDS) and 3' untranslated region (3'UTR) of *PER2* and *BMAL1* (hs.Ri.PER2.13.1, hs.Ri.PER2.13.2, hs.Ri.PER2.13.3, hs.Ri.BMAL1.13.1, hs.Ri.BMAL1.13.2, hs.Ri.BMAL1.13.3) were used. These oligos, alongside a negative control siRNA sequence (DsiRNA, 1nmol), were purchased from Integrated DNA Technologies (IDT) company, Coralville, IA, USA. Additionally, we used HPRT-S1 DS Positive Duplex and TYETM 563 DS as internal positive transfection controls. *In vitro* siRNA transfection was performed with Lipofectamine® 3000 reagent (Invitrogen, USA), according to manufacturing instructions. For each experiment, gene KD was confirmed after 72 hours of siRNA transfection, a reference time point for cell irradiation (0h).

Infinium Methylation EPIC Array

Bisulfite-converted DNA from 22Rv1 cell lines (22Rv1-P and 22Rv1-RR) was analysed using the Infinium Methylation EPIC Array (Illumina, San Diego, CA, USA). The array was processed on a HiScan system (Illumina), and intensity values were extracted with the GenomeStudio (V2011.1) Methylation Module (1.9.0) software. Data quality was evaluated through GenomeStudio and BeadArray Controls Reporter (Illumina) using internal control probes. Methylation levels for each CpG site were quantified as β -values, calculated as the ratio of the fluorescent signal from the methylated probe to the combined signal from methylated and unmethylated probes. β -values range from 0 (no methylation) to 1 (full methylation).

RNA sequencing

Total RNA from 22Rv1-P and 22Rv1-RR cells was sequenced by MVLS Polyomics facilities, University of Glasgow, UK. Briefly, RNA extraction was performed through RNeasy Mini Kit according to the manufacturing procedures (Qiagen). Before RNA sequencing, the integrity and quantity of each RNA sample were evaluated using Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Then, the libraries were prepared using the RNAseq_Library_Prep, QuantSeq (Lexogen) (24s) kit according to the manufacture procedures, and samples were sequenced on a NextSeq2000 using 1X100 read length and 10 million reads per sample.

Raw FASTQ files containing unaligned reads for each sample were quality-checked. Reads were aligned in the form of BAM files, and normalisation and raw expression estimation for gene/transcripts for each sample were performed. Differential Gene Expression analysis (DGE) was performed using DESeq2, and Gene/transcript lists showing statistical measures were annotated.

Statistical analysis

Data were checked for normal distribution and homogeneity of variances using the Shapiro-Wilk and Levene's tests, respectively. For comparisons between two groups, independent t-tests or Mann-Whitney U tests were used, depending on data distribution. For comparisons among multiple groups, one-way ANOVA or Kruskal-Wallis tests were applied. Post hoc analyses for multiple comparisons were conducted using Dunn's or Dunnett's tests. All results are presented as mean $\pm$ SEM.

The linear-quadratic (LQ) model ($S = e - (\alpha D + \beta D^2)$), was applied to analyse the radiobiological cell survival curves.

Statistical significance was established for $p \leq 0.05$ (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns, not significant). The statistical analyses were conducted using GraphPad Prism software (version 9.0).

RESULTS

Opposing circadian dynamics of *BMAL1* and *PER2* in prostate cancer cell lines

We assessed the expression profiles of *BMAL1* and *PER2* in three prostate cancer cell lines (PC3, 22Rv1-P and 22Rv1-RR) over 48 hours. During this period, transcript levels of core-clock genes *BMAL1* and *PER2* were assessed every 4 hours. Over the 48h period, significant differences were observed between the cell lines' total transcript levels of *BMAL1* and *PER2* (Figure 1A-B). *BMAL1* expression was highest in the 22Rv1-RR cells compared to the 22Rv1-P cells ($p < 0.0001$) and PC3 cells ($p < 0.01$). In contrast, *PER2* expression was significantly lower in radioresistant cells compared to the parental ($p < 0.0001$) and PC3 cells ($p < 0.01$). Temporal profiling over 48 hours revealed rhythmic expression patterns in PC3 cells, while 22Rv1-P cells showed consistently higher *PER2* levels and 22Rv1-RR cells exhibited persistently elevated *BMAL1* expression (Figure 1C-E).

PER2 and *BMAL1* expression modulates radiosensitivity in PC3 cells: insights from simulating 22Rv1-RR dynamics

Based on the aforementioned CR dynamics, we proceeded by simulating the radioresistant dynamics observed in 22Rv1-RR cells within PC3 cells regarding *PER2* and *BMAL1* expression patterns. Based on the expression levels of *PER2* and *BMAL1*, normalised to their mean values over 48 hours, in the PC3 heatmap (Figure 2A), we selected two critical time points: 4 hours, corresponding to a peak of *PER2* expression, while low *BMAL1* expression, and 44 hours, representing low *PER2* and high *BMAL1* expression. PC3 cells were irradiated at these selected time points, and colony formation assays were performed to assess the impact of CR in RT response.

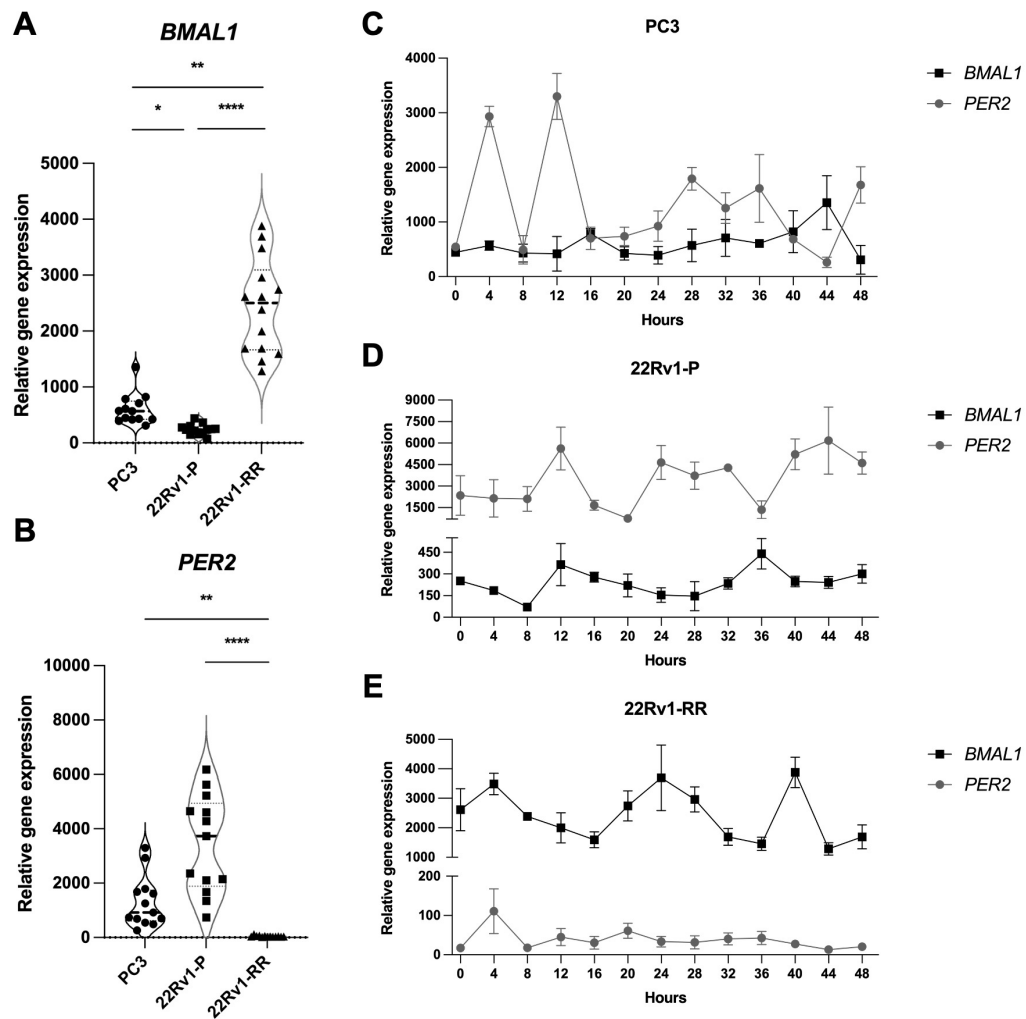


Figure 1. Distinct circadian rhythm patterns in PC3, 22Rv1-P and 22Rv1-RR cell lines. Relative gene expression levels of *BMAL1* (A) and *PER2* (B) after a 48h-period in PC3, 22Rv1-P and 22Rv1-RR cells. Relative *BMAL1* and *PER2* gene expression in PC3 (C), 22Rv1-P (D) and 22Rv1-RR (E) PCa cells over the 48 hour-period. Data are presented as mean $\pm$ SEM from at least three independent biological replicates of 22Rv1-RR vs 22Rv1-P or PC3 cell extracts. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. RR, radioresistant; P, parental.

Gene and protein expression levels of *PER2* and *BMAL1* were further validated at the 4-hour and 44-hour time points. Transcript analysis confirmed a significant reduction in *PER2* expression from 4 hours to 44 hours (mean $\pm$ SD values: 2005 $\pm$ 467.1 versus 943.5 $\pm$ 392.4, respectively; $p < 0.01$) (Figure 2B). Alongside, a significant decrease in *PER2* protein levels (mean $\pm$ SD values: 27.72 $\pm$ 10.21 versus 7.79 $\pm$ 3.57, respectively; $p < 0.05$) was observed (Figure 2C). Conversely, *BMAL1* transcript levels were higher at 44 hours compared to 4 hours, although the difference was not significant (Figure 2C). A similar trend

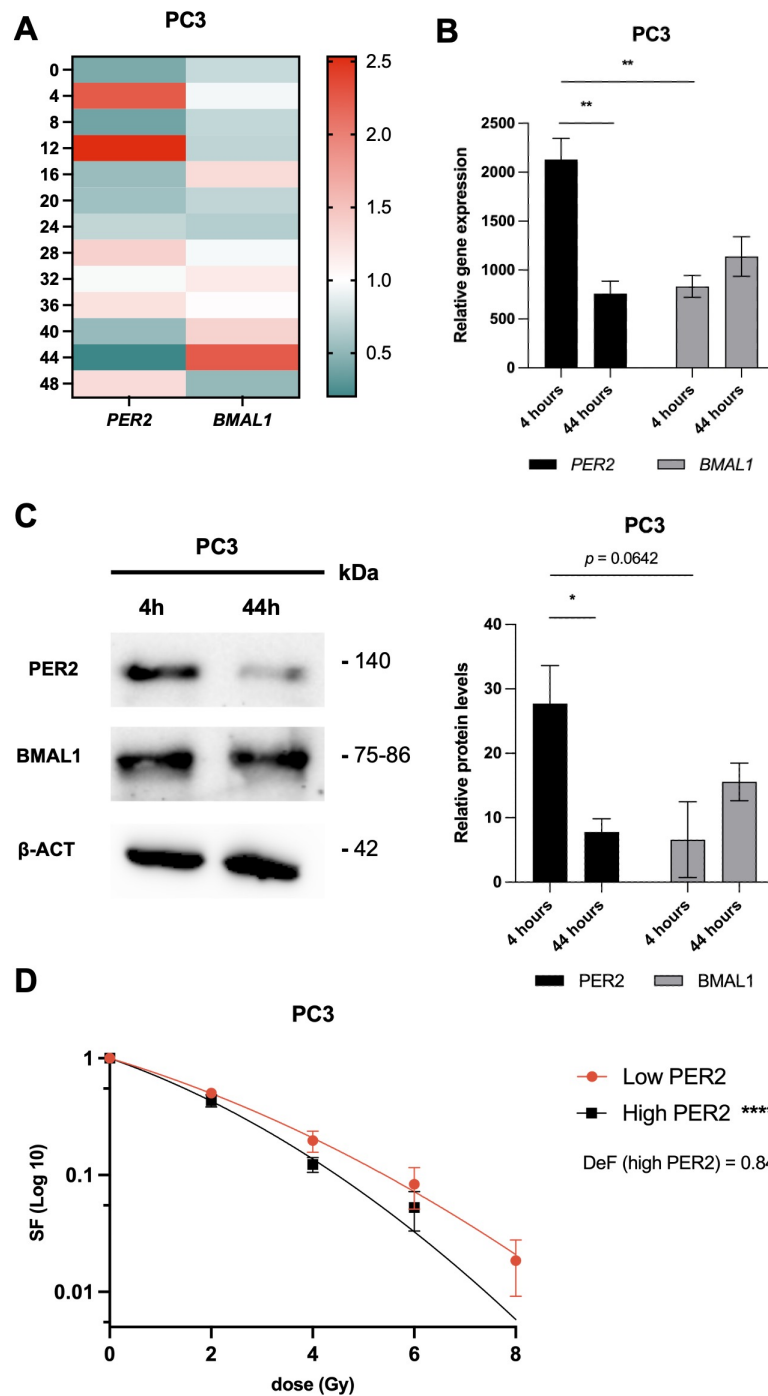


Figure 2. Timing radiotherapy for maximum impact based on *PER2* and *BMAL1* expression in PC3 cells.

(A) Heatmap of the relative gene expression of *PER2* and *BMAL1* in PC3 cells over a 48-hour period. *GUS- β* was used as the housekeeping gene. Transcript (B) and protein (C) levels of *PER2* (140kDa) and *BMAL1* (75-86kDa) at 4- and 44h-time points. (D) Cell survival fraction of PC3 cells irradiated at 4 hours (High *PER2*) and at 44 hours (Low *PER2*), represented through a linear-quadratic model (LQ = $S = e - (xD + BD^2)$). Data are presented as mean $\pm$ SEM from at least three independent biological replicates. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. DeF, dose enhancement factor; SF, survival fraction; RR, radioresistant.

was observed at the protein level, with *BMAL1* levels increasing from 4 hours to 44 hours (Figure 2C). When comparing *PER2* and *BMAL1* at the 4-hour time point, there were significant differences in transcript levels ($p < 0.01$), with *PER2* showing a higher expression, and a borderline significance was observed in protein levels ($p = 0.0642$). The clonogenic assay revealed that PC3 cells irradiated at the 44-hour time point (low *PER2*) exhibited significantly higher clonogenicity capacity compared to cells irradiated at the 4-hour time point (high *PER2*) (DeF = 0.84; $p < 0.0001$) (Figure 2D).

Silencing *BMAL1* enhances radiosensitivity in 22Rv1-RR cells

We proceeded with *BMAL1*-KD in 22Rv1-RR cells. A significant reduction in *BMAL1* was confirmed across all three silencing conditions at transcript and protein levels (Figure 3A, B). Subsequently, clonogenic assays revealed a significant radiosensitising effect in *BMAL1*-silenced cells ($p < 0.001$) (Figure 3C). The DeFs were 2.17, 2.11, and 1.78 for the three siRNAs, respectively. *PER2* levels were not significantly affected upon *BMAL1* silencing (Figure 3A and B), although a slight increase was observed with siRNA 13.1 compared to the negative control (NC).

