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Bladder Cancer Epidemiology in Latvia and Machine Learning-Based Evaluation of Urinary Exosomal microRNAs as Diagnostic Biomarkers

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Table of Contents

Abbreviations used in the Thesis 5

Introduction 7
Aim of the Thesis 9
Objectives of the Thesis 9
Hypotheses of the Thesis 9
Novelty of the Thesis 9
Author's contribution 10
Artificial intelligence (AI) Disclosure / Large language model (LLM) statement 10

1 Materials and methods 11
1.1 BC epidemiology in Latvia 11
1.1.1 Study group 11
1.1.2 Statistical methods 12
1.2 Urinary exosomal miRNAs biomarkers and their targets 12
1.2.1 Study Group and Inclusion Criteria 12
1.2.2 Clinical Data 14
1.2.3 Urine Sample Collection and Processing 15
1.2.4 Exosome Isolation and RNA Extraction 15
1.2.5 Reverse Transcription and Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) 15
1.2.6 RT-qPCR Data Analysis 16
1.2.7 miRNA Target and Pathway Analysis 16
1.2.8 Pilot Diagnostic Model 17
1.2.9 Machine Learning 17

2 Results 20
2.1 Incidence 20
2.2 Prevalence and years of potential life lost 21
2.3 Mortality 22
2.4 Survival 23
2.4.1 Relative survival 23
2.4.2 Overall survival (OS) and cancer specific survival (CSS) 23
2.5 Patient groups 24
2.6 Differentially expressed urinary exosomal miRNAs in bladder cancer patients 24
2.7 *TP53* and *PTEN* related exosomal miRNAs 30
2.8 Pathway enrichment analysis of exosomal miRNAs 31
2.9 Analysis of target genes of up-regulated miRNAs and their interactions 31
2.10 Enhancing bladder cancer diagnosis using machine learning on miRNA expression and clinical data 32

Conclusions 37

Proposals 38

List of publications, reports and patents on the topic of the Thesis 39

References 40

Abbreviations used in the Thesis

AAPC	Average Annual Percent Change
AI	Artificial Intelligent
AKT1	AKT serine/threonine kinase 1
APC	Annual Percent Change
ASR	Age-Standardised Rate
AUC	Area Under the Curve
BC	Bladder Cancer
cDNA	Complementary DNA
CI	Confidence Interval
CSS	Cancer-Specific Survival
CT	Cycle threshold
CTNNB1	Catenin Beta 1
CytoHubba	Cytoscape plugin for hub gene identification
CytoNCA	Cytoscape Network Centrality Analysis plugin
DCO	Death Certificate Only
DE	Differentially Expressed
EGFR	Epidermal Growth Factor Receptor
FDR	False Discovery Rate
FR	Fold Regulation
HG	High-Grade
ICD-10	International Classification of Diseases, 10th revision
LG	Low-Grade
LLM	Large Language Model
MCC	Maximal Clique Centrality
miEAA	miRNA Enrichment Analysis and Annotation
miRNA	MicroRNA
MNC	Maximum Neighbourhood Component

MNDR	Mammalian ncRNA-Disease Repository
mRNA	Messenger RNA
MTOR	Mechanistic target of rapamycin kinase
MYC	MYC proto-oncogene
NFKBIA	NFKB inhibitor alpha
OS	Overall Survival
PCR	Polymerase Chain Reaction
PTEN	Phosphatase and Tensin Homolog
RF	Random Forest
RNA	Ribonucleic Acid
ROC	Receiver Operating Characteristic
RS	Relative Survival
RT	Reverse Transcription
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
SD	Standard Deviation
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
SVM	Support Vector Machine
TNM	Tumour-Node-Metastasis
TP53	Tumour Protein p53
TURBT	Transurethral Resection of Bladder Tumour
UMAP	Uniform Manifold Approximation and Projection
UTR	Untranslated Region
WHO	World Health Organization
XGBoost	Extreme Gradient Boosting
YPLL	Years of Potential Life Lost

Introduction

Bladder cancer (BC) is a significant health challenge and is the tenth most common cancer worldwide (Bray et al., 2018). In approximately 30 % of cases, BC is diagnosed at a late stage when the tumour has already grown into the muscle layer. It is characterised by a high metastatic risk and, despite new treatment strategies, correlates with high mortality rates (Kaufman et al., 2009; Xie et al., 2017). Moreover, Latvia ranks second after Poland in terms of male mortality among other European countries (International Agency for Research on Cancer, 2022).

Effective diagnosis of bladder cancer is imperative for improving patient outcomes, yet the currently available non-invasive gold-standard methods for diagnosing and following-up BC are apparently are insufficient and have limited sensitivity. Urinary cytology has high sensitivity in the high-grade tumours including *carcinoma in situ* but may be negative in low- grade tumours (European Association of Urology [EAU], 2026), which can lead to missed diagnoses. In contrast, cystoscopy is a relatively expensive and invasive diagnostic method, despite the development of new technologies such as fluorescence and *narrow-band* imaging. Furthermore, it is not usually used in daily practice in patients without lower urinary tract symptoms or haematuria (Hong, 2016). *European guidelines* (EAU, 2026) consider the use of some specific biomarkers, but the main problem is that a number of noninvasive urine tests have failed to meet clinical expectations, because their diagnostic specificity is inferior to that of urinary cytology tests (Crocetto et al., 2022; EAU, 2026) and none of them has been accepted into routine practice by any clinical guidelines for primary diagnosis. Therefore, novel biomarkers for the diagnosis of BC need to be found and exosomal microRNAs (exomiRs) could fulfil this role.

Exosomes are small extracellular vesicles (EVs) ranging from 30 to 150 nm in diameter and produced by a variety of cells, including tumour cells, and found in a multitude of biological fluids (Mathieu et al., 2019; Wu et al., 2021). Although exosomes were recently considered to be a cellular waste bin, a growing number of studies now consider them to be important facilitators of intercellular communication and, thereby participants in the regulation of various biological processes, including cancer (Challagundla et al., 2015).

MiRNAs are small, non-coding ribonucleic acids (RNAs) that play an important role in tumourigenesis (Weber et al., 2010). They bind to the 3′-untranslated region (3′-UTR) of target messenger RNAs (mRNAs) and thus, post-transcriptionally regulate gene expression, which leads to degradation of the target mRNA or inhibition of its translation (Shukla et al., 2011). Notably, miRNAs are more stable than mRNAs in samples such as urine, making them less prone to degradation by nucleases due to their small size (Tilki et al., 2011; Weber et al., 2010).

Moreover, exosomal miRNAs are significantly more stable than plasma miRNAs and endure multiple freeze–thaw cycles, remaining practically indistinguishable from freshly obtained samples (Jacob et al., 2018). Given their dual roles in tumour promotion and suppression, miRNAs are regarded as promising biomarkers for cancer diagnosis and prognosis (Ling et al., 2016).

Multiple studies have sought miRNA-based biomarkers for bladder cancer, but results rarely overlap across cohorts and most miRNAs are reported in only one or two studies, with miR-96-5p as a notable exception (Grimaldi et al., 2022; Aveta et al., 2023). Despite this variability, a recent large mixed-cohort study developed a serum miRNA screening model using machine learning, achieving high accuracy for early bladder cancer detection (Lai et al., 2024), and similar pan-cancer work supports the diagnostic potential of circulating miRNAs (Wu et al., 2024). Beyond expression, miRNA editing events have been implicated in recurrent bladder cancer and may serve as prognostic or therapeutic targets (Qin et al., 2022).

Ongoing research aims to address the shortcomings of prior studies by incorporating machine learning and artificial intelligence. One effective approach is the evolutionary learning method CancerSig (Yerukala Sathipati et al., 2023), which couples the IBCGA feature-selection algorithm with a Support Vector Machine (SVM) classifier and a pan-cancer analysis of miRNA signatures to discriminate early- from advanced-stage disease across 15 cancer types with strong accuracy. Multimodal strategies have shown comparable promise in bladder cancer specifically: Moisoiu et al. combined urinary miRNA profiling with surface-enhanced Raman spectroscopy (SERS) in a liquid-biopsy setting, reporting improved point-of-care diagnosis and molecular stratification (Moisoiu et al., 2022). Building on this direction, the present study integrates urinary exosomal miRNA expression with demographic, laboratory and clinical data in a machine-learning model, hypothesising that fusing these data sources improves the diagnostic accuracy of non-invasive bladder cancer detection.

To improve understanding of the cell-to-cell communication of bladder cells in patients with low-grade (LG) and high-grade (HG) BC and the possible impact of urine exosomal miRNAs on the oncotransformation of normal cells, profiles of differentially expressed urine exosomal miRNAs were analysed. Striking differences of miRNA cargo in exosomes from the BC group as compared to the control group were identified. Further, miRNA expression levels were combined with laboratory data using advanced machine learning models and showed the potential of a multimodal approach to improve the accuracy of bladder cancer diagnosis.

Such an approach may enhance diagnostic performance and provide a broader view of tumour biology compared to single-site biopsy, thereby supporting more informed and timely clinical decision-making.

Aim of the Thesis

The aim of this study was to analyse epidemiological trends of bladder cancer in Latvia, and to evaluate the diagnostic potential of urinary exosomal miRNAs in combination with clinical and laboratory data using machine learning approaches, while also improving the understanding of cell-to-cell communication between bladder cells in patients with LG and HG bladder cancer and the potential role of urinary exosomal miRNAs in the oncotransformation of normal cells.

Objectives of the Thesis

1 To determine the dynamics of bladder cancer incidence, prevalence and mortality in Latvia from 1990 to 2017, overall and by age and sex groups.
2 To determine the dynamics of overall, cancer-specific and relative survival of bladder cancer patients in Latvia from 1990 to 2017.
3 To identify differentially expressed clinically significant urinary exosomal miRNA biomarkers and evaluate their potential molecular targets.
4 To apply machine learning approaches for the evaluation of urinary exosomal miRNAs in combination with clinical and laboratory data as a multimodal non-invasive diagnostic approach for bladder cancer.