In parallel, *PER2* was silenced in the parental 22Rv1 cell line to investigate whether its KD could be a potential driver of radioresistance. The silencing was unsuccessful, with varying degrees of transcript KD (71.4%, 46.6%, and 26.3% for the three siRNAs, respectively) (Figure 3D), while protein levels did not decrease correspondingly (Figure 3E). In fact, the first siRNA showed an unexpected increase in *PER2* protein levels compared to the negative control, although not statistically significant (Figure 3E). Despite the limited KD at the protein level, clonogenic assays revealed increased survival ($p < 0.01$ and $p < 0.05$) in the first two silencing conditions (si*PER2* 13.1 and 13.2), with DeFs of 0.81 and 0.67, respectively (Figure 3F). Interestingly, si*PER2* 13.3, which exhibited the least KD at transcript levels, displayed an inverse relationship with a DEF of 1.27, which might suggest that *PER2* expression sensitises PCa cells to ionising radiation (Figure 3F). Additionally, only the si*PER2* 13.1 condition showed a slight increase of *BMAL1* transcript levels, although any effect was observed at protein levels (Figure 3D and E).

Phosphorylation of H2AX (γ -H2AX) increased upon irradiation in *BMAL1* knocked down cells

γ -H2AX is a recognised biomarker of DNA double-strand breaks. Its phosphorylation works like an internal signal to recruit DNA damage repair proteins and activate the DNA damage repair cascade. Thus, we assessed γ -H2AX protein levels in both *BMAL1* and *PER2* silenced cells to see whether those markers may affect DNA damage in response to RT.

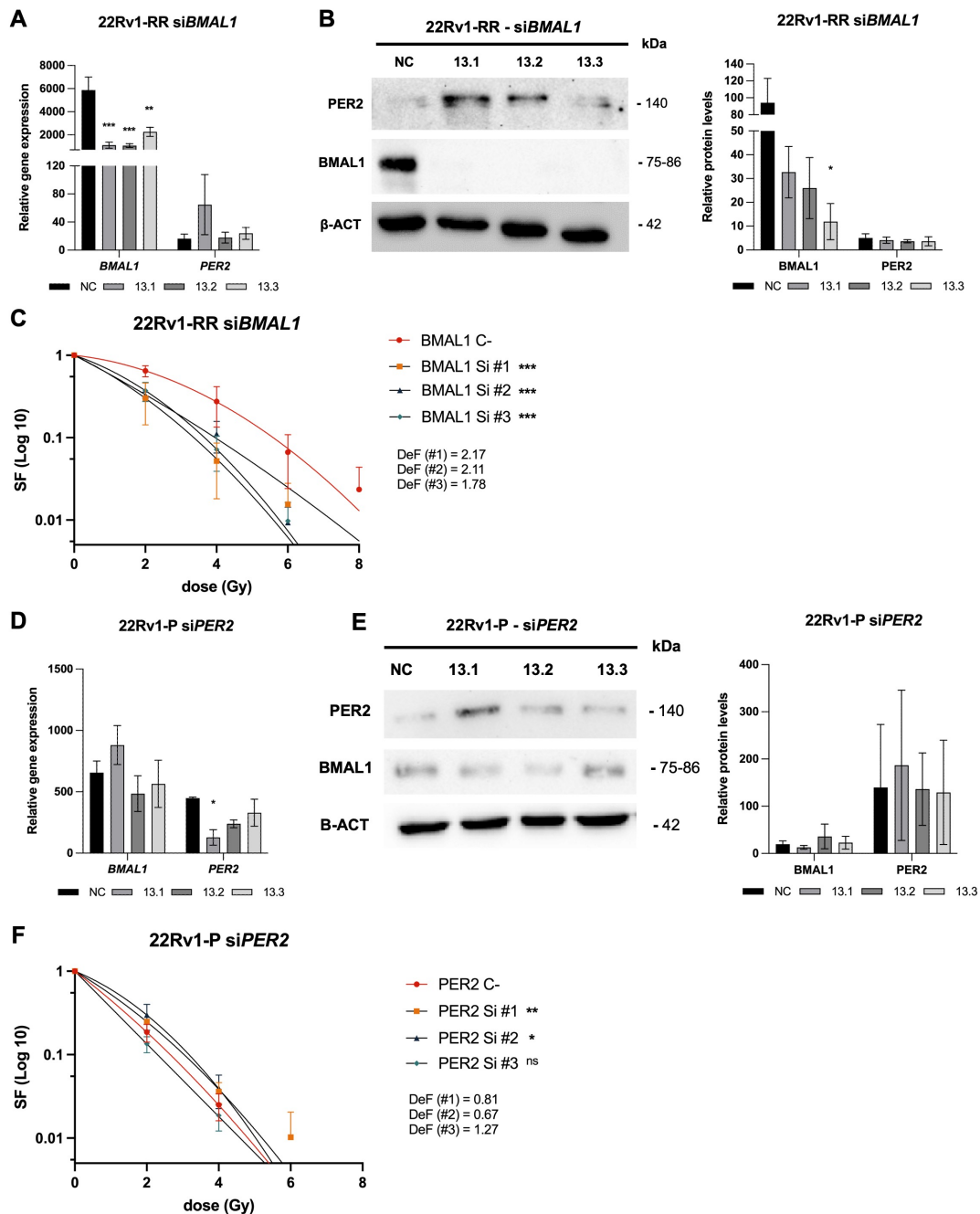


Figure 3. Silencing *BMAL1* led to a substantial radiosensitising effect in 22Rv1-RR. (A) *BMAL1* and *PER2* transcript levels across the 22Rv1-RR-negative control (NC) and *BMAL1*-KD clones 13.1 to 13.3 at 72 hours. (B) Corresponding protein levels of *BMAL1* and *PER2* across the 22Rv1-RR-negative control (NC) and *BMAL1*-KD clones 13.1 to 13.3 at 72 hours. (C) Cell survival fraction of 22Rv1-RR-negative control (*BMAL1* C-) and *BMAL1*-KD clones #1 to #3 transfected cells represented through linear-quadratic model ($LQ = (S = e - (xD + BD^2))$). (D) *BMAL1* and *PER2* transcript levels across the 22Rv1-P-negative control (NC) and *PER2*-KD clones 13.1 to 13.3 at 72 hours. (E) Corresponding protein levels of *BMAL1* and *PER2* across the 22Rv1-P-negative control (NC) and *PER2*-KD clones 13.1 to 13.3 at 72 hours. (F) Cell survival fraction of 22Rv1-P-negative control (*PER2* C-) and *PER2*-KD clones #1 to #3 transfected cells represented through linear-quadratic model ($LQ = (S = e - (xD + BD^2))$). Data are presented as mean $\pm$ SEM from at least three independent biological replicates. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, ns, not significant. DeF, dose enhancement factor; SF, survival fraction; KD, knockdown; RR, radioresistant; P, parental.

In the 22Rv1-RR-*BMAL1*-KD cells, γ -H2AX levels were notably increased at 30 minutes post-irradiation when normalised to both the control and 0 Gy baseline (Figure 4A). In the parental 22Rv1 cells, only a slight decrease in γ -H2AX levels was observed in the si*PER2* 13.1 silencing condition, while an increase was observed in si*PER2* 13.2 (Figure 4B). Nonetheless, we must interpret this data carefully since the transfection of *PER2* was quite unsuccessful.

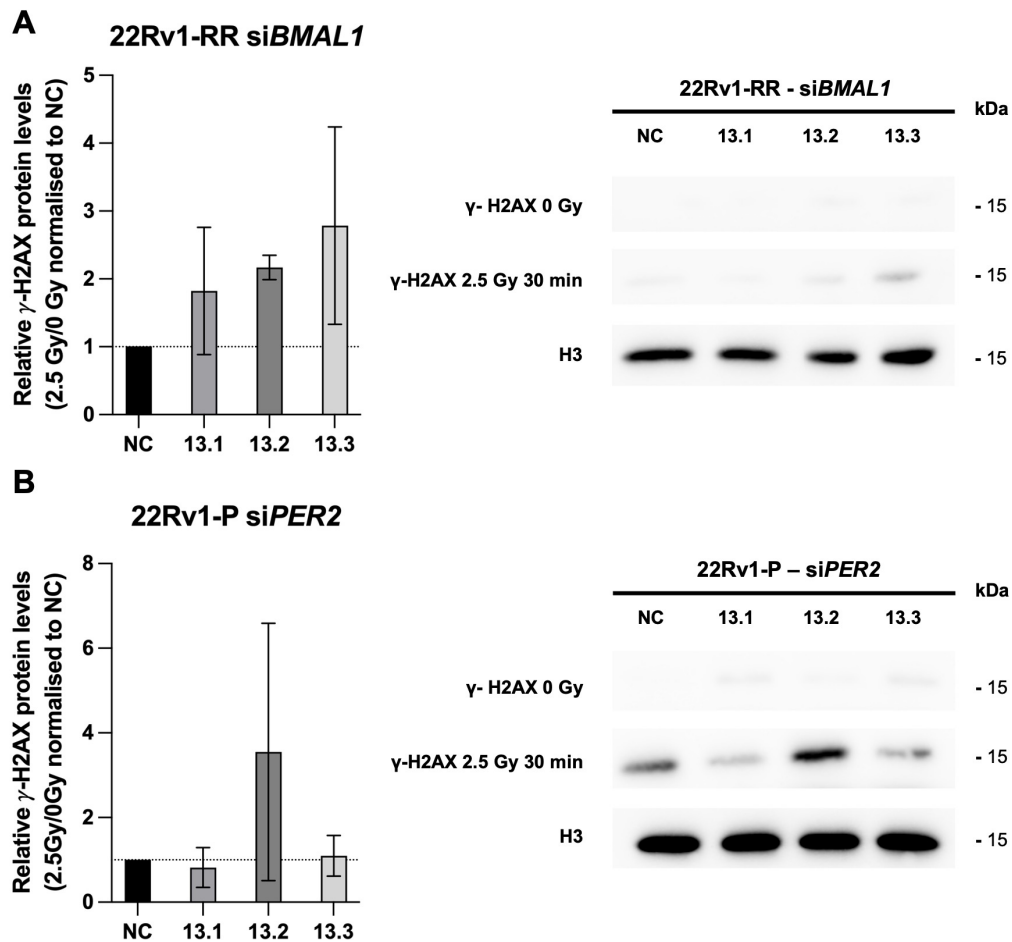


Figure 4. Phosphorylation of H2AX (γ -H2AX) in *BMAL1* and *PER2* knocked-down irradiated cells. (A) γ -H2AX protein levels across 22Rv1-RR-negative control (NC) and *BMAL1*-KD clones 13.1 to 13.3, normalise to NC and 0 Gy baseline. (B) γ -H2AX protein levels across 22Rv1-P-negative control (NC) and *PER2*-KD clones 13.1 to 13.3, normalise to NC and 0 Gy baseline. Data are presented as mean $\pm$ SEM from at least three independent biological replicates. KD, knockdown; RR, radioresistant; P, parental.

Silencing *BMAL1* and *PER2* disrupts DNA repair pathways: p53, KAT2B, and SIRT1 as key players

Notably, *TP53*, *KAT2B*, and *SIRT1* expression levels were reduced in 22Rv1-RR-*BMAL1*-KD cells compared to the negative control, although not statistically significant (Figure 5A). In contrast, 22Rv1-P *PER2* transfected cells significantly upregulated *KAT2B* and *SIRT1*

expression, although only with the si*PER2* 13.3 oligo ($p < 0.05$) (Figure 5B). Additionally, *PER2* transfection did not alter *TP53* expression (Figure 5B).

Moreover, remarkable variations were observed in the protein levels of the same players across the radioresistant cell line, although not statistically significant. Indeed, *BMAL1*-KD led to a decrease of KAT2B, SIRT1, and p53 protein levels compared to the negative control (Figure 5C). Conversely, *PER2* transfection led to increased p53 and SIRT1 levels, although no apparent variations for KAT2B protein levels (Figure 5D).

Epigenetic regulation of circadian rhythm dynamics in 22Rv1-P and -RR cell lines: Insights from DNA methylation analysis and RNA sequencing

CR dynamics might be potentially modulated by epigenetics mechanisms. Thus, we further explored whether in our RR model, epigenetic mechanisms, specifically DNA methylation, play a role in the regulation of CR gene transcription. For that, we performed the DNA methylation EPIC array analysis in 22Rv1-P and 22Rv1-RR cells. Pathway dysregulation analysis revealed circadian entrainment as one of the top ten dysregulated KEGG pathways in 22Rv1-RR cells ($p < 0.0001$) (Figure 6Ai). Notably, genes such as *RORs*, *PERs*, *BMAL1*, *DBP*, and *DEC* were highlighted as key components of both the circadian rhythm and circadian entrainment pathways which are deregulated in 22Rv1-RR compared to 22Rv1-P (Figure 6Aii).

Since aberrant methylation of CpG islands within gene promoters is a well-established contributor to carcinogenesis and gene expression modulation, our analysis focused on CpG sites located in the promoter regions (TSS200, TSS500, and TSS1500) of circadian-related genes. Interestingly, significant hypermethylated CR-related genes - *RORA*, *RORB*, *CSNK1E*, *CSNK1D*, and *PER2*- were identified in 22Rv1-RR compared to 22Rv1-P cell fraction (Figure 6B).

To further validate the biological significance of these findings at the RNA transcription level, we cross-referenced the methylation data with RNA sequencing results. RNA-sequencing analysis revealed differential expression of several CR-related genes between the parental and radioresistant cell lines (Figure 6C). Notably, *RORB* and *PER2*, which were hypermethylated, were also downregulated at the transcript level. In addition, genes such as *PER1*, *CRY1*, *CRY2*, *RORC*, *MYC*, and *NR1D2* were downregulated, while *TP53*, *SIRT1*, *BMAL1*, *RORA*, *CSNK1E*, and *KITLG* were upregulated in the radioresistant cells (Figure 6D).