Hypotheses of the Thesis

1 Between 1990 and 2017, bladder cancer incidence, prevalence, and mortality increased in Latvia, while survival outcomes improved and the proportion of advanced-stage bladder cancer cases decreased.
2 There is a significant difference in the expression of urinary exosomal miRNAs between bladder cancer patients and controls.
3 There is a significant difference in the expression levels of specific urinary exosomal miRNAs between patients with different tumour grades and stages.

Novelty of the Thesis

In this work, a large population-based analysis of bladder cancer in Latvia from 1990 to 2017 was performed. The following epidemiological indicators of BC were evaluated:

- Incidence, prevalence, mortality rates overall and by age groups;

- Relative, overall and cancer-specific survival, over the whole period and according to T-stage, including separate rates for early and advanced T-stages tumours, as well as by year of diagnosis.

These data complement and update the existing information on bladder cancer epidemiological trends in Latvia.

Differentially expressed urinary exosomal miRNAs were identified between the study and control groups in a Latvian population cohort, revealing potential associations with tumour-related molecular pathways and their possible involvement in cell-to-cell communication and oncotransformation processes.

Furthermore, this study integrated urinary exosomal miRNA expression profiles with clinical and laboratory data using machine learning approaches, demonstrating the potential of a multimodal non-invasive diagnostic framework for bladder cancer detection.

Author's contribution

The author was responsible for developing the study concept and planning, exosome isolation from urine, miRNA profiling, statistical data analysis, interpretation of results, and writing this dissertation. The author led the collection of urine samples and clinical data at the Pauls Stradiņš Clinical University Hospital in collaboration with colleagues from Riga East University Hospital, who assisted with sample collection and the acquisition of clinical data. The machine learning analysis was conducted in collaboration with researchers from Riga Technical University. This research was supported by the BioPhoT project LiqBio-BC: Liquid biopsy biomarkers for bladder cancer diagnostics (Project No. OSI_PIP_BioPhoT-2025/1-0038), the State Research Program in the field of biomedicine, medical technologies, and pharmacy (No. VPP-EM-BIOMEDICĪNA-2022/1-0001), the Latvian Council of Science (grant No. LZP-2020/2-0104), and an internal grant from Rīga Stradiņš University (No. RSU/PP/2020-07). The author assumes responsibility for the integrity of the work as a whole.

Artificial intelligence (AI) Disclosure / Large language model (LLM) statement

Given that the author is not a native English speaker, AI-based language models and editing tools were employed to refine the grammatical and stylistic aspects of this PhD Thesis. These technologies facilitated improvements in language use and readability but did not supplant essential authorial functions such as the generation of scientific or medical insights and the derivation of conclusions. The author has thoroughly reviewed and revised the manuscript as required, ensuring the veracity and integrity of the research presented.

1 Materials and methods

The study consists of two parts: 1) Description and analysis of BC epidemiology in Latvia; 2) Establishing the correlation between relative ratio of several specific miRNAs in urine exosomes and bladder cancer in Latvia using molecular profiling and machine learning. The research was conducted at the Oncology Institute of Rīga Stradiņš University. The first (epidemiological) part of the study was approved by the Ethics Committee for Clinical Research at the Development Society of Pauls Stradiņš Clinical University Hospital. The biomarker study was conducted in accordance with the Declaration of Helsinki and approved by the Central Medical Ethics Committee of Latvia (protocol No. 1/19-02-12, 12 February 2019, supplement No. 01-29.1.2/1352). Patient consent was not required for the BC epidemiology study in Latvia, as it used regularly collected anonymous electronic data. In the second part of the study all participants provided written informed consent.

1.1 BC epidemiology in Latvia

1.1.1 Study group

A retrospective cohort study was conducted using data from all patients diagnosed with bladder cancer (BC, n = 9589) categorised under the International Classification of Diseases (ICD-10, C67) between 1 January 1990 and 31 December 2017. The information was sourced from the Centre for Disease Prevention and Control (CDPC) of Latvia. A total of 13 158 patient records were received. After exclusion of 3569 (27.1 %) duplicate registrations, 9589 records of patients of all ages with confirmed BC were selected for analysis. BC patients were grouped and categorised in 20-year age bands within which age- specific incidence, prevalence, mortality and survival were analysed. Because patients younger than 40 years accounted for only 145 cases, they were excluded from the age-specific trend analysis due to statistical insufficiency, leaving the following analytical groups: 40–59, 60–79, and 80+ years. Cases registered only by death certificate (DCO) or diagnosed *post-mortem* (n = 392) were excluded from survival analyses.

In the stage-specific incidence and survival trend analysis were included following primary tumour stages: T1, T2, T3 and T4. Cancer-specific incidence, prevalence and mortality rates of the Latvian population were calculated as cases per 100 000 people. These calculations were derived from data sourced from the Central Statistical Bureau of Latvia (CSB) updated in 2018 (following the latest Population and Housing Census in 2011), which provided information on changes in the number and age structure of the Latvian population, as well as the annual total mortality rates, available on the website http://www.csb.gov.lv/dati/statistikas-datubazes-28270.html (Central Statistical Bureau of Latvia, 2011).

Cancer rates were subsequently age-adjusted to the World Health Organisation (WHO) standard population in 2000 (Ahmad et al., 2001). To calculate the relative survival rate, life expectancy table for the Latvian population was downloaded from the Human Mortality Database (CSB of Latvia).

1.1.2 Statistical methods

All data analyses were performed using SPSS (version 22.0, IBM Corp., USA). Descriptive statistics are presented as frequencies and percentages for categorical variables, and as mean ± standard deviation for continuous variables. To avoid year-to-year fluctuations, age and survival comparisons were grouped into three-year periods. The Mann-Whitney U test was used for quantitative data and chi-square test for qualitative variables.

Trend analysis was performed using the Joinpoint Regression Analysis programme (version 4.6.0.0, April 2018, Surveillance Research Program, US National Cancer Institute). Joinpoint software identifies the number and location of statistically significant trend changes (joinpoints). Log-transformation was applied to age-standardised rates (ASR) for male, female and both, and for all age groups. The Monte Carlo permutation method was used to select the optimal number of joinpoints (range: 0–4; significance level $p = 0.05$; 4,499 randomly permuted datasets) (Kim et al., 2000). The annual percentage change (APC) and average APC (AAPC) were calculated for each linear segment; a trend was considered statistically significant when $p < 0.05$.

For survival analysis, a cohort of BC patients with at least a 5-year follow-up was selected, and patients were stratified into seven time periods. Relative survival (RS) was calculated using the R statistical programme (version 4.0.3, R Foundation for Statistical Computing, Austria) and the 'relsurv' package. Expected survival was derived from life expectancy tables for the Latvian population (Human Mortality Database, Central Statistical Bureau of Latvia). The five-year RS for 1990–2012 data was calculated by cohort analysis using the Ederer I method; for 2013–2017 data, the Ederer II method was applied. Overall survival (OS) and cancer-specific survival (CSS) were analysed using Kaplan–Meier curves with Log-rank test, with follow-up from date of diagnosis until death, censoring, or 31 December 2017.

1.2. Urinary exosomal miRNAs biomarkers and their targets

1.2.1 Study Group and Inclusion Criteria

From 2019 to 2024, urine samples were prospectively collected from bladder cancer patients and control subjects at Pauls Stradiņš Clinical University Hospital and Riga East University Hospital. Participants were categorised into three groups: LG BC (LG, histologically

confirmed Ta or T1 tumour), HG BC (HG, T2 tumour), and healthy controls. Staging and grading were performed according to the TNM classification system and 2022 WHO criteria. Patients without a confirmed pathohistological diagnosis of bladder cancer were excluded from the analysis.

Table 1.1

Inclusion and exclusion criteria for the study

Criterion	Bladder Cancer Group	Control Group
Inclusion	Age ≥ 50 years; pathohistologically proven primary or recurrent bladder cancer after TURBT or cystectomy; without previously received systemic chemotherapy; signed informed consent; available medical records	Age-matched; no history of malignancy; urinalysis without signs of inflammation or infection; no significant blood test abnormalities; signed informed consent; available medical records
Exclusion	Any other oncological disease or previous upper urinary tract cancer; diabetes mellitus; inflammatory or autoimmune disease; non-urinary tract infection	Any other oncological disease or previous upper urinary tract cancer; diabetes mellitus; inflammatory or autoimmune disease; non-urinary tract infection

The discovery miRNA profiling cohort consisted of 7 healthy controls, 6 LG patients and 6 HG patients. The differential expression analysis of urinary exosomal miRNAs was performed on an initial cohort of 210 participants (111 BC patients and 99 controls). For the machine learning analysis, only participants with complete clinical data were retained, resulting in a final analytical cohort of 152 participants (89 BC patients and 63 controls); all three machine learning configurations were trained and evaluated on this 152-participant cohort. (Figure 1.1)

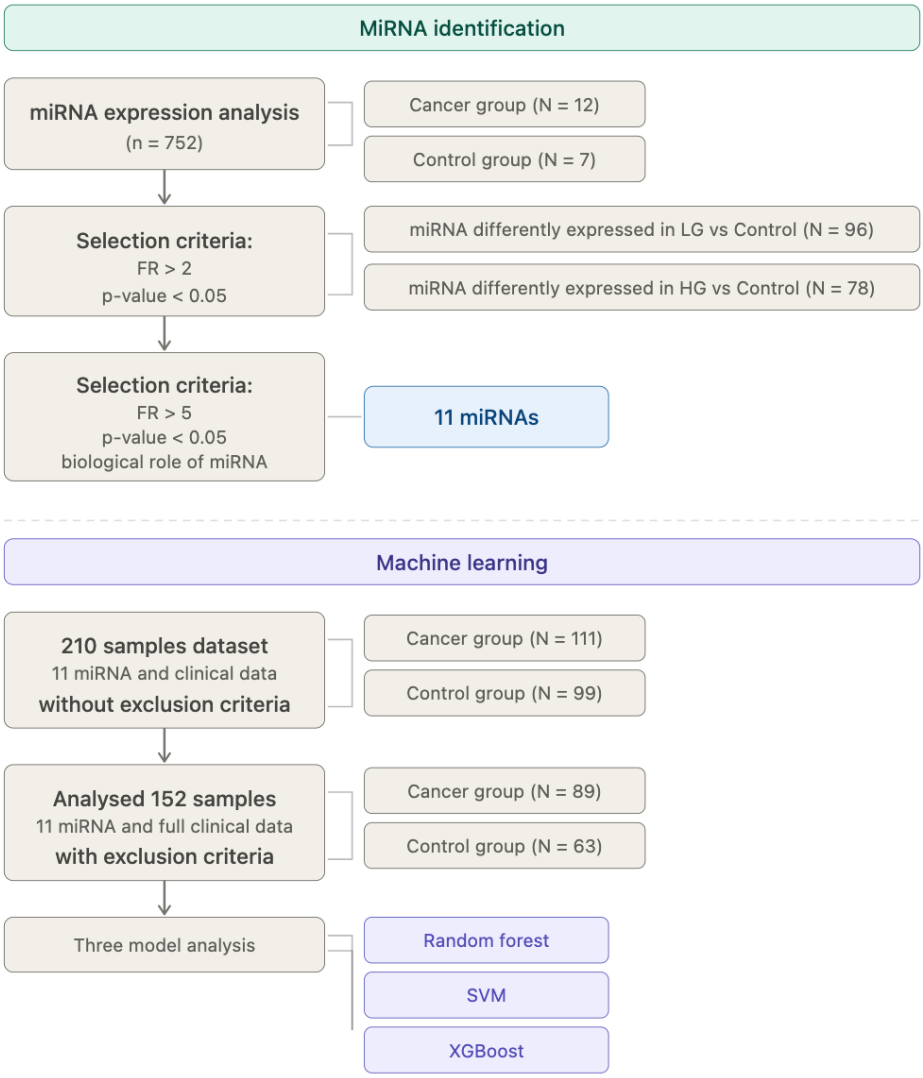


Figure 1.1 **Flowchart of data analysis**

FR – fold regulation; SVM – support vector machine; XGBoost – extreme gradient boosting.

1.2.2 Clinical Data

Clinical and demographic data were prospectively collected from patients' medical records and entered into a dedicated database. The database included: clinicopathological characteristics; surgical treatment received; chemotherapy; laboratory test results; family history; and smoking and occupational exposure history. For Ta and T1 stage patients, the European Organisation for Research and Treatment of Cancer (EORTC) risk calculator was applied to predict recurrence and progression. A non-invasive urinary ultrasound was offered to control group participants before enrolment. Urine samples for urinalysis, cytology and miRNA profiling were obtained from all BC patients before surgery; control group urine samples were collected for urinalysis and miRNA profiling only.

1.2.3 Urine Sample Collection and Processing

Spontaneous voided urine samples were collected from all participants. If immediate processing was not possible, samples were stored at 4 °C for up to 24 hours. Samples were centrifuged at 3.0 rcf (4.4 rpm) for 5 minutes 30 seconds at room temperature to remove cellular debris. The resulting supernatant was aliquoted and stored at −80 °C until analysis.

1.2.4 Exosome Isolation and RNA Extraction

Exosome isolation was performed according to the miRCURY Exosome Cell/Urine/CSF Kit protocol (Qiagen, Hilden, Germany; Cat. No. 76743). Stored samples were thawed on ice and subjected to a second centrifugation at 10,000 × g for 10 minutes at room temperature to remove residual cellular debris. Exosomes were then isolated from 1,960 µL of urine supernatant divided into two tubes.

Total RNA including miRNA was extracted from urinary exosomes using the miRNeasy Mini Kit (Qiagen, Hilden, Germany; Cat. No. 17004) according to the manufacturer's protocol. Each sample was spiked with a synthetic UniSp2, UniSp4, and UniSp5 RNA mixture (Qiagen, USA; Cat. No. 339390) for isolation quality control. Purified RNA was eluted in a single step with 50 µL of RNase-free water.

1.2.5 Reverse Transcription and Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Reverse transcription and polyadenylation of RNA to complementary DNA (cDNA) was performed using the miRCURY LNA RT Kit (Qiagen, USA; Cat. No. 339340) with a UniSp6 spike-in for process quality control. Incubation was carried out using the peqSTAR thermal cycler: 42 °C for 60 minutes, 95 °C for 5 minutes, held at 4 °C. An increased RNA template volume of 4 µL (instead of the standard 2 µL) was used to improve sensitivity.

For the discovery phase, genome-wide miRNA profiling was performed using the miRCURY LNA miRNA miRNome Pre-designed PCR Panels (Qiagen; Cat. No. 339322). For the machine learning phase, a custom pre-designed panel (miRCURY LNA miRNA Custom PCR Panel YCA34464; Qiagen; Cat. No. 339330/339332) targeting 11 candidate miRNAs (see Table 1.2) was used. Both phases employed the miRCURY LNA SYBR Green PCR Kit (Qiagen, USA; Cat. No. 339347). RT-qPCR was performed on the Applied Biosystems ViiA 7 Real-Time PCR System. Automatic threshold and baseline settings were applied to record CT values for all miRNAs.

Table 1.2

Configuration of miRCURY LNA ™ miRNA Custom PCR Panel YCA34464

Position	Assay Name
A01-A24	UniSp6
B01-B24	UniSp3
C01-C24	UniSp2
D01-D24	UniSp5
E01-E24	hsa-miR-30c-5p
F01-F24	hsa-miR-126-3p
G01-G24	hsa-miR-1260a
H01-H24	hsa-miR-20a-3p
I01-I24	hsa-miR-28-3p
J01-J24	hsa-miR-28-5p
K01-K24	hsa-miR-30b-3p
L01-L24	hsa-miR-324-5p
M01-M24	hsa-miR-339-5p
N01-N24	hsa-miR-429
O01-O24	hsa-miR-455-3p
P01-P24	hsa-miR-502-5p

1.2.6 RT-qPCR Data Analysis

To analyse the data obtained from miRNA panels, the miRCURY miRNA PCR Data Analysis programme (version 1.0) was used. Data normalisation was performed using the global mean normalisation method for discovery phase analysis. Differences in miRNA expression levels between control and cancer groups were assessed by calculating p-values based on a parametric Student's t-test of replicate values of 2 ^ (−ΔCT) (two-sample equal variance, unpaired, two-tailed distribution). GeNorm, integrated within the miRCURY analysis software, was used to identify the most stable internal control miRNAs. The minimum sample size for the miRNA panel was calculated based on Lee & Whitmore (2002): statistical power 90 %, two-fold difference detection across ~700 miRNAs, significance level $\alpha = 0.05$; minimum n = 5 per group.

Differentially expressed miRNAs in LG and HG groups were compared to the control group and filtered by fold change and p-value threshold.

1.2.7 miRNA Target and Pathway Analysis

The list of the statistically significant, differentially expressed miRNAs was submitted to the miEAA tool (miRNA Enrichment Analysis and Annotation) for miRNA enrichment analysis (Kern et al., 2020). The results were filtered by the false discovery rate (FDR) and the subcategories capturing the top 5 highest numbers of observed miRNAs were selected. Prediction of the miRNA targets was performed by miRTargetLink 2.0, applying strong validation criteria (Kern et al., 2021). Protein–protein interaction networks of predicted target genes were constructed and visualised using STRING (Szklarczyk et al., 2021). Hub gene

analysis was performed in Cytoscape (Shannon et al., 2003) using Cytoscape Network Centrality Analysis plugin (CytoNCA) and Cytoscape plugin for hub gene identification (CytoHubba) (Tang et al., 2015) with the following centrality measures: degree, betweenness, closeness and eigenvector (CytoNCA); Edge Percolated Component (EPC), Maximal Clique Centrality (MCC), Maximum Neighbourhood Component (MNC) and stress (CytoHubba).

1.2.8 Pilot Diagnostic Model

Prior to the main machine learning analysis, a pilot neural network model (caret package, R) was constructed on 23 controls and 32 BC patients using 9 differentially expressed (DE) miRNAs (fold change > 3 in either LG or HG; $p < 0.05$) and 3 reference miRNAs (expressed equally in BC patients and controls by using NormFinder. Delta CT values (CT of normalisation miRNA − CT of target miRNA) were calculated for each miRNA. The combination of six DE miRNAs (miR-429, miR-30b-3p, miR-126-3p, miR-28-5p, miR-324-5p and miR-455-3p), normalised to miR-1260a, yielded an accuracy of 0.889 (95 % CI 0.653–0.986), sensitivity of 0.800 and specificity of 1.000 ($p = 0.003$). This panel was therefore taken forward into the full machine learning analysis.

1.2.9 Machine Learning

Dataset and Features

The machine learning dataset comprised miRNA expression levels for 11 candidate miRNAs across an initial cohort of 210 participants (111 BC patients, 99 controls) for the differential expression analysis. For the machine learning analysis, only participants with complete clinical data were retained, resulting in a final analytical cohort of 152 participants (89 BC patients, 63 controls); all three machine learning configurations were trained and evaluated on this 152-participant cohort. The combined feature set comprised 24 variables: 11 miRNA expression levels and 13 clinical/demographic variables.

The 13 clinical variables used in modelling were:

- Demographic: age (years), sex, weight (kg), height (cm);
- Urinalysis: erythrocyturia, leukocyturia, nitrite test (dipstick);
- Risk factors: smoking history (current/former), aromatic amine exposure;
- Symptoms/history: dysuria, chronic urinary tract infection, family oncological history
- and an exclusion criteria indicator as a binary covariate.

Missing blood creatinine values were imputed using the mean value of the respective group (cancer or control); advanced imputation methods were considered but not applied due to the risk of overfitting in smaller datasets.

Machine Learning Algorithms and Hyperparameter Optimisation

Three complementary machine learning classifiers were evaluated, each with distinct methodological strengths suited to biomedical data of moderate size:

- Random Forest (RF): an ensemble of decision trees using bootstrap aggregation (bagging) to reduce overfitting; captures non-linear interactions and provides inherent feature importance ranking.
- Support Vector Machine (SVM): identifies the optimal hyperplane maximising the margin between classes; effective with high-dimensional data and smaller sample sizes due to its focus on the most informative support vectors, making it less prone to overfitting.
- XGBoost (Extreme Gradient Boosting): builds an ensemble of decision trees sequentially, with each tree correcting errors of the previous; handles missing values natively and achieves high accuracy through gradient-based optimisation.