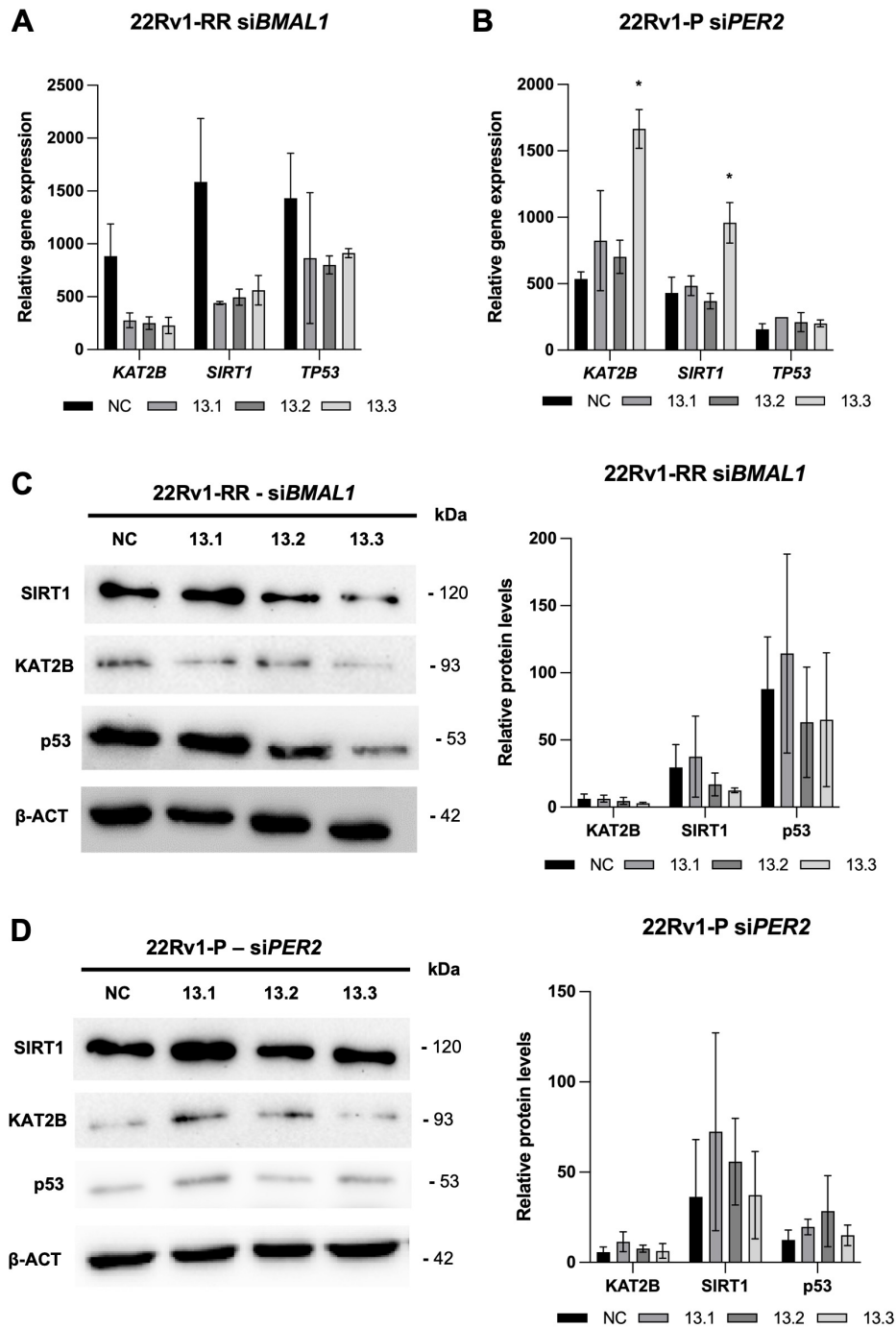


Figure 5. Silencing *BMAL1* and *PER2* disrupts DNA repair pathways: p53, KAT2B, and SIRT1 as key players. Transcript levels of *TP53*, *KAT2B* and *SIRT1* across the 22Rv1-RR-negative control (NC) and *BMAL1*-KD clones 13.1 to 13.3 at 72 hours (A) and across the 22Rv1-P-negative control (*PER2* C-) and *PER2*-KD clones #1 to #3 at 72 hours (B). Protein levels of KAT2B, SIRT1, and p53 across the 22Rv1-RR-negative control (NC) and *BMAL1*-KD clones 13.1 to 13.3 at 72 hours (C) and across the 22Rv1-P-P-negative control (*PER2* NC) and *PER2*-KD clones #1 to #3 at 72 hours (D). Data are presented as mean $\pm$ SEM from at least three independent biological replicates. *p < 0.05. KD, knockdown; RR, radioresistant; P, parental.

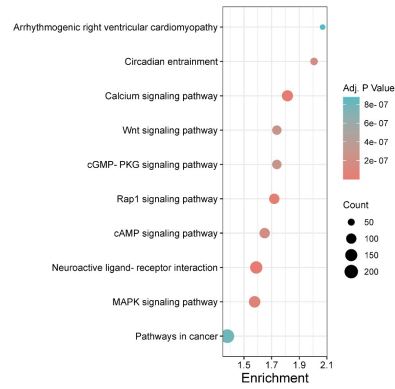
DISCUSSION AND CONCLUSION

RT remains a cornerstone in PCa treatment, but one of the major challenges is the development of resistance, which means cases with recurrent or progressive disease post-RT [23, 24]. Their high rates emphasise the need to find new therapeutic strategies or refine the conventional ones [25, 26]. The primary target of IR is the nuclear DNA, and double-strand breaks represent the most lethal form of damage. These breaks activate cellular repair mechanisms that can either facilitate repair or induce cell death depending on the extent of the damage and the functional state of the cell [25]. Recent findings have shown that CR is an important regulator of cellular processes, including the cell cycle and DNA repair mechanisms [27]. Nonetheless, the influence of CC genes on PCa EBRT response remains largely underexplored. Given their role in tumour progression and DNA repair, with this study, we aimed to investigate two key CC genes, *BMAL1* and *PER2*, and their potential to modulate radioresistance in PCa cell lines.

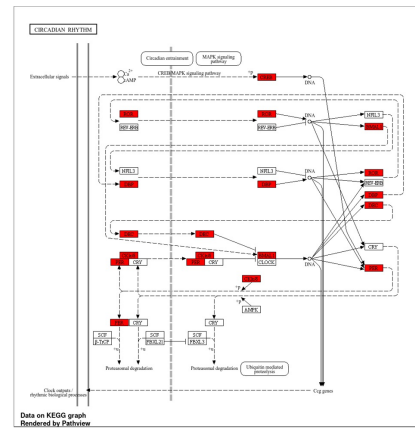
Our results revealed distinct expression patterns of *BMAL1* and *PER2* across the PCa cell lines, with both reduced *PER2* expression and *BMAL1* upregulation being associated with increased radioresistance. These findings agree with previous literature on the role of *PER2* as a tumour suppressor and the oncogenic potential of *BMAL1* [8, 9, 12]. In nasopharyngeal carcinoma (NPC), *BMAL1* overexpression was found to inhibit cell proliferation and promote p53 levels, while *BMAL1*-KD led to reduced p53 levels [28]. This relation was also confirmed by other studies in other tumour models [29, 30]. Accordingly, in our study on PCa, p53 expression was significantly reduced with *BMAL1*-KD. This was accompanied by elevated γ -H2AX levels at 30 minutes post-IR, suggesting that *BMAL1* may drive radioresistance in PCa by influencing DNA repair mechanisms via p53-dependent pathways.

In contrast to the NPC study, where lower p53 levels were linked to increased radioresistance, our data suggest that reduced p53 levels enhance radiosensitivity in PCa. Previous work from our group demonstrated that p53 is crucial in signalling DNA damage and aiding cell recovery after prolonged IR exposure, being associated with a radioresistant phenotype [21]. Higher p53 levels likely enable more efficient DNA repair, promoting cell survival after RT [31]. Likewise, in our model, *BMAL1* appears to mediate radioresistance, and its reduction sensitises cells to radiation by limiting repair capacity. Our findings are further supported by research on *BMAL1*'s role in cell-cycle regulation [32]. *BMAL1*-KD in prostate epithelial cells has been shown to increase the proportion of cells in the G0/G1 phase and reduce the number in S-phase [32]. This shift in cell-cycle distribution may help explain the heightened radiosensitivity observed in *BMAL1*-KD cells since the S-phase is the most radioresistant phase. Furthermore, recent evidence showed that *BMAL1* directly promotes DNA double-strand break repair [14].

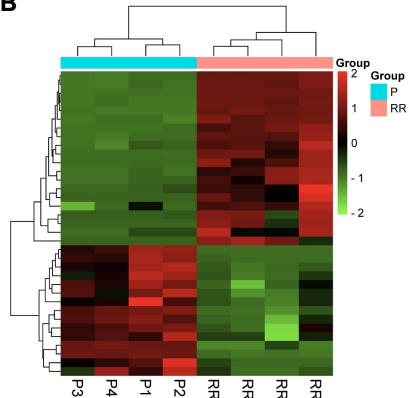
Ai



Aii



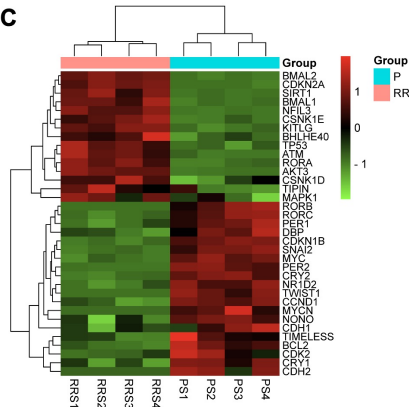
B



Most significant CpG sites within promoter region of genes.

Gene	Number of hypermethylated CpGs
<i>RORA</i>	5
<i>RORB</i>	5
<i>CSNK1E</i>	2
<i>CSNK1D</i>	1
<i>PER2</i>	1

C



D

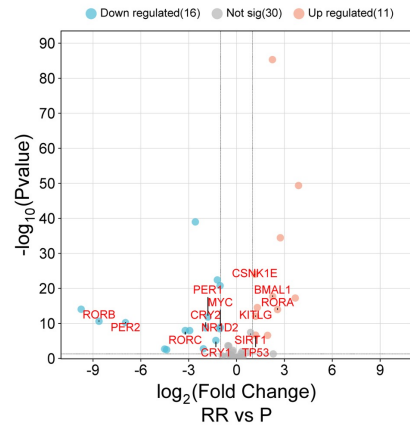


Figure 6. Epigenetic regulation of circadian rhythm dynamics in 22Rv1-P and 22Rv1-RR cell lines: Insights from DNA methylation analysis and RNA sequencing. (Ai) KEGG pathway analysis for the top ten dysregulated pathways in 22Rv1-RR cells compared to 22Rv1-P on DNA methylation data. (Aii) Diagram of the circadian rhythm pathway, with red-marked genes representing those deregulated in 22Rv1-RR compared to 22Rv1-P. (B) Heatmap showing significantly hypermethylated CpG sites ($p < 0.05$) within the promoter regions of circadian-related genes in 22Rv1-RR versus 22Rv1-P cells. (C) Heatmap of RNA-sequencing data displaying significant ($p < 0.05$) differential expression of circadian-related genes between 22Rv1-P and 22Rv1-RR cells. (D) Volcano plot illustrating differentially expressed genes in 22Rv1-RR versus 22Rv1-P cells. Genes such as *PER1*, *CRY1*, *CRY2*, *RORC*, *MYC*, and *NR1D2* were downregulated (blue dots), while *TP53*, *SIRT1*, *BMAL1*, *RORA*, *CSNK1E*, and *KITLG* were upregulated (red dots).

Consequently, *BMAL1*-KD cells exhibit impaired homologous recombination and accumulate unrepaired double-strand breaks [14], further explaining their increased radiosensitivity.

Additionally, *PER2* has been considered a key player in the process of radiation-induced upregulation of CC-related genes, which frequently led to tumour suppression and increased patient survival [33]. In glioma, *PER2* high levels led to a cytostatic effect. Rat glioma cells were arrest at G2/M transition of the cell cycle, making them more sensitive to RT [34, 35]. Our findings are in accordance with these results, as *PER2*-KD led to an increased radioresistance, aligned with lower *PER2* expression in 22Rv1-RR cells compared with parental fraction. Nevertheless, the inefficiency of siRNA-mediated *PER2* silencing observed in our experiments could be attributed to several factors. These include suboptimal siRNA design, compensatory cellular mechanisms, a possible long half-life of *PER2* protein, and post-transcriptional or translational regulation that maintains *PER2* protein levels. Feedback loops may also impact both *PER2* expression and protein stability, contributing to incomplete KD. To address these issues, we plan to optimise KD efficiency in the 22Rv1-P cell line and explore the effects of *PER2* overexpression in 22Rv1-RR cells to assess whether increased *PER2* levels enhance radiosensitivity. These experiments will provide a deeper understanding of *PER2*'s role in modulating radiotherapy response in PCa.

In addition to *BMAL1*, *PER2*, and *p53*, we studied two other key players with established links to the CC and radiation response. *SIRT1*, a well-known deacetylase, contributes to radioresistance by protecting against radiation-induced damage and promoting DNA repair, partly by deacetylating *p53* [36, 37]. Studies show that *SIRT1* enhances cell survival following radiation by maintaining genomic integrity and preventing apoptosis [38]. In our study, *BMAL1*-KD led to a reduction in *SIRT1* levels, which may explain the observed increase in radiosensitivity and reduced cell survival, as diminished *SIRT1* likely impaired DNA repair mechanisms. *KAT2B*, on the other hand, acts as a co-activator of *p53*, enhancing its transactivation potential through acetylation, which promotes apoptosis and cell arrest [39]. We observed a decrease in *KAT2B* expression following *BMAL1*-KD, that could further contribute to the increased radiosensitivity of RR cells. The reduction in *KAT2B* might weaken *p53*-mediated transcriptional activity, thus promoting a more pronounced apoptotic response post-radiation. These findings suggest that *BMAL1* modulates the radiation response not only through *p53* but also by influencing *SIRT1* and *KAT2B* activity.

The methylation analysis revealed epigenetic modulation of circadian genes, which may explain the differential gene expression patterns observed between the 22Rv1-P and 22Rv1-RR cell lines. DNA methylation seems to act as a regulatory mechanism in

radioresistance. Thus, combining RT with epigenetic modulators that target circadian gene dysregulation could offer a promising approach to overcoming radioresistance. In fact, epigenetic drugs, such as HDAC inhibitors (e.g., Panobinostat, Vorinostat, Valproate) and DNMT inhibitors (e.g., Hydralazine), are being studied to enhance RT efficacy. Furthermore, methylation markers could be used to monitor therapy response through non-invasive methods (e.g., liquid biopsies). Previous work from our group showed that DNA methylation biomarkers successfully detected oesophageal cancer pre- and post-neoadjuvant chemoradiation [40]. To further explore the epigenetic regulation of the CC, we plan to perform chromatin immunoprecipitation (ChIP), providing insight into their role in radiosensitivity.