To maximise model performance, hyperparameter tuning was performed, focusing on the following parameters: n_estimators and max_depth for Random Forest and XGBoost; C and gamma for SVM. For n_estimators, ranges from 5 to 105 were tested in increments of 20 during the initial coarse search and then refined to 80–90 in increments of 1 for fine-tuning. For max_depth, ranges from 3 to 50 were tested in increments of 10 during the initial search and then refined to 3–10 in increments of 1. In SVM, the ranges were as follows: C [0.1..10], gamma [0.001..1].

Feature Importance and Dimensionality Reduction

Feature importance was assessed for each classifier to identify the most discriminative variables. For the Random Forest, the mean decrease in Gini impurity was used. For the SVM, feature weights derived from the linear kernel coefficients were applied. For XGBoost, gain-based feature importance scores were calculated. Results were compared across all three classifiers to identify consistently important predictors.

To visualise the separability of cancer and control groups in a two-dimensional space, Uniform Manifold Approximation and Projection (UMAP) dimensionality reduction was applied to the combined dataset, using the Jaccard distance metric . UMAP preserves both local and global data structure and was used as a supplementary visual confirmation of classifier performance.

All machine learning analyses were conducted in Python. All AI-generated contributions were thoroughly reviewed and approved by medical experts to ensure accuracy and conformity to scientific standards.

Model Evaluation and Validation

Model performance for each hyperparameter combination was evaluated by Leave-One-Out Cross-Validation (LOOCV), implemented with parallel processing.

To ensure result stability and independence from random initialisation, each model was trained and evaluated 20 times using different random seeds. Performance metrics were averaged across all 20 repetitions, confirming that model performance was not dependent on any particular random seed. The following performance metrics were calculated: accuracy; sensitivity (Recall); specificity; F1-Score; Area Under the Curve (AUC)

Three model configurations were evaluated for each classifier on the same 152-participant cohort: (1) miRNA expression data only; (2) clinical and demographic data only; (3) combined miRNA + clinical data.

2 Results

Between 1990 and 2017, 9589 patients with newly diagnosed BC were identified (75 % male, 25 % female; gender ratio 3:1). The mean age at diagnosis was 68.8 years (SD 11.5; males 67.8, females 72.0) and increased significantly over the study period from 67.6 to 71.2 years ($p < 0.001$), driven mainly by the male subgroup. Of 7182 patients who died during the review period, 67 % (n = 4790) had BC-related deaths; mean age at death increased from 71.3 (SD 11.0) to 76.0 (SD 9.4) years.

2.1 Incidence

The age-standardised incidence rate (ASR, per 100 000) of BC increased from 6.8 in 1990 to 10.8 in 2017 (males 15.1→21.5; females 1.9→3.9), with a statistically significant APC of 2.6 % from 1990 to 2014 and a non-significant decline of −3.6 % thereafter (Table 2.1). Significant increases were observed in both sexes and in the 80+ age group (APC 2.6 %). The T1-stage subgroup showed the largest APC (+8.0 %, joinpoint in 2013), while T3-stage incidence significantly decreased (APC −2.3 % from 1995 to 2017).

Table 2.1

Bladder cancer incidence and mortality trends in Latvia 1990–2017
(joinpoint analysis: age-standardized, by age, gender and stage groups)

	Trend 1		Trend 2		AAPC (95 % CI)
	Years	**APC**	**Years**	**APC**	**1990–2017**
Incidence	1990–2014	2.6*	2014–2017	−3.6	1.9* (0.9 to 2.9)
Gender					
Male	1990–2017	2.1*			2.1* (2.5 to 10.1)
Female	1990–2017	2.7*			2.7* (2.0 to 3.5)
Age group, years					
40–59	1990–2017	1.7*			1.7* (0.8 to 2.5)
60–79	1990–2017	2.5*			2.5* (2.1 to 2.8)
80+	1990–2017	2.6*			2.6* (2.0 to 3.1)
T stage					
1	1990–2013	9.7*	2013–2017	−1.3	8.0* (6.9 to 9.7)
2	1990–2017	0.4			0.4 (−0.3 to 1.1)
3	1990–1995	6.9	1995–2017	−2.3*	−0.7 (−2.2 to 0.9)
4	1990–1995	1.3*			1.3* (0.2 to 2.5)

Table 2.1 continued

	Trend 1		Trend 2		AAPC (95 % CI)
	Years	**APC**	**Years**	**APC**	**1990–2017**
Mortality	1990–2017	0.5*			0.5*(0.1 to 0.9)
Gender					
Male	1990–2017	0.5*			0.5* (0.1 to 1.0)
Female	1990–2017	0.2			0.2 (−0.8 to 1.3)
Age group, years					
40–59	1990–2017	−1.1*			−1.1* (−2.2 to –0.1)
60–79		0.7*			0.7* (0.2 to 1.3)
80+		1.9*			1.9* (1.2 to 2.5)

APC, annual percent change; AAPC, average annual percent change; ASR, age standardised rate per 100 000.

*p < 0.05

2.2 Prevalence and years of potential life lost

At the beginning of the period under review, the number of prevalent BC cases was 969; by 2017, this had nearly tripled to 2,834 cases. When the largest numbers of patients by age group were counted during the reference period, in 2017 they were in the 80+ age group with 720 cases (25 %) and in the 60–79 age group with 1689 cases (60 %). Dividing patients into gender groups, males comprised 72 % (n = 2,030) and females 28 % (n = 804) of prevalent cases in 2017.

The standardised prevalence rates (per 100 000) increased from 25.2 in 1990 to 69.7 in 2017 with AAPC 3.8 % (95 % CI 3.1 to 4.6) during the whole period, and 3 join points were observed in 1999, 2002 and 2015 (Figure 2.1). The prevalence of BC in 1990 was 25.2 (per 100 000 inhabitants per year), which is the lowest value over the considered time period. A statistically significant increase in prevalence was observed between 1990 and 1999 (APC 4.6) reaching a prevalence of 38.1 (per 100 000 inhabitants per year) in 1999. Subsequently, no statistically significant increase in prevalence was observed between 1999 and 2002. From 2002 until 2015, a statistically significant increase in prevalence was noted reaching a prevalence of 68.6 (per 100 000 inhabitants per year) in 2015 with an APC of 4.3. After that no statistically significant increase was observed until 2017 (Figure 2.1).

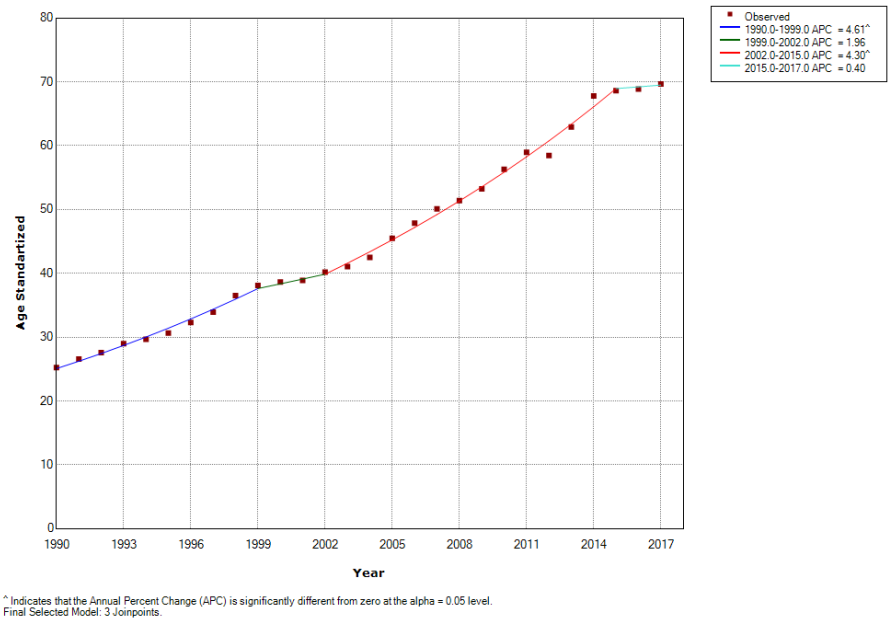


Figure 2.1 **Bladder cancer prevalence trends using joinpoint analysis**

Both genders experienced an increase (per 100 000) in the standardised prevalence (men, 53.4 in 1990 to 132.5 in 2017 versus women, 8.9 in 1990 versus 29.2 in 2017) throughout the study period. Only in the 80+ year-old age group were observed statistically significant reduction with APC −7.6 % from 2013 to 2017

The total number of years of potential life lost (YPLL) due to BC in Latvia over 28 years was 7450. Comparing the YPLL by periods, 959 years (12.1 per 100 000 general population) were lost between 1990 and 1992, and then decreased to 474 years (7.9 per 100 000) between 2015 and 2017. Every person who died due to BC in the beginning of the period, lost an average of 8 years of life, while in the end of the period, this factor fell to 6.5 years.

2.3 Mortality

The annual number of deaths among BC patients rose from 186 in 1990 to 360 in 2017. Cancer-specific deaths increased from 155 to 208 per year over the same period. Standardised overall mortality rates (per 100 000) also increased from 4.6 in 1990 to 7.0 in 2017.

Age-standardised overall mortality rates (per 100 000) also increased from 4.6 in 1990 to 7.0 in 2017. Females and the 40–59-year age group was the only one showing a statistically insignificant minimal increase in the mortality. (Table 2.1).

Overall cancer-specific age-standardised BC mortality rates (per 100 000) rose from 3.9 (8.5 for men, 1.3 for women) in 1990 to 4.4 (9.7 for men, 1.2 for women) in 2017. These rates slightly increased, with APC 0.5 % (Table 2.1) and statistically significantly decreased in the 40–59 year age group, with an APC of −1.1 % from 1990 to 2017. However, BC mortality rates significantly increased in the age group > 80 years (APC 1.9 %, 95 % CI 1.2 to 2.5) (Table 2.1)

2.4 Survival

2.4.1 Relative survival

Over the whole period, the 5-year relative survival (RS) was 59.9 % (95 % CI 58.4 to 61.3. A statistically significant improvement was observed across the three study periods: 55.0 % (1990–2000), 61.9 % (2001–2012), and 59.0 % (2013–2017; p = 0.006).

Age-group analysis revealed a clear inverse relationship between age and survival. In the 40–59-year age group, 5-year RS improved continuously across all periods, reaching 76.9 % (95 % CI: 69.4–85.2) in 2013–2017. In the 80+ group, survival remained consistently the poorest, at 43.4–50.2 % across periods.

Gender-stratified survival analysis showed that women consistently demonstrated higher 5-year RS than men, although the difference was not statistically significant (all periods p > 0.05).

The 5-year RS in the T2 and T3 stage groups has significantly deteriorated compared to 1990–2000 and 2001–2012 and 2013–2017 (Figure 2.2).

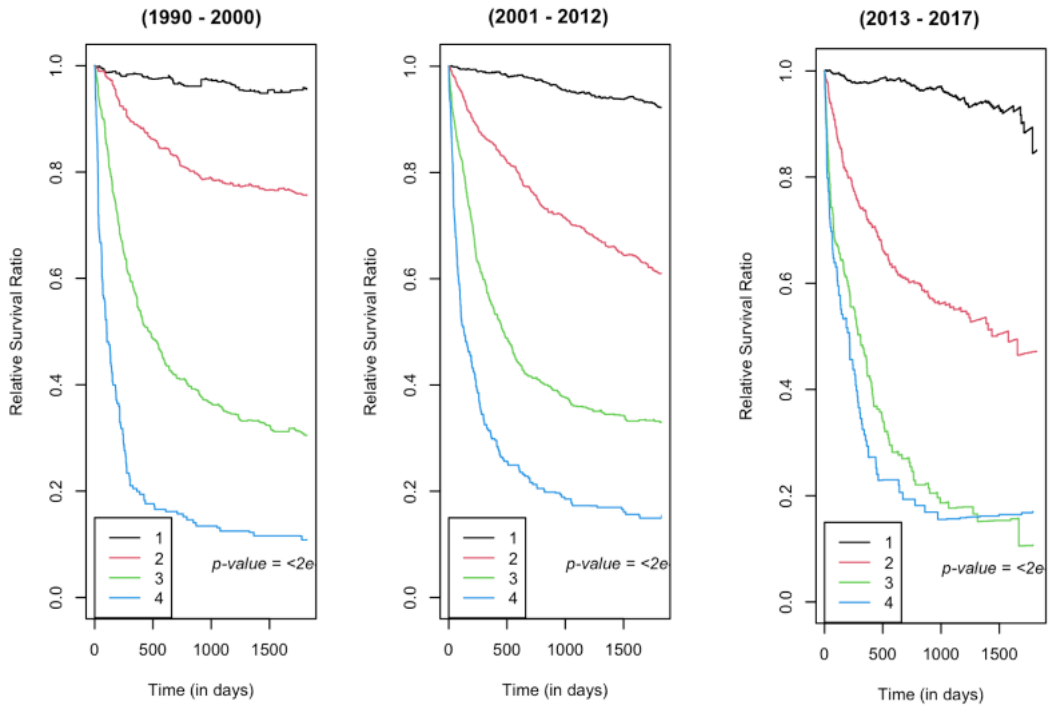


Figure 2.2 **T stage-specific 5-year relative survival of bladder cancer by three time periods**

2.4.2 Overall survival (OS) and cancer specific survival (CSS)

Over the whole period, the median CSS was 8.45 years (95 % CI, 7.562–9.348).

The five- and ten-year OS rates were the lowest in the first analysis period following bladder cancer diagnosis: 38.5 % (95 % CI 35.3–41.8) between 1996 and 1998 and 24.1 % (95 % CI 21.2–27.0). Five- and ten-year OS rates improved during the assessment period, until

the five-year OS rate reached 48.6 % (95 % CI 45.6–51.6) between 2008 and 2010 and ten-year OS reached 31 % (95 % CI 28.2–34.0) between 2005–2007.

The lowest survival rates for cancer-specific 5 and 10 years were 47.2 % (95 % CI 43.4–51.0) and 41.3 % (95.5 % CI 37.5–45.1) for the period that was considered at the beginning of the period from 1990 to 1992. The survival rates for five and ten years of cancer have improved throughout the period studied, until the five year CSS reached 62.0 % (95 % CI 58.9–65.0) between 2008 and 2010 and ten-year CSS reached 52.4 % (95 % CI 48.8; 56.0) between 2005 and 2007.

When considering OS and CSS by gender, the five-year survival improved in all gender groups. At the end of the reference period, women had better survival than men, which is in stark contrast to the initial period ($p < 0.05$). (Table 2.2).

Table 2.2

5-year survival rates in different gender groups

Survival rates		1990–1992	1993–1995	1996–1998	1999–2001	2002–2004	2005–2007	2008–2010
Men	OS	42.3 (38.2 to 46.5)	40.1* (36.0 to 44.2)	37.7 (33.9 to 41.4)	38.8* (35.0 to 42.6)	39.3 (35.7 to 43.0)	45.2* (41.7 to 48.7)	48.3 (44.9 to 51.7)
	CSS	48.5 (44.3. to 52.8)	46.8* (42.5 to 51.0)	48.7 (44.7 to 52.8)	52.6* (48.5 to 56.6)	53.0* (49.1 to 57.0)	58.9 (55.2 to 62.5)	61.7 (58.2 to 65.2)
Female	OS	37.8 (30.0 to 45.5)	46.4* (39.2 to 53.7)	41.0 (34.5 to 47.6)	43.3* (36.7 to 49.9)	47.4 (41.0 to 53.8)	53.8* (47.6 to 60.0)	49.8 (43.5 to 56.1)
	CSS	42.2 (34.1 to 50.2)	52.5* (45.1 to 60.0)	48.6 (41.8 to 55.4)	55.1* (48.2 to 61.9)	61.5* (55.0 to 68.0)	62.5 (56.3 to 68.7)	62.8 (56.3 to 69.2)

2.5 Patient groups

For urinary exosomal miRNA analysis, samples were collected from 19 participants: 6 patients with LG BC (LG, ≤ pT1; all men, median age 71.5 years), 6 patients with HG BC (HG, ≥ pT2; 2 men, 4 women, median age 71.5 years), and 7 healthy control subjects (6 men, 1 woman, median age 66 years). Staging and grading were performed according to the TNM classification system and the 2022 WHO criteria.

2.6 Differentially expressed urinary exosomal miRNAs in bladder cancer patients

After comparing the overall profiles of exosomal miRNAs in the urine samples of cancer patients, miRNAs presented differently with more than 2-fold regulation (FR) or less than -2-FR were analysed compared to the controls. The number of miRNAs corresponding to these cut-off values was 96 miRNAs in the LG group (93 up-regulated and 3 down-regulated) and 78 in the HG group (72 up-regulated and 6 down-regulated), respectively. (Tables 2.3 and 2.4;

Figures 2.3 and 2.4). Among these miRNAs presented differently in the exosome, 42 miRNAs were found in both LG and HG groups, and these miRNAs were all up-regulated in cancer patients.

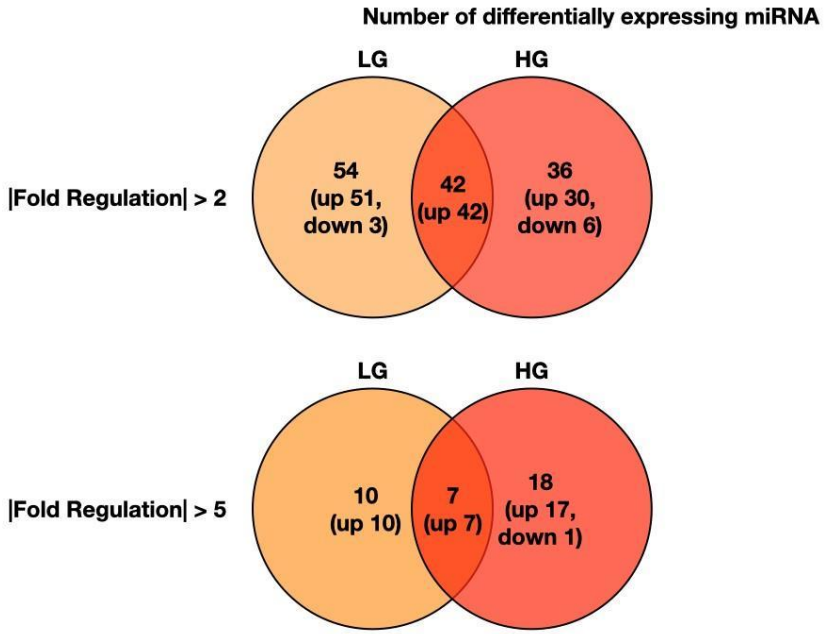


Figure 2.3 **Number of differentially expressing miRNA in low grade and high grade groups**