In conclusion, our study highlights the roles of BMAL1 and PER2 in modulating radiosensitivity in PCa. The differential expression and methylation patterns observed between radioresistant, and parental cell lines underscore the significance of circadian regulation in determining RT outcomes. These findings contribute to growing evidence that CC genes hold promise as therapeutic targets. Future studies should focus on chronotherapy and epigenetic modulators to enhance RT efficacy in PCa, paving the way for more personalised treatment approaches.

Currently, we are conducting experiments with melatonin (MLT), which is thought to act as a radiosensitiser and influence DNA methylation [41, 42]. Our next step is to treat the 22Rv1-RR cell line with MLT to investigate its potential to enhance radiosensitivity by affecting methylation levels. Additionally, since MLT is known to increase PER2 expression while reducing BMAL1 [11], we hypothesise whether MLT uptake sensitises cells to radiation by shifting the balance of these key circadian regulators. High doses of MLT have also been shown to increase SIRT1 expression [43].

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CHAPTER 6

Prospective cohort study

Impact of circadian rhythm-based radiotherapy timing on toxicities in localised prostate cancer patients: a prospective cohort study protocol

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Protocol ID: CIRCA-PREDICT (CES. 157/023).

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ABSTRACT

Background: Prostate cancer (PCa) remains a significant health concern worldwide in men. Radiotherapy is one of the main treatment modalities; however, the therapeutic benefits of RT may be accompanied by potentially debilitating side effects that impair patients' functional capacity and quality of life (QoL). Clinical evidence suggests that circadian rhythms (CR) and individual chronotypes may influence RT response and toxicity profiles, but prospective evidence is limited.

Objectives: This study aims to evaluate the RT-related acute and late toxicities in a prospective study of PCa patients based on the individual chronotype.

Methods: This prospective, observational cohort study will recruit men with localised PCa scheduled for External Beam Radiotherapy. Participants will be characterised by their chronotype using the Munich ChronoType Questionnaire and salivary melatonin levels to determine dim light melatonin onset. Primary endpoints include acute and late RT-related toxicities graded with the RTOG acute and late morbidity scoring schema. Secondary endpoints encompass QoL and fatigue assessments. Statistical analyses will explore associations between circadian metrics, toxicity outcomes, and QoL changes, adjusting for confounders.

Discussion: This study will provide evidence on personalised chronotype-driven RT strategies to minimise toxicities and improve QoL for PCa patients. Findings could inform clinical protocols that integrate circadian biology into RT.

Keywords: Prostate Cancer, Radiotherapy, Circadian Rhythms, Toxicity, Chronotype, Salivary Melatonin, Quality of Life

RATIONALE OF THE STUDY

Prostate cancer epidemiology and treatment remarks

Prostate cancer (PCa) remains a significant health concern worldwide in men. Radiotherapy (RT) is a relevant first-line therapeutic modality, with nearly 30% of all PCa patients treated with definitive RT [1, 2]. Remarkably, the Portuguese Institute of Oncology of Porto (IPO Porto) is a reference centre for RT. It receives around 900 newly diagnosed PCa patients annually, half of whom undergo definitive RT. In 2021, 488 patients with urological cancer, mostly PCa, were treated with RT. However, one-third of these patients progressed to locally advanced disease. Additionally, while surgery remains a treatment option, there has been a decline in its adherence. Androgen Deprivation Therapy (ADT) is commonly prescribed in cases of intermediate and high-risk, as neoadjuvant or concomitant treatment with RT [3].

Advancements and challenges in RT for PCa

RT, especially when conventional fractionation is prescribed, demands multiple hospital visits, resulting in increased patient transportation costs and prolonged linear accelerator occupancy [4]. As a result, clinical trials have showcased the viability of escalating daily radiation doses to 2.4–3.4 Gy, effectively shortening the overall treatment duration [5]. Notably, this approach retains its cell-killing efficacy while mirroring similar toxicity profiles. Improvements in External Beam Radiation Therapy (EBRT), incorporating techniques such as Intensity-Modulated Radiation Therapy (IMRT) and Volumetric Modulated Arc Therapy (VMAT), have enabled the modulation of radiation beam intensity. Simultaneously, Image-Guided Radiation Therapy (IGRT) has bolstered dose accuracy, enabling more precise targeting [4].

However, the therapeutic benefits of RT may be accompanied by potential debilitating side effects that impair patients' functional capacity and quality of life (QoL) [6]. Importantly, it is imperative to investigate the underlying mechanisms contributing to radioresistance in PCa and the associated radiotoxicity. This understanding is crucial for improving the precision and efficacy of PCa treatment.

Understanding circadian rhythms and their potential influence on RT response

Circadian rhythm (CR) is an approximately 24-hour cycle that governs numerous processes between day and night in living organisms. It plays a key function in responding to environmental changes and can reset internal clocks [7]. The central clock in the hypothalamic suprachiasmatic nucleus synchronises with external stimuli and communicates with the peripheral clocks of different tissues. In reaction to environmental changes, these peripheral clocks independently regulate molecular, biochemical, and

physiological processes that affect metabolism, cell cycle, hormone production, sleep patterns and body temperature [8].

The circadian clock (CC) is created and driven by three autoregulatory transcriptional-translational feedback loops involving multiple genes and proteins [9]. The CC regulates rhythmic events in DNA repair, apoptosis, and the immune system. Melatonin (MLT) is primarily secreted by the pineal gland. This hormone regulates the sleep-wake cycle and synchronises the CRs. Its secretion typically rises 2-3 hours before the onset of nocturnal sleep and peaks during the night in dim light conditions [10, 11]. The onset of MLT secretion, particularly under dim light (known as dim light melatonin onset or DLMO), is considered the gold standard for assessing the circadian phase in humans [12]. Besides its role in sleep regulation, MLT exhibits antioxidant properties, modulates immune responses, and regulates various cellular processes. Importantly, MLT has been studied for its oncostatic effects, including inhibiting cancer cell proliferation, inducing apoptosis, and enhancing DNA repair mechanisms [13]. The observation of MLT receptors in prostate epithelial cells further supports the potential influence of CR in these cells [14].

Timing of radiotherapy and treatment response

Previous studies indicate that the timing of RT in PCa patients impacts both the response to treatment and the degree of treatment-related toxicity. In a retrospective cohort study, PCa patients who received EBRT in the evening experienced worse freedom from grade ≥ 2 late gastrointestinal (GI) toxicities [15]. Additionally, in patients with T2b-T3, the 5-year biochemical-free survival rate was significantly worse in the evening group [15]. Similarly, Negoro et al. [16] found that morning RT for localised PCa improved QoL and reduced the worsening of lower urinary tract symptoms (LUTS). Thus, clinical evidence suggests that the timing of RT profoundly impacts treatment response and toxicity.

Research gaps

Despite the existing knowledge on the influence of RT timing on treatment outcomes, there are no prospective cohort studies investigating how individual circadian preferences, or chronotypes, can guide the timing of RT and, consequently, affect its associated toxicities in PCa patients. There is a pressing need to understand whether aligning treatment schedules with patients' CRs can minimise toxicities and improve outcomes. Addressing this gap could lead to personalised RT schedules that optimise therapeutic results while minimising adverse effects. Nonetheless, based on existing evidence, it is anticipated that, in a prospective study, the afternoon group may experience higher rates of acute toxicities.

Study Importance

This study aims to evaluate the association between circadian biomarkers (e.g., chronotype), RT timing, and radiotoxicity in PCa patients. It will contribute to the body of evidence on personalised chronotype-driven RT strategies in the future, potentially reducing toxicities and improving patient QoL.

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STUDY DESIGN

A monocentric, prospective, uncontrolled, non-randomised observational study was chosen to explore the impact of CRs on EBRT-related toxicities in PCa patients, providing insights into potential personalised treatment strategies based on circadian biology. The enrolment and data collection period were set from September 2023 to December 2026.

STUDY POPULATION - INCLUSION AND EXCLUSION CRITERIA

Inclusion criteria include males with a confirmed diagnosis of localised prostate adenocarcinoma scheduled for definitive EBRT (VMAT or IMRT) and able to provide informed consent.

Patients will be excluded if they have metastatic disease, a history of cancer within five years of the PCa, prior prostate surgery, or an inability to adhere to study protocols (e.g., saliva collection). In addition, patients taking medications that could potentially interfere with MLT secretion will not be eligible for participation in the study. Such medications include benzodiazepines and their antagonists, fluvoxamine, vitamin B12 supplements, nonsteroidal anti-inflammatory drugs (e.g., aspirin, ibuprofen, and indomethacin), prostaglandin inhibitors, calcium channel blockers, dexamethasone, and certain antidepressants.

ENDPOINTS

The primary endpoints include the measurement of acute and late EBRT-related toxicities, such as GI and genitourinary (GU) toxicities, radiodermatitis, and other common PCa-related toxicities. Toxicities will be classified as acute if appear within three months after the start of EBRT, and as late if they occur beyond this period. The secondary endpoint focuses on assessing QoL and fatigue levels before and after RT.

ANALYSED CRITERIA

The individual chronotypes will be defined by the MCTQ to assess individual chronotypes by inquiring about sleep-wake times on work and non-workdays to determine the mid-point of sleep during off days. This information acts as a reliable indicator of an individual's circadian phase and is validated for the Portuguese population. The Direct Saliva Melatonin RIA kit Novolytix® (RK- DSM2) will be used to determine the MLT concentration in saliva samples. This tool will enable the calculation of the DLMO. DLMO is the gold standard for

estimating the circadian phase and the most stable anchor point of the biological clock. It represents the point when circulating MLT has risen two standard deviations above the low baseline concentrations present during the day. For a more accurate estimation of the DLMO, a spline was fitted to model the melatonin concentration curve, allowing for a smooth approximation of the inflection point where melatonin levels begin to rise. Salivettes® from Sarsted will be used as a reliable tool, user-friendly and non-invasive method for collecting saliva samples.

To assess the QoL of PCa patients, the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire Core-30 (EORTC QLQ C30) will be used. This tool comprises various scales and items and is designed to evaluate physical, psychological, and social functions among cancer patients. It is a comprehensive tool for assessing the QoL and is validated for the Portuguese population. Patients' fatigue will be evaluated with the Functional Assessment of Chronic Illness Therapy – Fatigue (FACIT-F) scale. It consists of 13 items scored on an ordinal scale of 0-4 and measures self-reported fatigue and its impact on daily activities. This is a reliable tool for assessing fatigue, an acute toxicity that can be challenging to measure accurately. However, it has not been validated for the Portuguese population.

VISITS AND EXAMINATIONS

Follow-up will be conducted at regular intervals according to the institute's protocol, with Prostate-Specific Antigen (PSA) measurements and toxicity assessments at specified intervals. Patients treated with ADT are monitored at six-month intervals for five years, while those who did not receive ADT have a follow-up every four months during the first year and every six months until the fifth year. After five years, all patients are followed annually.

DATA COLLECTION

The data collection will encompass demographic, clinical, and treatment-related information extracted from electronic medical records. Upon the initial appointment, participants will receive comprehensive information and will be provided with the opportunity to give informed consent. Those enrolled will receive three questionnaires administered solely on the first and last day of treatment. These questionnaires include the MCTQ, EORTC QLQ C30, and the FACIT-F. All questionnaires are validated for the Portuguese population, and permissions for their use have been obtained from all relevant authors. Saliva samples will be collected concurrently with scheduled RT treatment at three specific time points: at the beginning, middle, and end of the treatment - under dim light conditions. Then, the results

from the MCTQ will be cross-referenced with the DLMO. A simplified and brief chronogram can be found in Appendix IV: Supplementary Figure 1.

A comprehensive flyer was designed, containing crucial information to facilitate the collection process (e.g., instructions, collection times, precautions) (Appendix IV: Supplementary Figure 2). Moreover, a timesheet was provided to record the collection times (Appendix IV: Supplementary Figure 3). Eight specific collection time points were established following a thorough review of existing literature, encompassing periods starting five hours before the participants' usual bedtime, at hourly intervals, at the participants' usual bedtime, one-hour post-bedtime, and upon awakening. Each Salivette® used in the collection process is colour- and number-coded, simplifying the procedure and ensuring accuracy.

Salivette® devices were centrifuged at 2500 g for 2 minutes at 4°C. A volume of 900 µL of saliva supernatant was transferred for melatonin analysis. The assay followed a competitive binding principle, where 300 µL of each sample, calibrators, and controls were incubated with 75 µL of melatonin-specific antibody and 75 µL of radiolabeled 125I-melatonin. After incubation at 4°C for 20±4 hours, a second solid-phase antibody was added, followed by centrifugation at 2000 g for 2 minutes at 4°C. The supernatants were aspirated, and the remaining precipitates were counted in a gamma counter for 2 minutes. Results were calculated against a standard curve.

Radiation Oncologists will use the Radiation Therapy Oncology Group (RTOG) Acute and Late Radiation Morbidity Scoring Schema to assess toxicities already validated for clinical practice. Within Appendix IV, a detailed Supplementary Table 1 encapsulates the RTOG acute and late morbidity scoring scheme specifically addressing acute and late GU and GI toxicities. Other toxicities will be evaluated, such as radiodermatitis. Patients will be allocated into specific chronotypes, including morning-type, evening-type, more morning than evening-type, and more evening than morning-type.

SAFETY AND ETHICAL CONSIDERATIONS

The study will comply with the ethical standards set forth by the institutional review board (Comissão de Ética para a Saúde, CES. 157/023) of the Portuguese Oncology Institute of Porto and follow the principles of the Declaration of Helsinki. All participants will provide written informed consent. The study poses minimal risks to participants, as it primarily involves observational data collection and non-invasive procedures, such as saliva sampling and questionnaires.

STATISTICAL ANALYSIS

Statistical analysis will be performed using GraphPad Prism version 9.0 software (GraphPad Software Inc., La Jolla, CA, USA) and RStudio (R software, version 4.3.1 for macOS) (PBC, Boston, MA, USA). Data will be checked for normal distribution and homogeneity of variances using the Shapiro-Wilk's and Levene's tests, respectively. Primary analysis will be done to analyse toxicity outcomes using logistic regression models adjusted for potential confounders, including patient age, group risk, use of ADT, RT dose, and fractionation scheme. A secondary analysis will associate chronotype with toxicity outcomes and QoL measures using multivariable linear and logistic regression models. The 2-tailed alpha level was set at 0.05, with a confidence interval of 95% for all statistical tests.

EXPECTED RESULTS

The study anticipates finding a relationship between patients' chronotypes and the severity of radiotoxicities. On top of that, it is expected that aligning treatment schedules with CRs will reduce toxicities, improving the QoL for PCa patients. This study could potentially guide future clinical protocols for personalised RT.

PRELIMINARY DATA

Currently, our cohort consists of eight patients with complete data collection, who are under follow-up. The patients have a median age of 70.5 years (range: 57-78), and the majority present a BMI in the overweight range (median: 26.7 kg/m² range: 24.0-38.2). Most have PSA levels below 10 ng/mL, with 62.5% of patients having ISUP grade 2 tumours and 62.5% with clinical stage T2, indicating a generally favourable prognosis (Table 1).

Table 1. Patient clinicopathological characteristics.

Characteristics		n (%)
Total		8
Age		
	median (range)	70.5 (57.0-78.0)
BMI (kg/m ²)		
	median (range)	26.7 (24.0-38.2)
Clinical tumour stage		
	T1c	2 (25.0)
	T2	5 (62.5)
	T3	1 (12.5)
ISUP grade		

	2	5 (62.5)
	3	3 (37.5)
Initial PSA (ng/mL)		
	<10	6 (75.0)
	10-20	1 (12.5)
	>20	1 (12.5)
Risk group*		
	Low risk	2 (25.0)
	Favourable intermediate risk	3 (37.5)
	Unfavourable intermediate risk	2 (25.0)
	High risk	1 (12.5)
ADT		
	None	5 (62.5)
	Short-term	2 (25.0)
	Long-term	1 (12.5)
ECOG performance status		
	0	5 (62.5)
	1	3 (37.5)
Technique		
	VMAT	8 (100.0)
Fractionation scheme		
	Moderate hypofractionation	8 (100.0)
Treated volume		
	Prostate and SV	7 (87.5)
	Prostate, SV and Pelvic lymph nodes	1 (12.5)
Tabacco status		
	Never	3 (37.5)
	Ex-smoker	5 (62.5)
Regular bowel movements		
	Yes	6 (75.0)
	No	2 (25.0)
Chronotype		
	Morning-type	2 (25.0)
	More a morning-type than an evening-type	2 (25.0)
	More an evening-type than a morning-type	1 (12.5)
	Evening-type	3 (37.5)
DLMO (hh:mm)		
	Median (range)	19:36 (18:50-00:00)

*Risk classification is based on clinical tumour stage, ISUP grade and initial PSA serum levels, according to the National Comprehensive Cancer Network (NCCN) guidelines.

Abbreviations: BMI, body mass index; ISUP, International Society of Urological Pathology; PSA, prostate-specific antigen; ADT, androgen deprivation therapy; ECOG, Eastern Cooperative Oncology Group; SV, seminal vesicles; DLMO, dim light melatonin onset.

All patients experienced acute grade 2 GU toxicities, while GI toxicities were less prevalent, affecting only 37.5% of the cohort (Table 2). Moreover, three patients reported erectile dysfunction, and three developed grade 1 radiodermatitis (Table 2). In terms of patient-reported outcomes, the median QoL score declined substantially from 87.5 to 62.5 post-treatment (Table 3). However, fatigue levels remained largely unchanged, with median FACIT-F scores of 43.5 before and 43 after treatment.

A slight decrease in chronotype scores was observed (median MCTQ change: -0.16), suggesting minimal impact on CR during the treatment period. The distribution of chronotypes revealed that 25% were morning-type, 25% more morning than evening-type, 12.5% more evening than morning-type, and 37.5% evening-type. When grouping in morning and evening chronotypes, the latter exhibited a greater decline in QoL post-treatment (mean QoL difference: -12.5) compared to the former (mean QoL difference: -6.3).

Table 2. Incidence of acute gastrointestinal and genitourinary toxicities, radiation dermatitis and erectile dysfunction.

Radiotoxicity	Grade <2	Grade ≥2
Acute toxicities		
Gastrointestinal	7 (87.5)	1 (12.5)
Genitourinary	0	8 (100.0)
Radiation dermatitis		
	8 (100.0)	0
n (%)		
Erectile dysfunction		
Yes	4 (50.0)	
No	4 (50.0)	

Table 3. Fatigue, quality of life and chronotype scores before and after treatment.

Questionnaires scores	Median (range)
FACIT-F scores	
Before treatment	43.5 (31.0-51.0)
After treatment	43.0 (35.0-51.0)
QoL scores	
Before treatment	87.5 (33.3-100.0)
After treatment	62.5 (41.7-100.0)
MCTQ scores	
Before treatment	4.1 (1.8-4.5)
After treatment	4.0 (2.8-5.0)

Abbreviations: FACIT-F, Functional Assessment of Chronic Illness Therapy – Fatigue; QoL, Quality of Life; MCTQ, Munich ChronoType Questionnaire.

Regarding MLT concentration, Figure 1 illustrates the mean MLT levels for the eight patients across the three treatment moments. Furthermore, the median DLMO was 19:36 (range: 18:50-00:00). These preliminary results hint at potential trends, such as a decline in QoL post-treatment by chronotype, but they remain based on a small cohort of only 8 patients. While no definitive patterns have emerged, further data collection is essential to support robust conclusions.

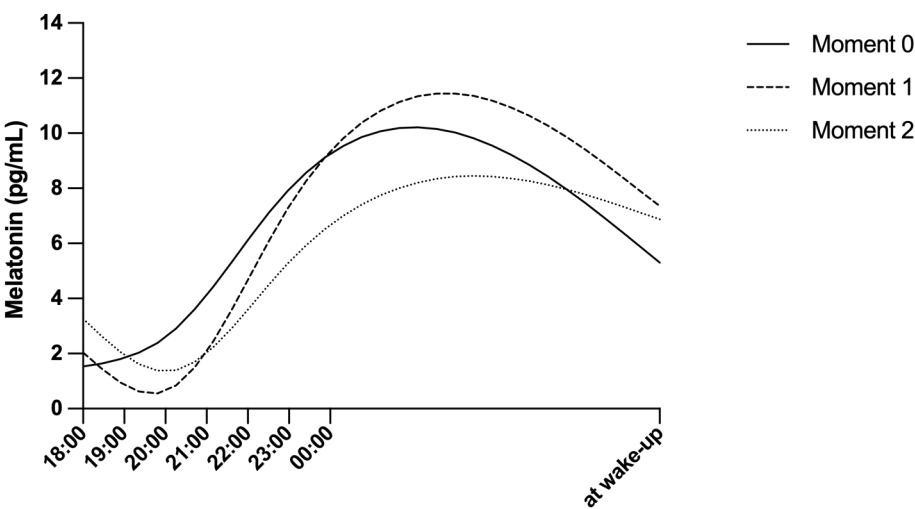


Figure 1. Mean melatonin levels for the three collection moments (before, middle and after treatment).

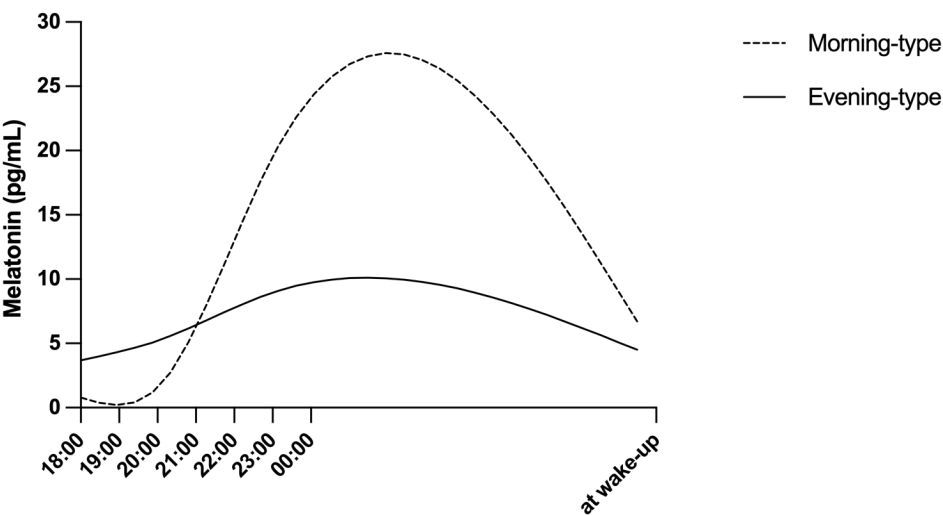


Figure 2. Representative MLT curves for a morning-type and evening-type chronotypes.

CHAPTER 7

General discussion

General Discussion

In this master's dissertation, we explored the relationship between circadian rhythms (CRs) and radiotherapy (RT) outcomes in PCa through three complementary studies: a retrospective cohort study, a molecular study, and a prospective cohort study. Collectively, these studies underscore the potential of chronoradiotherapy in enhancing treatment efficacy while minimising radiotoxicities.

Notably, the retrospective cohort study showed that patients treated in the morning had a significantly lower risk of metastasis after treatment and a lower risk of higher grades of late gastrointestinal (GI) and genitourinary (GU) toxicities compared to those treated in the afternoon. These clinical results align with existing literature, reinforcing that the timing of RT is linked to patient outcomes, with morning treatments leading to better long-term disease control and fewer late adverse effects.

The molecular study provided mechanistic insights by investigating two key circadian regulators, *BMAL1* and *PER2*, in PCa cell lines. *BMAL1*, associated with cell cycle progression and DNA repair, was upregulated in radioresistant cells, while *PER2*, a tumour suppressor, downregulated compared to the most responsive cell lines. Silencing *BMAL1* increased radiosensitivity, whereas *PER2* downregulation resulted in less radio responsiveness. These findings suggest that the timely-dependent effects observed in the retrospective cohort may be explained, in part, by circadian variations due to the differential expression of these genes.

Based on these results, the prospective cohort study aims to explore whether aligning treatment with individual circadian profiles can optimise RT outcomes. Preliminary data from eight patients revealed a lower QoL post-treatment in those with a morning chronotype compared to an evening chronotype. As the study progresses, it will offer further insights into the potential of personalised RT schedules based on circadian biomarkers to reduce treatment-related toxicities.

In conclusion, this dissertation contributes to the growing body of evidence of the importance of chronoradiotherapy in improving PCa therapeutic management. Additionally, the ongoing prospective study will offer valuable insights into whether individualised treatment schedules based on chronotypes can further reduce radiotoxicity. Integrating CR into clinical practice could pave the way for more personalised, effective, and less toxic treatments for PCa patients.

APPENDIX

Appendix

APPENDIX I

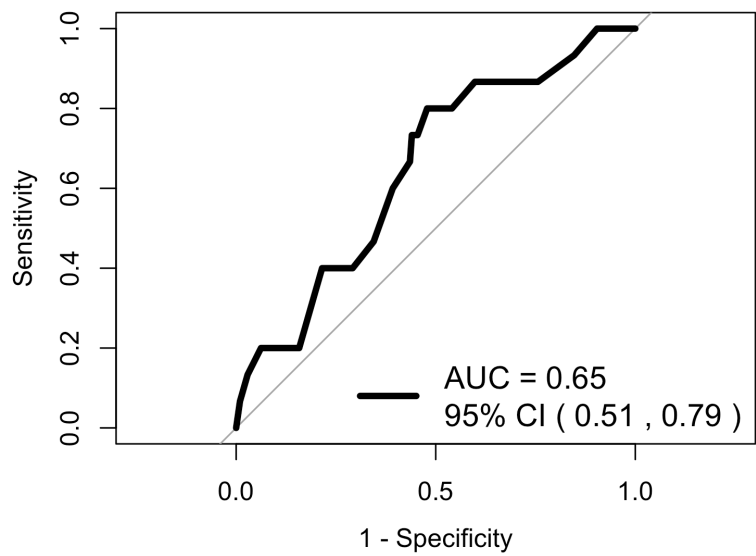
Supplementary Table 1. Histological International Society of Urological Pathology (ISUP) Grade Groups for prostate cancer.

Gleason pattern	Gleason score	ISUP grade
≤3+3	≤6	1
3+4	7	2
4+3	7	3
4+4; 3+5; 5+3	8	4
4+5; 5+4; 5+5	9 or 10	5

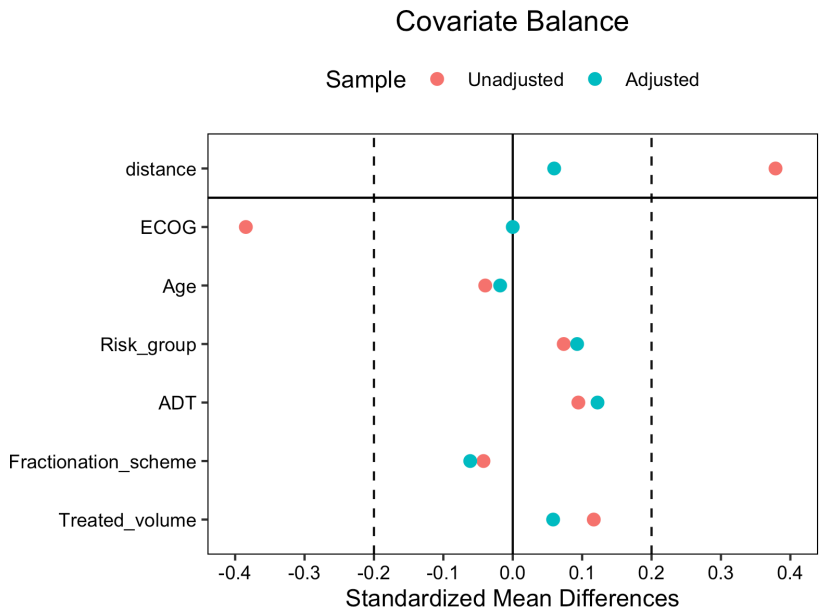
Supplementary Table 2. TNM classification of prostate cancer according to the 8th edition of the American Joint Committee on Cancer (AJCC) guidelines.