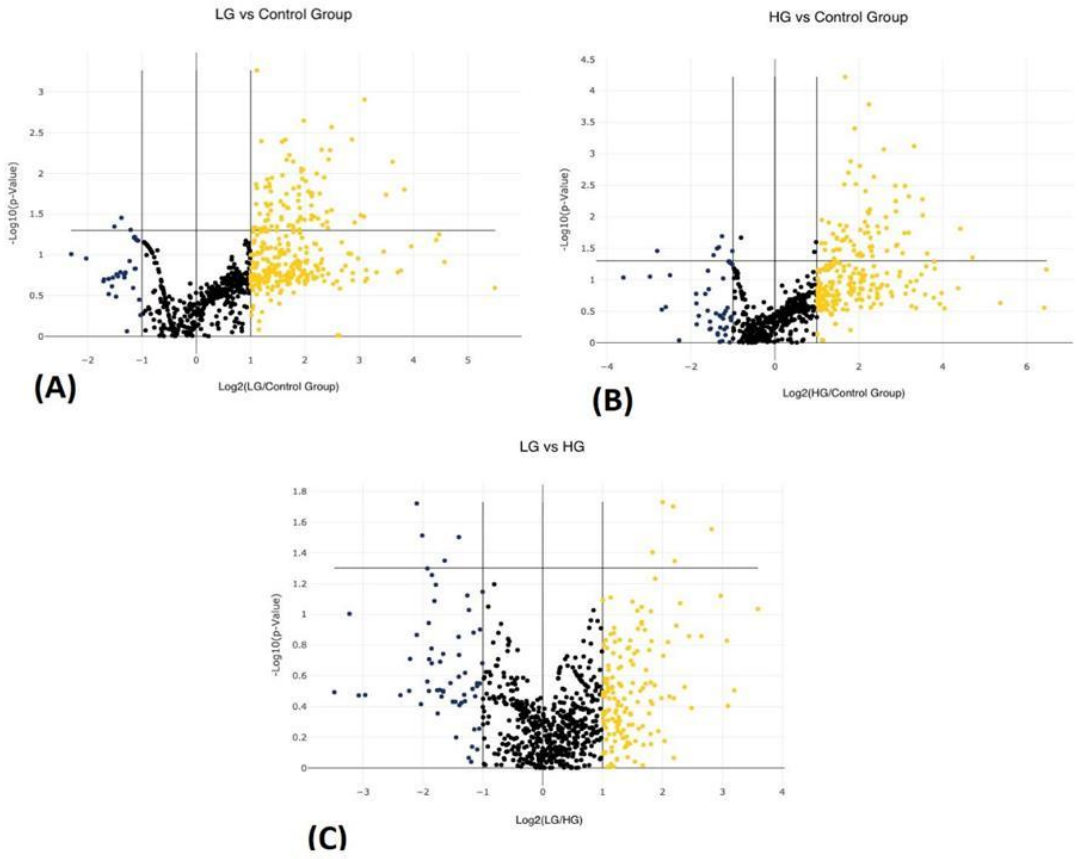


Figure 2.4 **Volcano plots of comparison. (A) wild type(wt) low grade to control group, (B) wt high grade to control group, (C) wt low grade to high grade group**

Table 2.3

Differentially expressed urine exosomal miRNAs and corresponding target genes in LG bladder cancer patients group

miRNA	Fold regulation	p-value	Target gene
Up-regulated			
hsa-miR-21-3p	14.25	0.016	*PTEN*
hsa-miR-324-5p	12.24	0.007	
hsa-miR-31-3p	11.27	0.018	
hsa-miR-30b-3p	8.57	0.001	
hsa-miR-188-5p	8.50	0.034	
hsa-miR-769-5p	8.15	0.033	
hsa-miR-132-3p	7.55	0.040	*EGFR*
hsa-miR-628-3p	7.30	0.004	
hsa-miR-126-3p	5.62	0.003	*AKT1*
hsa-miR-221-3p	5.62	0.028	*PTEN*
hsa-miR-455-3p	5.51	0.005	
hsa-miR-34a-3p	5.42	0.007	*AKT1, CTNNB1, MYC*
has-let-7f-2-3p	5.37	0.047	
hsa-miR-28-5p	5.30	0.039	*TP53*
hsa-miR-210-3p	5.29	0030	
hsa-miR-20a-3p	5.11	0.011	*PTEN, MYC*
hsa-miR-10b-3p	5.01	0.043	
hsa-miR-196b-3p	4.94	0.005	
hsa-miR-652-3p	4.84	0.018	

Table 2.3 continued

miRNA	Fold regulation	p-value	Target gene
Up-regulated			
hsa-miR-582-5p	4.73	0.036	
hsa-miR-15b-5p	4.64	0.010	
hsa-miR-27b-3p	4.49	0.034	
hsa-miR-29a-5p	4.39	0.018	
hsa-miR-193b-5p	4.36	0.019	
hsa-miR-301a-3p	4.36	0.033	*PTEN*
hsa-miR-450a-5p	4.35	0.018	
hsa-miR-29c-5p	4.34	0.025	
hsa-miR-135b-5p	4.33	0.037	
hsa-miR-21-5p	4.32	0.018	*PTEN, EGFR*
hsa-miR-152-3p	4.18	0.017	*PTEN*
hsa-miR-16-5p	4.05	0.012	*TP53*
hsa-miR-200a-5p	3.99	0.037	*PTEN, CTNNB1*
hsa-miR-320b	3.94	0.002	
hsa-miR-151a-3p	3.90	0.009	
hsa-miR-20b-3p	3.87	0.012	
hsa-miR-18a-5p	3.82	0.009	*PTEN*
hsa-miR-425-5p	3.82	0.009	*PTEN*
hsa-miR-148b-3p	3.78	0.034	
hsa-miR-31-5p	3.75	0.036	
hsa-miR-15a-5p	3.74	0.027	
hsa-miR-129-5p	3.73	0.049	
hsa-miR-27a-3p	3.71	0.015	
hsa-miR-376c-3p	3.71	0.018	
hsa-miR-101-3p	3.67	0.022	
hsa-miR-197-3p	3.65	0.039	
has-let-7e-3p	3.46	0.015	
hsa-miR-24-2-5p	3.46	0.049	
hsa-miR-181a-3p	3.45	0.044	
hsa-miR-664a-3p	3.44	0.007	
hsa-miR-106b-3p	3.30	0.043	*PTEN*
hsa-miR-23a-3p	3.28	0.006	
hsa-miR-331-3p	3.24	0.044	
hsa-miR-185-5p	3.23	0.027	*AKT1*
hsa-miR-99b-5p	3.18	0.026	
hsa-miR-28-3p	3.17	0.007	*TP53*
hsa-miR-501-3p	3.16	0.018	
hsa-miR-191-5p	3.12	0.004	
hsa-miR-502-3p	3.04	0.024	
hsa-miR-151a-5p	3.00	0.011	
hsa-miR-378a-3p	2.99	0.004	
hsa-miR-200a-3p	2.97	0.034	*PTEN, CTNNB1, EGFR*
hsa-miR-324-3p	2.97	0.037	
hsa-miR-532-3p	2.93	0.022	
hsa-miR-500a	2.80	0.045	
hsa-miR-222-3p	2.80	0.050	*PTEN*
hsa-miR-141-3p	2.62	0.011	*PTEN*
hsa-miR-24-3p	2.61	0.045	*TP53, MYC*
hsa-miR-19a-3p	2.60	0.024	*PTEN*
hsa-miR-22-3p	2.59	0.017	*AKT1*
hsa-miR-25-3p	2.59	0.028	*TP53*

Table 2.3 continued

miRNA	Fold regulation	p-value	Target gene
Up-regulated			
hsa-miR-200c-3p	2.55	0.014	*PTEN*
hsa-miR-200b-3p	2.54	0.013	
hsa-miR-125a-5p	2.51	0.050	*AKT1*
hsa-miR-320c	2.50	0032	
hsa-miR-423-5p	2.50	0.037	
hsa-miR-33a-5p	2.48	0.025	*MYC*
hsa-miR-429	2.47	0.012	*PTEN*
hsa-miR-320d	2.43	0.023	
hsa-miR-26b-5p	2.43	0.038	*PTEN*
hsa-miR-181a-5p	2.42	0.015	*PTEN*
hsa-miR-19b-3p	2.42	0.038	*TP53*
hsa-miR-320a	2.40	0.046	*PTEN*
hsa-miR-424-5p	2.38	0.046	
hsa-miR-10b-5p	2.38	0.047	*TP53*
hsa-miR-203a-3p	2.29	0.004	
hsa-miR-107	2.26	0.048	
hsa-miR-374b-5p	2.24	0.031	
hsa-miR-934	2.17	0.001	
hsa-miR-361-5p	2.17	0.0150	
hsa-miR-93-5p	2.15	0.014	*PTEN*
hsa-miR-29c-3p	2.15	0.024	*PTEN*
hsa-miR-103a-3p	2.14	0.047	*PTEN*
hsa-miR-628-5p	2.09	0.046	
Down-regulated			
hsa-miR-1247-5p	−2.31	0.049	
hsa-miR-492	−2.60	0.035	*PTEN*
hsa-miR-135b-3p	−2.84	0.045	

Table 2.4

Differentially expressed urine exosomal miRNAs and corresponding target genes in high grade bladder cancer patients group

miRNA	Fold regulation	p-value	Target gene
Up-regulated			
hsa-miR-150-5p	26.15	0.044	*TP53, CTNNB1*
hsa-miR-126-3p	21.35	0.015	*AKT1*
hsa-miR-205-5p	12.37	0.038	*PTEN*
hsa-miR-324-5p	11.47	0.009	
hsa-miR-628-3p	11.42	0.005	
hsa-miR-28-5p	9.95	0.001	*TP53*
hsa-miR-18a-5p	9.14	0.005	*PTEN*
hsa-miR-21-3p	9.10	0.018	*PTEN*
hsa-miR-24-1-5p	8.57	0.003	
hsa-miR-342-3p	8.49	0.018	
hsa-miR-130b-3p	8.24	0.038	*PTEN*
hsa-miR-197-3p	7.54	0.028	
hsa-miR-376c-3p	7.41	0.029	
hsa-miR-182-5p	7.38	0.008	*PTEN*
hsa-miR-20a-3p	7.36	0.006	*PTEN*
hsa-miR-590-3p	7.30	0.003	
hsa-miR-21-5p	6.38	0.035	*PTEN, EGFR*
hsa-let-7f-2-3p	6.31	0.015	