Clinical primary tumour (cT)	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
T1	Clinically inapparent tumour that is not palpable
T1a	Tumour incidental histologic finding in 5% or less of tissue resected
T1b	Tumour incidental histologic finding in more than 5% of tissue resected
T1c	Tumour identified by needle biopsy found in one or both sides, but not palpable
T2	Tumour is palpable and confined within prostate
T2a	Tumour involves one-half of one side or less
T2b	Tumour invades more than one-half of one side but not both sides
T2c	Tumour invades both sides
T3	Extraprostatic tumour that is not fixed or does not invade adjacent structures
T3a	Extraprostatic extension (unilateral or bilateral)
T3b	Tumour invades seminal vesicle(s)
T4	Tumour is fixed or invades adjacent structures other than seminal vesicles such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall
Pathological primary tumour (pT)	
T2	Organ confined
T3	Extraprostatic extension
T3a	Extraprostatic extension (unilateral or bilateral) or microscopic invasion of bladder neck
T3b	Tumour invades seminal vesicle(s)
T4	Tumour is fixed or invades adjacent structures other than seminal vesicles such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall
Regional lymph node (N)	
Nx	Regional lymph nodes cannot be assessed
N0	No positive regional nodes
N1	Metastases in regional node(s)
Distant metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis
M1a	Nonregional lymph node(s)
M1b	Bone(s)
M1c	Other site(s) with or without bone disease

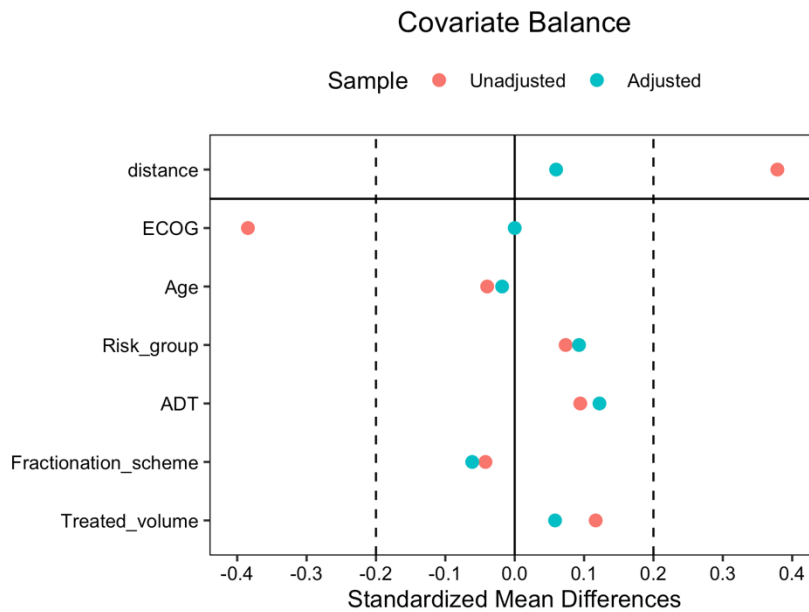
APPENDIX II



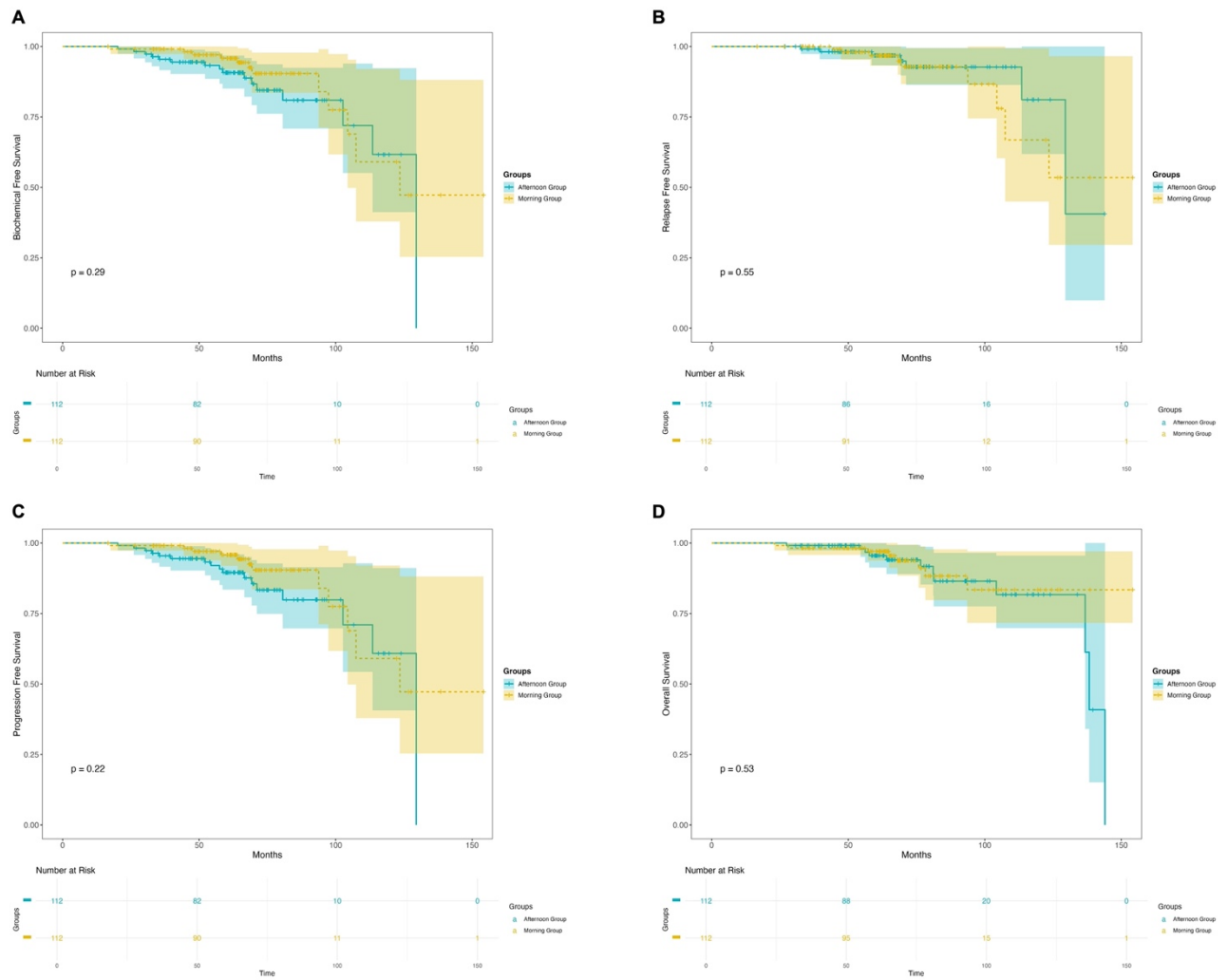
Supplementary Figure 1. ROC curve analysis for determining the optimal cut-off point of hour of treatment in predicting metastasis-free survival. The optimal cut-off was determined using Youden's Index (sensitivity + specificity - 1). AUC, area under the curve; CI, confidence interval.



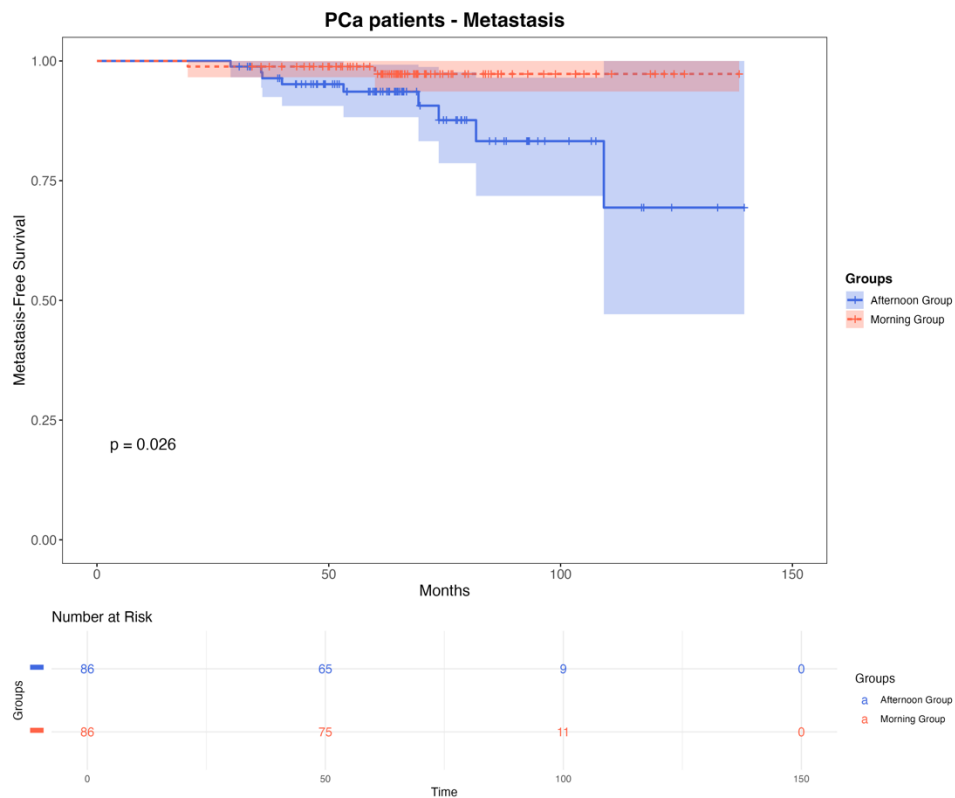
Supplementary Figure 2. Covariate balance before and after matching for the first cohort. Standardized mean differences (SMD) for all covariates used in the propensity score matching process. The red dots represent the unadjusted SMDs before matching, while the blue dots depict the adjusted SMDs after matching. The dashed vertical lines indicate the thresholds for acceptable covariate balance (SMD range of -0.2 to 0.2). Covariate balance was achieved when the blue dots fell within these limits, indicating successful adjustment and minimal residual confounding between treatment groups. ECOG, Eastern Cooperative Oncology Group; ADT, androgen deprivation therapy.



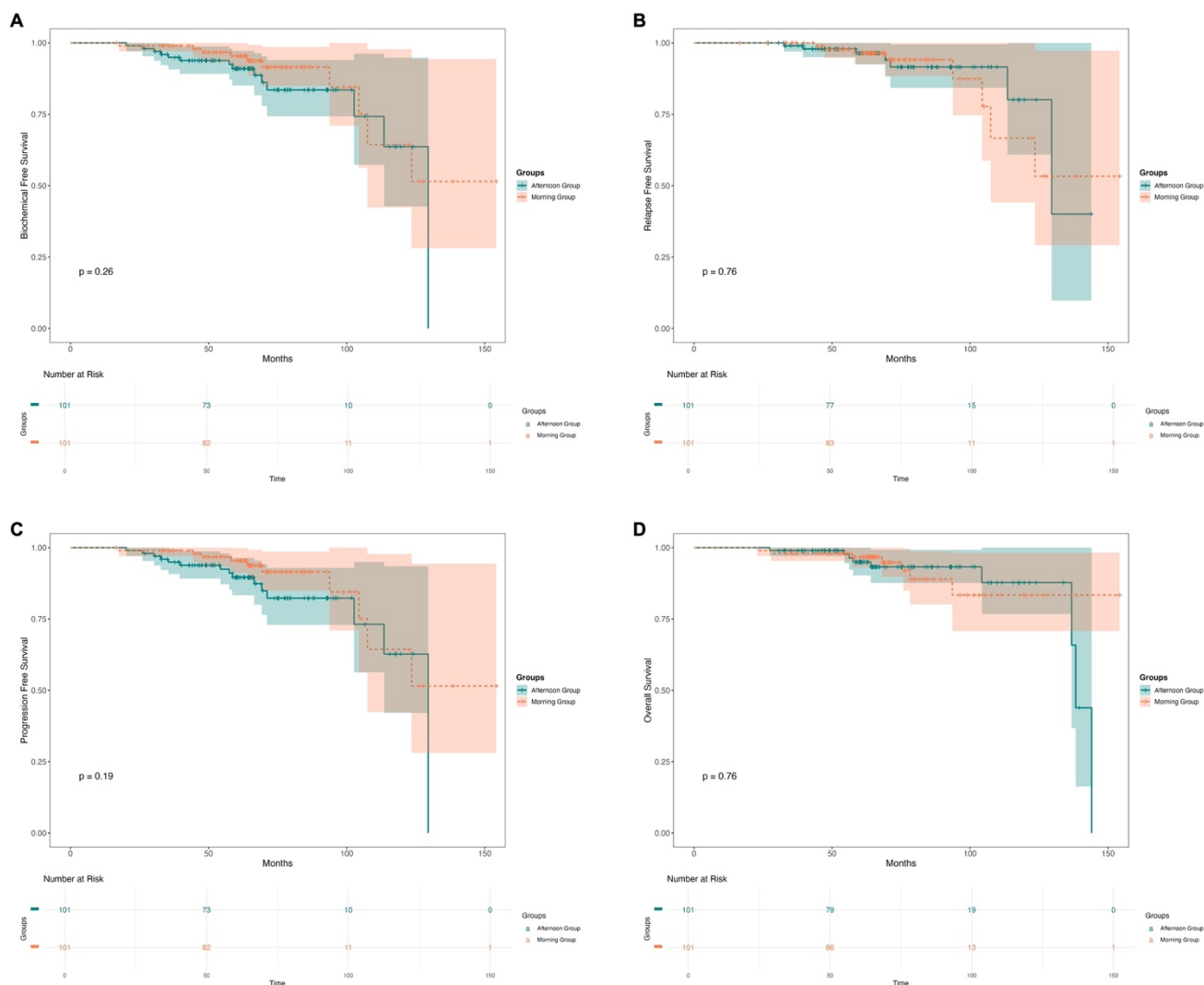
Supplementary Figure 3. Covariate balance before and after matching for the second cohort. Standardized mean differences (SMD) for all covariates used in the propensity score matching process. The red dots represent the unadjusted SMDs before matching, while the blue dots depict the adjusted SMDs after matching. The dashed vertical lines indicate the thresholds for acceptable covariate balance (SMD range of -0.2 to 0.2). Covariate balance was achieved when the blue dots fell within these limits, indicating successful adjustment and minimal residual confounding between treatment groups. ECOG, Eastern Cooperative Oncology Group; ADT, androgen deprivation therapy.



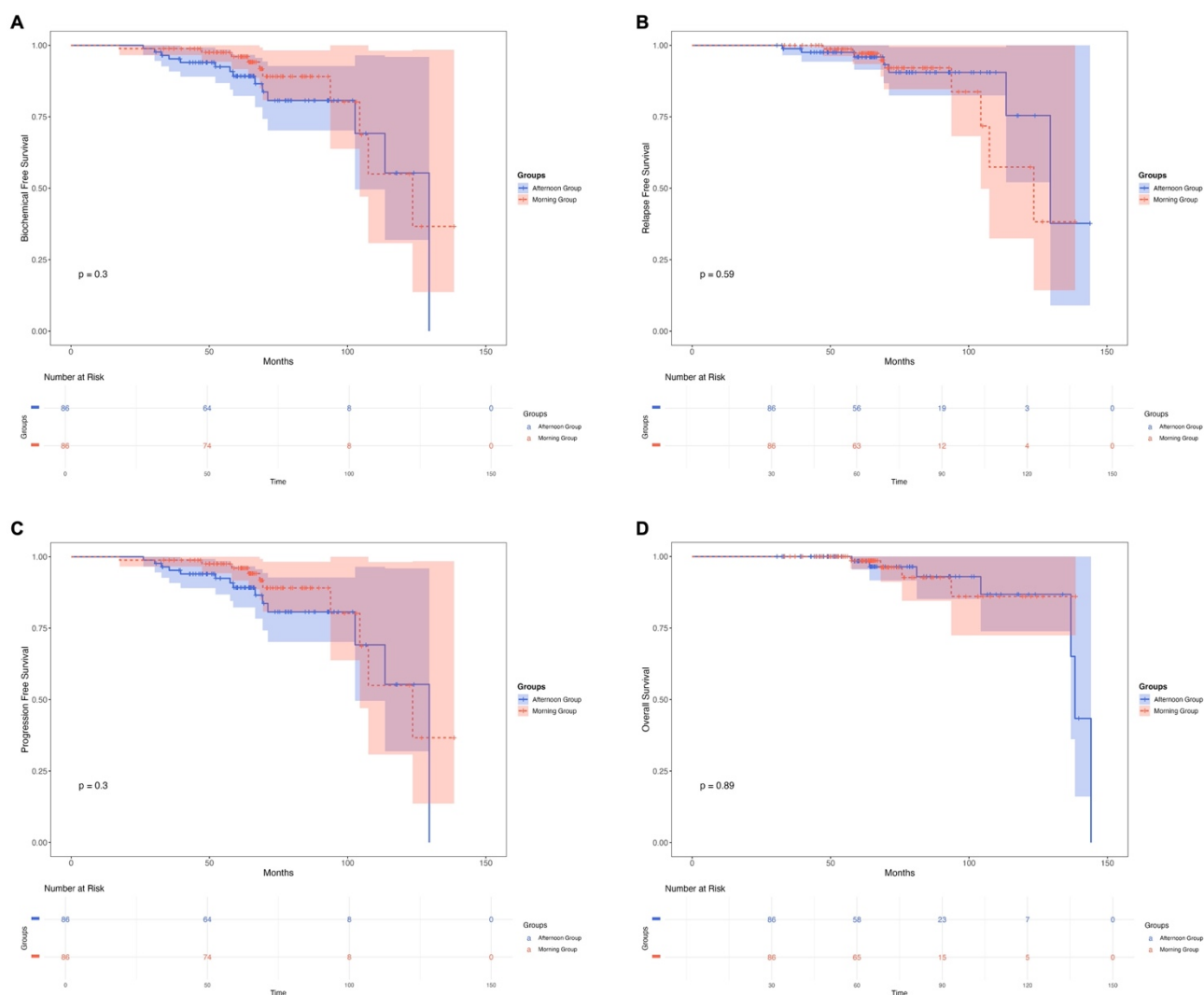
Supplementary Figure 4. (upper) Kaplan-Meier curves of biochemical-free survival (BFS) (A), relapse-free survival (RFS) (B), progression-free survival (C), and overall-survival (D) in morning (n = 112) and afternoon (n = 112) external beam radiotherapy groups for the unmatched first approach. The analysis demonstrates no statistically significant difference in survival rates for any of the outcomes (Log-rank test: $p > 0.05$). (lower) Number of patients at risk within the afternoon and morning groups, along the time.



Supplementary Figure 5. (upper) Kaplan-Meier curves of metastasis-free survival (MFS) in morning ($n = 86$) and afternoon ($n = 86$) external beam radiotherapy groups for the unmatched first approach. The analysis demonstrates a statistically significant difference in MFS favoring the morning treatment group (Log-rank test $p = 0.026$). (lower) Number of patients at risk within the afternoon and morning groups, along the time.



Supplementary Figure 6. (upper) Kaplan-Meier curves of biochemical-free survival (BFS) (A), relapse-free survival (RFS) (B), progression-free survival (C), and overall-survival (D) in morning (n = 86) and afternoon (n = 86) external beam radiotherapy groups for the matched second cohort. The analysis demonstrates no statistically significant difference in survival rates for any of the outcomes (Log-rank test: $p > 0.05$). (lower) Number of patients at risk within the afternoon and morning groups, along the time.



Supplementary Figure 7. (upper) Kaplan-Meier curves of biochemical-free survival (BFS) (A), relapse-free survival (RFS) (B), progression-free survival (C), and overall-survival (D) in morning ($n = 86$) and afternoon ($n = 86$) external beam radiotherapy groups for the matched second cohort. The analysis demonstrates no statistically significant difference in survival rates for any of the outcomes (Log-rank test: $p > 0.05$). (lower) Number of patients at risk within the afternoon and morning groups, along the time.

Supplementary Table 1. Patient clinicopathological characteristics and survival endpoints in the second matched approach.

Characteristics	2 nd matched approach		<i>p</i> -value [†] (effect size) [‡]
	08:30 AM - 11:30 AM	15:00 AM - 20:00 AM	
	n (%)	n (%)	
Total	86	86	
Age			0.671
median (range)	69.5 (51-79)	69.0 (56-84)	
Clinical tumour stage			0.190
T1c	11 (12.8)	25 (29.1)	
T2	70 (81.4)	54 (62.8)	
T3	5 (5.8)	7 (8.1)	
ISUP grade			0.766
1	16 (18.6)	15 (17.4)	
2	54 (62.8)	56 (65.1)	
3	8 (9.3)	11 (12.8)	
4	6 (7.9)	3 (3.5)	
5	2 (2.3)	1 (1.2)	
Initial PSA (ng/mL)			0.264
<10	58 (67.4)	58 (67.4)	
10-20	23 (26.7)	27 (31.4)	
>20	5 (5.8)	1 (1.2)	
Risk group*			0.660
Low risk	1 (1.2)	3 (3.5)	
Favourable intermediate risk	65 (75.6)	64 (74.4)	
Unfavourable intermediate risk	7 (8.1)	9 (10.5)	
High risk	13 (15.1)	10 (11.6)	
ADT			0.438
None	71 (82.6)	76 (88.4)	
Short-term	5 (5.8)	2 (2.3)	
Long-term	10 (11.6)	8 (9.3)	
ECOG performance status			>0.999
0	80 (93.0)	80 (93.0)	
1	6 (7.0)	6 (7.0)	
2	0	0	
Technique			>0.999
IMRT	22 (25.6)	21 (24.4)	
VMAT	64 (74.4)	65 (75.6)	
Fractionation scheme			0.836
Conventional	15 (17.4)	13 (15.1)	

Moderate hypofractionation	71 (82.6)	73 (84.9)	
Treated volume			0.937
Prostate	39 (45.3)	41 (47.7)	
Prostate and SV	42 (48.8)	41 (47.7)	
Prostate, SV and Pelvic lymph nodes	5 (5.8)	4 (4.7)	
Endpoints	n (%)	n (%)	p-value [†] (effect size) [‡]
Biochemical recurrence			0.509
Yes	10 (11.6)	14 (16.3)	
No	76 (88.4)	72 (83.7)	
Locoregional recurrence			>0.999
Yes	8 (9.3)	8 (9.3)	
No	78 (90.7)	78 (90.7)	
Metastasis			0.062
Yes	2 (2.3)	9 (10.5)	
No	84 (97.7)	77 (89.5)	
Progression			0.509
Yes	10 (11.6)	14 (16.3)	
No	72 (83.7)	76 (88.4)	
Deaths			0.533
Yes	4 (4.7)	7 (8.1)	
No	82 (95.3)	79 (91.9)	
BFS (months)			0.172
median (range)	66.1 (17.6-138.5)	63.0 (26.1-129.5)	
LRFS (months)			0.639
median (range)	66.4 (33.4-138.5)	65.1 (30.7-143.9)	
MFS (months)			0.176
median (range)	66.4 (19.6-138.5)	63.6 (28.8-139.6)	
PFS (months)			0.172
median (range)	66.1 (17.6-138.5)	63.0 (26.1-129.5)	
OS (months)			0.815
median (range)	67.5 (33.4-138.5)	65.5 (30.7-144.1)	

*Risk classification is based on clinical tumour stage, ISUP grade and initial PSA serum levels, according to the National Comprehensive Cancer Network (NCCN) guidelines.

[†]Statistical tests applied: Mann-Whitney U test for continuous variables, chi-squared test for categorical variables, Fisher's exact test when expected cell counts were less than 5, and chi-squared test with continuity correction for 2x2 contingency tables.

[‡]Effect size (Cramer's V) was computed for categorical variables when the p-value<0.05.

Bold values indicate statistical significance (p-value<0.05).

Abbreviations: PM, *post meridiem*; ISUP, International Society of Urological Pathology; PSA, prostate-specific antigen; AJCC, American Joint Committee on Cancer; ADT, androgen deprivation therapy; ECOG, Eastern Cooperative Oncology Group; SV, seminal vesicles; BFS, biochemical failure-free survival; LRFS, locoregional recurrence-free survival; MFS, metastasis-free survival; PFS, progression-free survival; OS, overall survival.

Supplementary Table 2. Multivariable Cox regression analysis of predictors of survival endpoints, in the first unmatched approach.

Endpoint	Variables	Coefficient (S.E.)	HR (95% CI)	Z	p-value
Biochemical-free survival					
	Treatment group	-0.4082 (0.3950)	0.6648 (0.3065-1.4420)	-1.0330	0.3010
	ECOG performance status	-0.3955 (0.7520)	0.6733 (0.1542-2.9400)	-0.5260	0.5990
	Age	0.0217 (0.0314)	1.0219 (0.9610-1.0870)	0.6910	0.4890
	*Risk group	0.1429 (0.0314)	1.1536 (0.5676-2.3450)	0.3950	0.6930
	ADT	-0.1918 (0.4319)	0.8255 (0.3541-1.9240)	-0.4440	0.6570
	Fractionation scheme	-0.0057 (0.6293)	0.9943 (0.2896-3.4140)	-0.0090	0.9930
	Treated volume	0.0443 (0.4156)	1.0453 (0.4629-2.3600)	0.1070	0.9150
Locoregional recurrence-free survival					
	Treatment group	0.0463 (0.4993)	1.0470 (0.3936-2.7870)	0.0930	0.9260
	ECOG performance status	-18.1800 (6734.0000)	<0.0001 (NE)	-0.0030	0.9980
	Age	0.0305 (0.0423)	1.0310 (0.9488-1.1200)	0.7200	0.4720
	*Risk group	-0.4848 (0.5526)	0.6158 (0.2085-1.8190)	-0.8770	0.3800
	ADT	0.1733 (0.6799)	1.1890 (0.3137-4.5080)	0.2550	0.7990
	Fractionation scheme	0.9226 (1.0020)	2.5160 (0.3532-17.9190)	0.9210	0.3570
	Treated volume	0.2593 (0.5496)	1.2960 (0.4414-3.8050)	0.4720	0.6370

Progression-free survival

Treatment group	-0.4869 (0.3895)	0.6146 (0.2864-1.3180)	-1.2500	0.2110
ECOG performance status	-0.4470 (0.7498)	0.6396 (0.1471-2.7800)	-0.5960	0.5510
Age	0.0208 (0.0310)	1.0210 (0.9609-1.0850)	0.6720	0.5020
*Risk group	0.0764 (0.3565)	1.0794 (0.5366-2.1710)	0.2140	0.8300
ADT	-0.1772 (0.4313)	0.8376 (0.3597-1.9500)	-0.4110	0.6810
Fractionation scheme	0.0556 (0.6276)	1.0572 (0.3090-3.6170)	0.0890	0.9290
Treated volume	0.1574 (0.4063)	1.1704 (0.5278-2.5950)	0.3870	0.6990

Overall survival

Treatment group	0.3065 (0.5172)	1.3587 (0.4930-1.7125)	0.5930	0.5534
ECOG performance status	1.5933 (0.5384)	4.9198 (1.7125-14.1340)	2.9590	0.0031
Age	0.0766 (0.0429)	1.0796 (0.9925-1.1740)	1.7850	0.0742
*Risk group	0.0741 (0.5682)	1.0769 (0.3536-3.2800)	0.1300	0.8962
ADT	0.1206 (0.6058)	1.1282 (0.3441-3.69909)	0.1990	0.8422
Fractionation scheme	-0.2539 (0.6406)	0.7758 (0.2210-2.7230)	-0.3960	0.6919
Treated volume	-0.6060 (0.4946)	0.5455 (0.2069-1.4380)	-1.2250	0.2205

*Risk classification is based on clinical tumour stage, ISUP grade and initial PSA serum levels, according to the National Comprehensive Cancer Network (NCCN) guidelines.

Bold values indicate statistical significance (p -value<0.05).

Abbreviations: ECOG, Eastern Cooperative Oncology Group; ADT, androgen deprivation therapy; HR, hazard ratio; S.E., standard error; CI, confidence interval; NE, Not Estimable.

Supplementary Table 3. Univariable Cox regression analysis of predictors of survival endpoints, in the matched approaches.

Approach	Endpoint	Variables	Coefficient (S.E.)	HR (95% CI)	Z	p-value
1st approach						
	Biochemical-free survival	Treatment group	-0.4675 (0.4166)	0.6266 (0.2769-1.4180)	-1.1220	0.2620
	Locoregional recurrence-free survival	Treatment group	0.0113 (0.5027)	1.0110 (0.3776-2.7090)	0.0230	0.9829
	Progression-free survival	Treatment group	-0.5371 (0.4107)	0.5844 (0.2613-1.3070)	-1.3080	0.1910
	Overall survival	Treatment group	-0.1575 (0.5071)	0.8543 (0.3162-2.3080)	-0.3110	0.7560
2nd approach						
	Biochemical-free survival	Treatment group	-0.4294 (0.4147)	0.6509 (0.2887-1.4670)	-1.0350	0.3010
	Locoregional recurrence-free survival	Treatment group	0.1273 (0.5037)	1.1360 (0.4232-3.0480)	0.2530	0.800
	Metastasis-free survival	Treatment group	-1.5747 (0.7820)	0.2071 (0.0447-0.9589)	-2.0140	0.0440
	Progression-free survival	Treatment group	-0.4294 (0.4147)	0.6509 (0.2887-1.4670)	-1.0350	0.3010
	Overall survival	Treatment group	-0.0923 (0.6613)	0.9118 (0.2495-3.3330)	-0.1400	0.8890

Bold values indicate statistical significance (p -value<0.05).

Abbreviations: HR, hazard ratio; S.E., standard error; CI, confidence interval.

Supplementary Table 4. Incidence of all grades of acute and late gastrointestinal, genitourinary and radiation dermatitis toxicities, in the first unmatched approach.

	Before 12:45 PM (n=112)					After 12:45 PM (n=112)				
	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Acute toxicities										
Gastrointestinal	90 (80.4)	25 (22.3)	1 (0.9)	6 (5.4)	0	73 (65.2)	25 (22.3)	8 (7.1)	6 (5.4)	0
Genitourinary	34 (30.4)	53 (47.3)	20 (17.9)	5 (4.5)	0	33 (29.5)	43 (38.4)	28 (25.0)	8 (7.1)	0
Late toxicities										
Gastrointestinal	81 (72.3)	29 (25.9)	2 (1.8)	0	0	83 (74.1)	17 (15.2)	11 (9.8)	1 (0.9)	0
Genitourinary	52 (46.4)	46 (41.1)	12 (10.7)	2 (1.8)	0	38 (33.9)	41 (36.6)	27 (24.1)	6 (5.4)	0
Radiation dermatitis										
	73 (65.2)	37 (33.0)	2 (1.8)	0	0	72 (64.3)	34 (30.4)	6 (5.4)	0	0

Abbreviations: PM, *post meridiem*.