Table 2.4 continued

miRNA	Fold regulation	p-value	Target gene
Up-regulated			
hsa-miR-200b-5p	6.26	0.010	
hsa-miR-374a-5p	6.05	0.001	
hsa-miR-7-1-3p	5.56	0.018	*EGFR*
hsa-miR-130b-5p	5.44	0.014	
hsa-miR-505-5p	5.14	0.002	
hsa-miR-183-5p	5.01	0.042	
hsa-miR-320b	4.97	0.029	
hsa-miR-105-5p	4.91	0.035	*AKT1*
hsa-miR-27b-3p	4.85	0.021	
hsa-miR-450a-5p	4.81	0.008	
hsa-miR-454-3p	4.79	0.030	
hsa-miR-28-3p	4.73	0.000	*TP53*
hsa-miR-146b-5p	4.71	0.009	
hsa-miR-425-5p	4.66	0.018	*PTEN*
hsa-miR-191-5p	4.65	0.008	
hsa-miR-301a-3p	4.62	0.014	*PTEN*
hsa-miR-151a-3p	4.47	0.004	
hsa-miR-320c	4.43	0.012	
hsa-miR-18a-3p	4.43	0.018	
hsa-miR-148b-3p	4.27	0.041	
hsa-miR-342-5p	4.12	0.042	
hsa-miR-664a-3p	4.10	0.016	
hsa-miR-455-3p	4.09	0.038	
hsa-miR-574-3p	4.06	0.002	*EGFR*
hsa-miR-618	3.89	0.040	
hsa-miR-365a-3p	3.81	0.003	
hsa-miR-200b-3p	3.74	0.000	
hsa-miR-181d-5p	3.69	0.034	
hsa-miR-193b-5p	3.68	0.028	
hsa-miR-200c-3p	3.50	0.001	*PTEN*
hsa-miR-23b-3p	3.48	0.024	*PTEN*
hsa-miR-374b-5p	3.37	0.002	
hsa-miR-92a-3p	3.29	0.021	*PTEN*
hsa-miR-320d	3.26	0.014	
hsa-miR-429	3.19	0.000	*PTEN, MYC*
hsa-miR-151a-5p	3.15	0.003	
hsa-miR-339-3p	3.15	0.023	
hsa-miR-26b-5p	3.12	0.023	*PTEN*
hsa-miR-99b-5p	3.11	0.016	
hsa-miR-489-3p	3.03	0.024	
hsa-miR-361-5p	2.92	0.014	
hsa-miR-320a	2.91	0.033	*PTEN*
hsa-miR-34a-3p	2.79	0.022	*AKT1, CTNNB, MYC*
hsa-miR-26a-5p	2.78	0.021	*PTEN*
hsa-miR-187-5p	2.77	0.033	
hsa-miR-218-5p	2.66	0.045	*EGFR*
hsa-miR-532-3p	2.66	0.047	
hsa-let-7d-3p	2.63	0.034	
hsa-let-7d-5p	2.61	0.019	
hsa-miR-125a-5p	2.43	0.012	*AKT1*
hsa-miR-106b-5p	2.30	0.026	*PTEN*

Table 2.4 continued

miRNA	Fold regulation	p-value	Target gene
Up-regulated			
hsa-let-7g-5p	2.29	0.033	
hsa-miR-423-3p	2.17	0.011	
hsa-miR-423-5p	2.15	0.026	
Down-regulated			
hsa-miR-548m	−2.02	0.035	
hsa-miR-1247-5p	−2.39	0.020	
hsa-miR-492	−2.52	0.030	*PTEN*
hsa-miR-302b-3p	−2.60	0.032	
hsa-miR-135b-3p	−2.75	0.041	
hsa-miR-1-3p	−6.99	0.035	

2.7 *TP53* and *PTEN* related exosomal miRNAs

Most miRNAs in the LG and HG group profiles are up-regulated (Tables 2.3 and 2.4). It suggests that many of these miRNAs may be involved in downregulating tumour suppressors. In the LG group, seven miRNAs (miR-28-5p, miR-16-5p, miR-28-3p, miR-24-3p, miR-25-3p, miR-19b-3p, and miR10b-5p) and in HG group three miRNAs (miR-150-5p, miR-28-5p, and miR28-3p) targeting TP53 were found in the miRTargetLink database with strong validation (Figure 2.5). Two of these miRNAs, miR-28-5p and miR-28-3p, were significantly up-regulated in both groups and reached higher fold regulation in the HG than in the LG group, whereas miR-150-5p was significantly up-regulated only in the HG group and did not reach statistical significance in the LG group.

There are 22 exosomal miRNAs in the LG group and 17 exosomal miRNAs in the HG group that directly target PTEN with strong validation. (Figure 2.6)

When focusing on exosomal miRNAs with greater than 5FR, the HG group had more miRNAs related to *TP53* and *PTEN* (two *TP53* targeting miRNAs and seven *PTEN* targeting miRNAs) than the LG group (one *TP53* targeting and three *PTEN* targeting miRNAs). This observation indicates *that TP53* and *PTEN* are more strongly suppressed in the HG group than in the LG group.

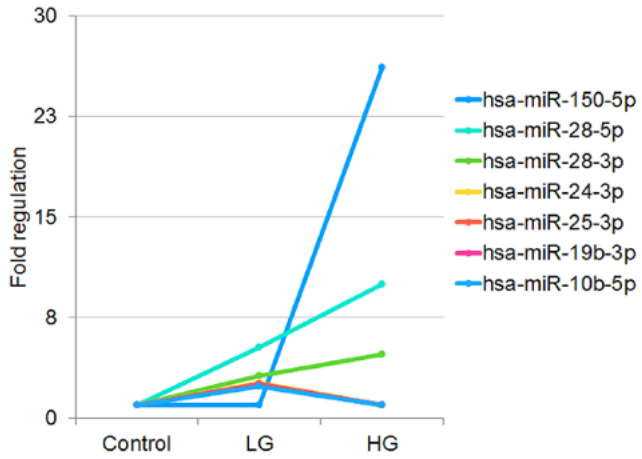


Figure 2.5 **Changes in levels of *TP53*-targeted exosomal miRNAs during bladder cancer progression**

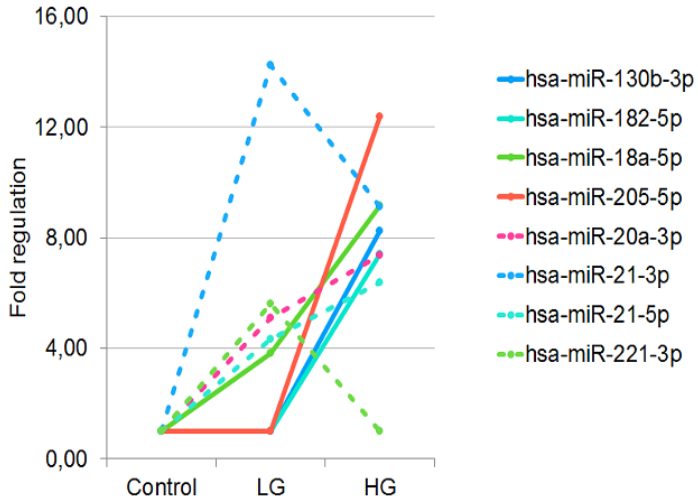


Figure 2.6 **Changes in levels of *PTEN*-targeted exosomal miRNAs during bladder cancer progression**

2.8 Pathway enrichment analysis of exosomal miRNAs

The enriched localisation of miRNAs were exosome, microvesicle and circulated with high significance in both LG and HG groups. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and MNDR disease categories mainly found cancer-related subcategories. We could not find statistically significant enriched pathways using the list of down- regulated miRNAs due to the small numbers of miRNAs in each group.

2.9 Analysis of target genes of up-regulated miRNAs and their interactions

A total of 1425 predicted target genes for 91 upregulated miRNAs in the LG group and 805 target genes for 71 upregulated miRNAs in the HG group were identified. Using these genes, regulatory network interactions between genes were created. To select the hub genes,

the top 50 genes were compared using 4 CytoNCA and CytoHubba methods. In total, 19 hub genes from the LG-regularised miRNA target group and 32 from the HG-regularised miRNA target group were extracted. The 17 target genes were overlapping in both groups. In both hub gene analysis with each of the four methods, *TP5*3 was ranked first, with the exception of MCC from CytoHubba (ranked 34th), and *PTEN* was ranked between 6 and 11 in LG and 7–10 in HG.

2.10 Enhancing bladder cancer diagnosis using machine learning on miRNA expression and clinical data

The machine learning analysis was performed on a cohort of 152 participants (89 bladder cancer patients and 63 healthy controls) who met all inclusion criteria and had complete clinical data. The clinical–pathological characteristics of this cohort are summarised in Table 2.5.

Table 2.5

Clinical–pathological parameters of the machine learning study cohort

Variable	Study Groups	
	Control (N = 63)	**Cancer (N = 89)**
Age (years)		
Median	66	74
Gender		
Male	39	70
Female	24	19
Pathological stage		
≤ pT1	–	80
≥ pT2	–	9
Grade		
LG	–	52
HG	–	37
Eritrocituria		
Yes	4	50
No	59	39
Smoking history		
Present/Past	30	47
Never	33	42

N – number; LG – low grade; HG – high grade.

Eight miRNAs from the discovery phase (mir-30b-3p, mir-28-3p, mir-339-5p, mir-20a-3p, mir-126-3p, mir-429, mir-28-5p and mir-324-5p) showed significant differential expression between cancer and control groups ($p < 0.05$).

Used as the sole input, miRNA data achieved the following diagnostic performance across the three classifiers (Figure 2.7.).