Supplementary Table 5. Incidence of all grades of acute and late gastrointestinal, genitourinary and radiation dermatitis toxicities, in the first unmatched approach.

	Before 12:45 PM (n=101)					After 12:45 PM (n=101)				
	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Acute toxicities										
Gastrointestinal	80 (79.2)	15 (14.9)	1 (1.0)	5 (5.0)	0	67 (66.3)	21 (20.8)	7 (6.9)	6 (5.9)	0
Genitourinary	30 (29.7)	47 (46.5)	19 (18.8)	5 (5.0)	0	30 (29.7)	39 (38.6)	25 (24.8)	7 (6.9)	0
Late toxicities										
Gastrointestinal	72 (71.3)	27 (26.7)	2 (2.0)	0	0	75 (74.3)	15 (14.9)	11 (10.9)	0	0
Genitourinary	44 (43.6)	43 (42.6)	12 (11.9)	2 (2.0)	0	35 (34.7)	37 (36.6)	25 (24.8)	4 (4.0)	0
Radiation dermatitis										
	66 (65.3)	34 (33.7)	1 (1.0)	0	0	66 (65.3)	30 (29.7)	5 (5.0)	0	0

Abbreviations: PM, *post meridiem*.

Supplementary Table 6. Incidence of all grades of acute and late gastrointestinal, genitourinary and radiation dermatitis toxicities, in the second matched approach.

	Before 12:45 PM (n=86)					After 12:45 PM (n=86)				
	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Acute toxicities										
Gastrointestinal	70 (81.4)	11 (12.8)	1 (1.2)	4 (4.7)	0	59 (68.6)	18 (20.9)	5 (5.8)	4 (4.7)	0
Genitourinary	28 (32.6)	41 (47.7)	14 (16.3)	3 (3.5)	0	23 (26.7)	34 (39.5)	23 (26.7)	6 (7.0)	0
Late toxicities										
Gastrointestinal	65 (75.6)	21 (24.4)	0	0	0	63 (73.3)	13 (15.1)	9 (10.5)	1 (1.2)	0
Genitourinary	40 (46.5)	36 (41.9)	8 (9.3)	2 (2.3)	0	32 (37.2)	28 (32.6)	25 (29.1)	1 (1.2)	0
Radiation dermatitis										
	56 (65.1)	29 (33.7)	1 (1.2)	0	0	57 (66.3)	26 (30.2)	3 (3.5)	0	0

Abbreviations: PM, *post meridiem*.

Supplementary Table 7. Comparison of acute and late gastrointestinal, genitourinary and radiation dermatitis toxicities by grade (<2 vs. ≥2) between treatment groups in the second matched approach.

	08:30 AM - 11:30 AM (n=86)		15:00 AM - 20:00 AM (n=86)		<i>p</i> -value [†] (effect size) [‡]
	Grade <2	Grade ≥2	Grade <2	Grade ≥2	
Acute toxicities	81 (94.2)	5 (5.8)	77 (89.5)	9 (10.5)	0.403
Gastrointestinal	69 (80.2)	17 (19.8)	57 (66.3)	29 (33.7)	0.058
Genitourinary					
Late toxicities	86 (100)	0	76 (88.4)	10 (11.6)	0.003 (0.248)
Gastrointestinal	76 (88.4)	10 (11.6)	60 (69.8)	26 (30.2)	0.005 (0.229)
Genitourinary					
Radiation dermatitis	85 (98.8)	1 (1.2)	83 (96.5)	3 (3.5)	0.621
	81 (94.2)	5 (5.8)	77 (89.5)	9 (10.5)	0.403

[†]Statistical tests applied: Fisher's exact test when expected cell counts were less than 5, and chi-squared test with continuity correction.

[‡]Effect size (Cramer's V) was computed for categorical variables when the *p*-value<0.05.

Bold values indicate statistical significance (*p*-value<0.05).

Abbreviations: PM, *post meridiem*.

Supplementary File 1. Logistic regression results for toxicities: [Available here](#)

APPENDIX III

Supplementary Table 1. RT-qPCR (Fw and Rv) primers details.

Application	Gene name	Fw sequence (5'→3')	Rv sequence (5'→3')	Primers, concentration (μM), Fw+Rv	Annealing temperature (°C)
RT-qPCR	<i>BMAL1</i>	GCTCAGGAGAACCCAGGTTATC	GCATCTGCTTCCAAGAGGCTCA	0.2	64
	<i>PER2</i>	GCCAAGTTTGTGGAGTTCCTG	CTTGACCTTGACCAGGTAGG	0.2	64
	<i>TP53</i>	CGCTTCGAGATGTTCCGAGA	CTTCAGGTGGCTGGAGTGAG	0.5	60
	<i>SIRT1</i>	GCGGGAATCCAAAGGATAAT	GCACCTAGGACATCGAGGAA	0.5	60
	<i>KAT2B</i>	GCCAAGAAACCCCGAGTTAT	AAAGACGAGCCGTGTGATGT	0.5	60
	<i>GUS-β</i>	CACTGAAGAGTACCAGAAAAGTC	TCTCTGCCGAGTGAAGATCC	0.5	60

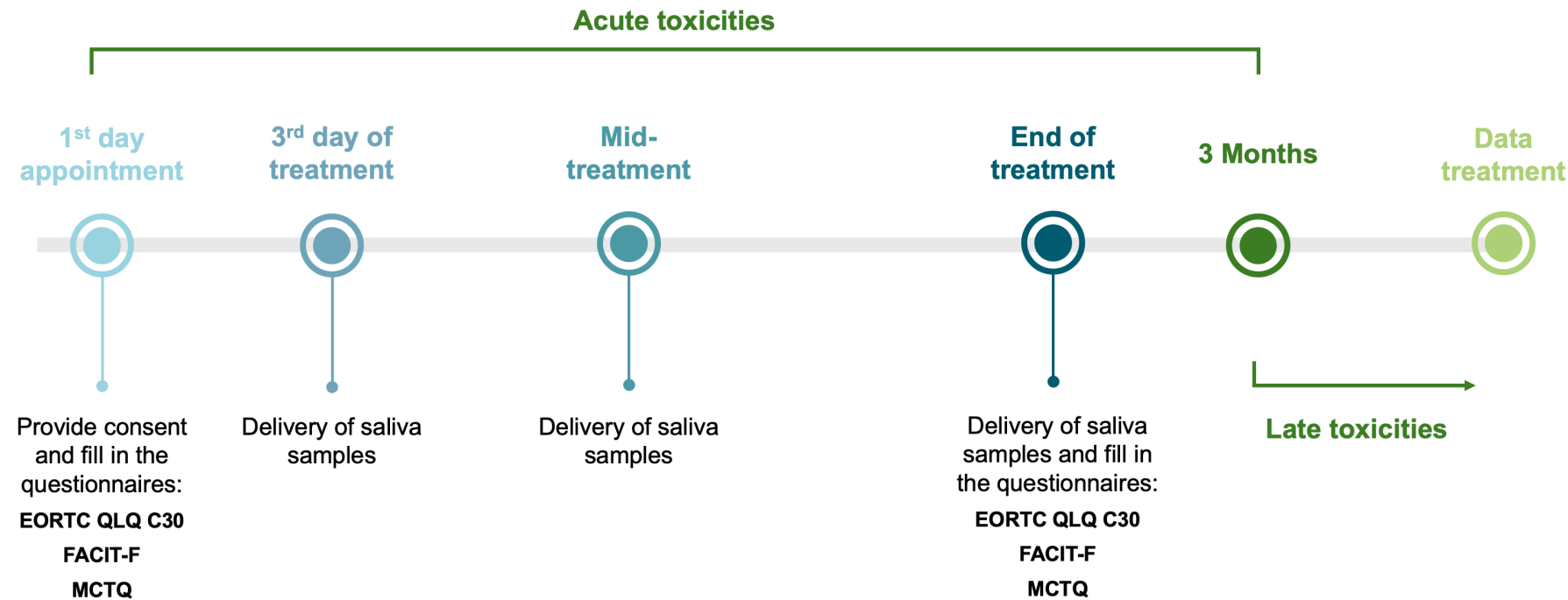
Abbreviations: RT-qPCR, reverse transcriptase quantitative polymerase chain reaction; Fw, forward; Rv, reverse.

Supplementary Table 2. Antibodies details for western blot (WB).

Antibody	Reference	WB Dilution	Second antibody
β-actin	Sigma-Aldrich, A1978,	1:10000; 5% milk in TBS/T	Anti-mouse
H3	Abcam, ab1791	1:3000; 5% BSA in TBS/T	Anti-rabbit
BMAL1	Santa Cruz Biotechnology, sc-365645	1:1000; 5% BSA in TBS/T	Anti-mouse
PER2	ThermoFisher, MA535644	1:2000; 5% BSA in TBS/T	Anti-rabbit
p53	M700129-2 (DO-7) Lusopalex	1:1000; 5% BSA in TBS/T	Anti-mouse
SIRT1	Abcam, ab32441	1:1000; 5% milk in TBS/T	Anti-rabbit
KAT2B	Cell Signaling, #3378	1:1000; 5% BSA in TBS/T	Anti-rabbit
γ-H2AX	Cell Signaling, 2577s	1:1000; 5% BSA in TBS/T	Anti-rabbit

APPENDIX IV

Supplementary Figure 1. Chronogram of the collection process.



Supplementary Figure 2. Instructions flyer (front and back) in Portuguese.

AMOSTRAS DE SALIVA

Neste panfleto pode encontrar todas as informações que precisa para a recolha das amostras de saliva.



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Investigamos para cuidar de si!



INSTRUÇÕES

- 30 minutos antes da recolha **NÃO PODE**:
 - Beber
 - Comer
 - Fumar
 - Mascar pastilha elástica
- 1 hora antes da recolha **NÃO PODE**:
 - Escovar os dentes
- Se tiver consumido comida entre as recolhas **TEM** que bochechar a boca com água 15 minutos antes de recolher a saliva.
- **NÃO PODE** comer bananas.
- **DEVE** realizar as recolhas em baixa luz.
- Idealmente **DEVE** utilizar luz amarela de baixa intensidade.

QUANDO?

- Terá de recolher 8 amostras de saliva ao longo do dia:
 - 5 horas antes da hora normal de se deitar, recolher de hora em hora;
 - Na hora a que se costuma a deitar;
 - 1 hora após a hora a que se costuma a deitar;
 - Ao acordar.

Exemplo

Hora de Recolha	17:00	18:00	19:00	20:00	21:00	22:00	23:00	08:00
Hora de dormir habitual	-5 horas	-4 horas	-3 horas	-2 horas	-1 hora	Hora normal de deitar	+1 hora	Ao acordar
Amostra	1	2	3	4	5	6	7	8

Nota: Este quadro serve meramente de exemplo. Deve ajustar às horas que normalmente se vai deitar.

APÓS A COLHEITA, O QUE FAZER?

Deve entregá-la, no próximo dia de tratamento, na sua sala de tratamento do serviço de Radioterapia do IPO Porto.



INSTRUÇÕES

- 1) Recolha a saliva nos **horários** escritos no verso deste panfleto.
- 2) Segure o Salivette® pela extremidade do recipiente de suspensão (c) e retire a rolha (a). A remoção da rolha é mais fácil se a empurrar ligeiramente para o lado ao abrir a o recipiente de suspensão.
- 3) Retire agora o esfregaço (b) do Salivette®.
- 4) Recolha a saliva de acordo com as seguintes instruções: mastigue suavemente o esfregaço durante 2 MINUTOS e mantenha o esfregaço na boca até sentir que já não consegue evitar engolir a saliva produzida.
- 5) Volte agora a colocar o esfregaço saturado (b) no recipiente de suspensão (c) e feche firmemente o Salivette® com a rolha (a).
- 6) Preencha as informações solicitadas na etiqueta do recipiente de recolha da centrifugação (d).
- 7) Não se esqueça de indicar a **HORA DA COLHEITA** e a **HORA A QUE JANTOU**.
- 8) O Salivette® pode agora ser devolvido. Como tem de recolher várias amostras de saliva, deve manter os Salivette® usados num FRIGORÍFICO.





(a) Rolha



(b) Esfregaço



(c) Recipiente de suspensão



(d) Recipiente de recolha da centrifugação

Supplementary Figure 3. Time sheet to record the collection times in Portuguese.



REGISTO DAS HORAS DE RECOLHA

Hora de dormir habitual	-5 horas	-4 horas	-3 horas	-2 horas	-1 hora	Hora normal de deitar	+1 hora	Ao acordar
Hora de Recolha	—:—	—:—	—:—	—:—	—:—	—:—	—:—	—:—
Amostra	1	2	3	4	5	6	7	8

Supplementary Table 1. RTOG/EORTC acute and late urinary and lower gastrointestinal radiation morbidity scoring schema.

Toxicities	Grade 1	Grade 2	Grade 3	Grade 4
Acute Urinary Toxicity	Frequency of urination or nocturia twice pretreatment habit / dysuria, urgency not requiring medication	Frequency of urination or nocturia that is less frequent than every hour. Dysuria, urgency, bladder spasm requiring local anaesthetic (e.g., Pyridium)	Frequency with urgency and nocturia hourly or more frequent / dysuria, pelvis pain or bladder spasm requiring regular, frequent narcotic / gross haematuria with/without clot passage	Haematuria requiring transfusion / acute bladder obstruction not secondary to clot passage, ulceration, or necrosis
Late Urinary Toxicity	Slight epithelial atrophy; minor telangiectasia (microscopic haematuria)	Moderate frequency; generalised telangiectasia; intermittent macroscopic haematuria	Severe frequency & dysuria; severe telangiectasia (often with petechiae); frequent haematuria; reduction in bladder capacity (<150 cc)	Necrosis/contracted bladder (capacity < 100 cc); severe haemorrhagic cystitis
Acute Lower GI Toxicity	Increased frequency or change in quality of bowel habits not requiring medication, rectal discomfort not requiring analgesics	Diarrhoea requiring parasympatholytic drugs, mucous discharge not necessitating sanitary pads, rectal or abdominal pain requiring analgesics	Diarrhoea requiring parenteral support, severe mucous or blood discharge necessitating sanitary pads\abdominal distension (flat plate radiograph demonstrates distended bowel loops)	Acute or subacute obstruction, fistula, or perforation; gastrointestinal bleeding requiring transfusion; abdominal pain or tenesmus requiring tube decompression or bowel diversion
Late Lower GI Toxicity	Mild diarrhoea; mild cramping; bowel movement five times daily; slight rectal discharge or bleeding	Moderate diarrhoea and colic; bowel movement more than five times daily; excessive rectal mucus or intermittent bleeding	Obstruction or bleeding requiring surgery	Necrosis/perforation fistula