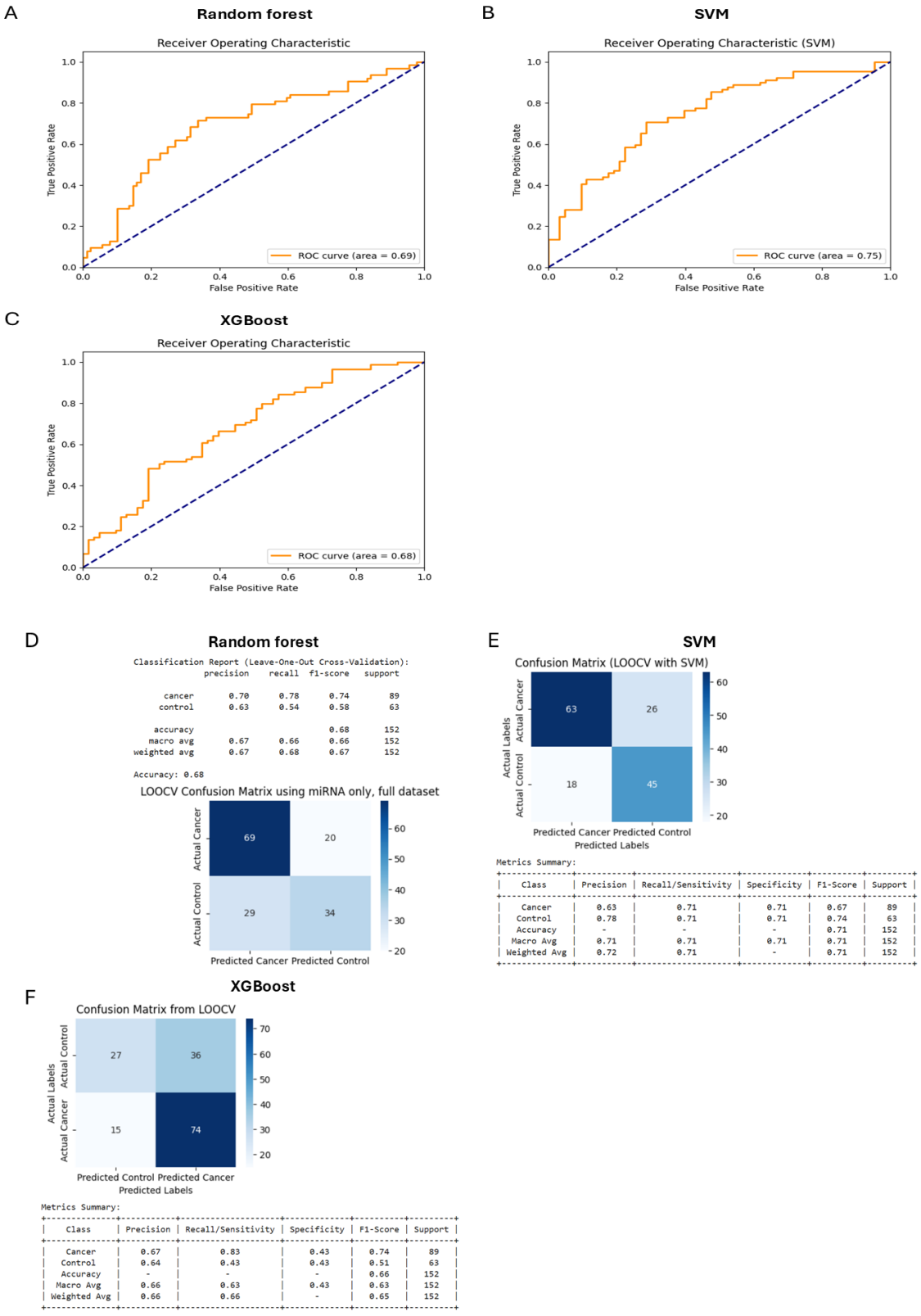


Figure 2.7 **Receiver-operating characteristic (ROC) curve analyses (A–C) and confusion matrices (D–F) for models trained on differentially expressed miRNA data using Random Forest (A, D), SVM (B, E), and XGBoost (C, F)**

A model trained exclusively on a set of clinical and demographic variables, such as age, sex, weight, height, erythrocyturia, leukocyturia, nitrite test, smoking history, aromatic amine

exposure, dysuria, chronic urinary tract infection, family oncological history was evaluated for 152 patients with complete records (Figure 2.8.)

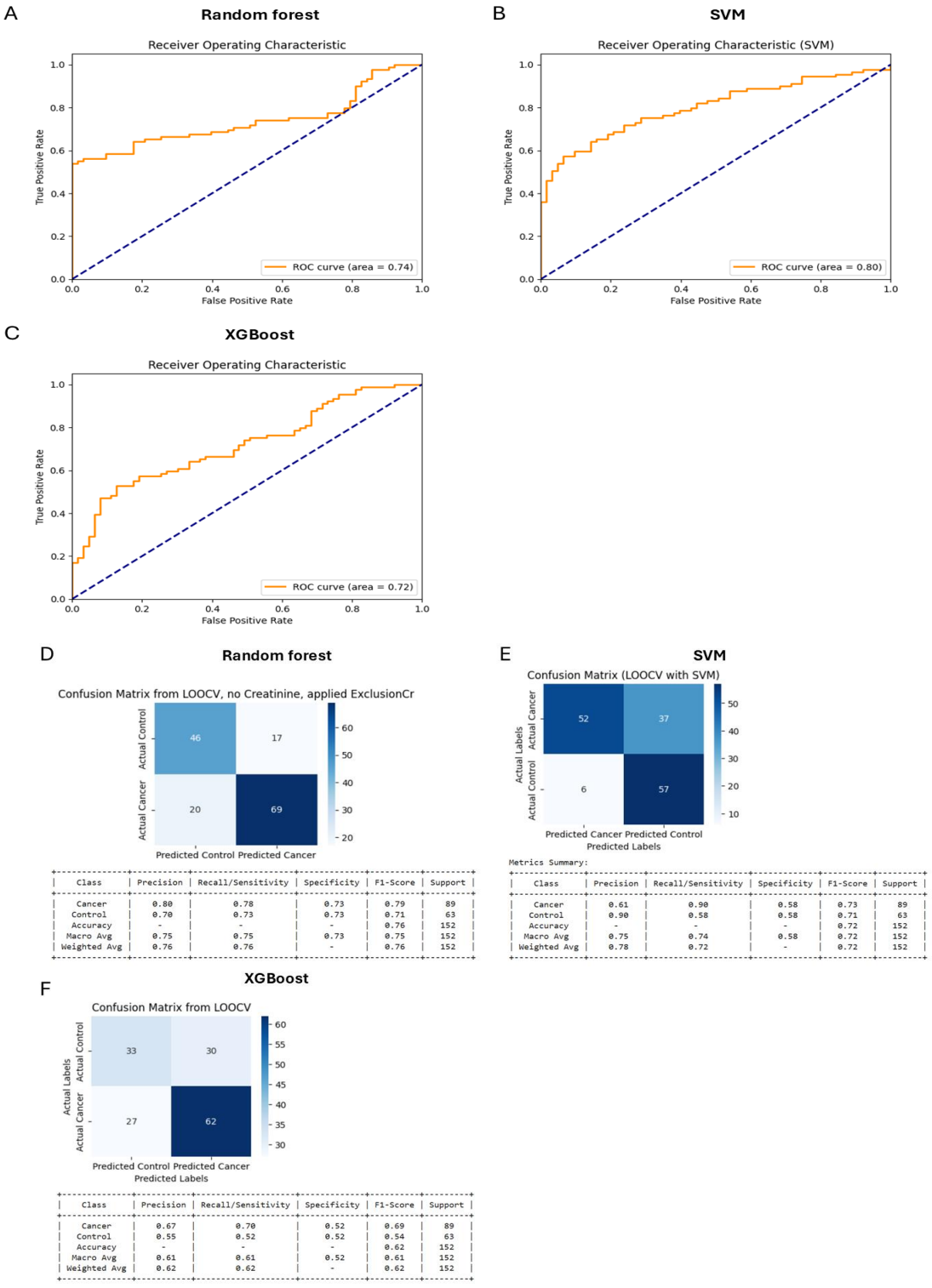
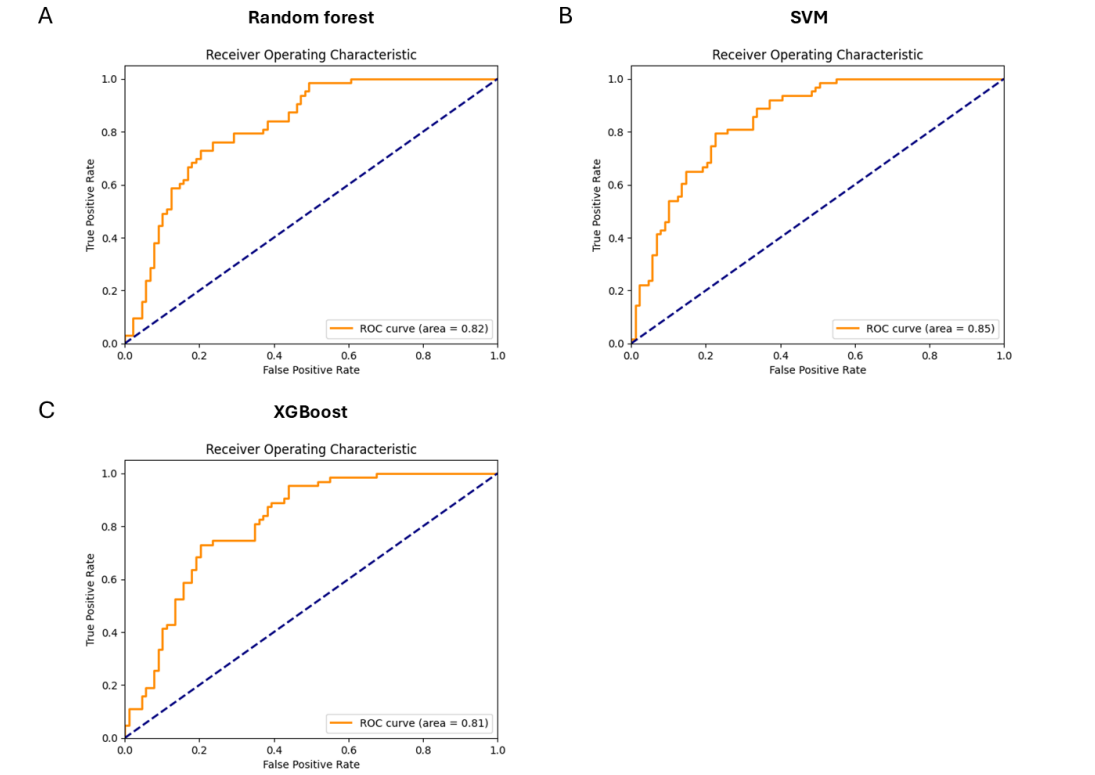
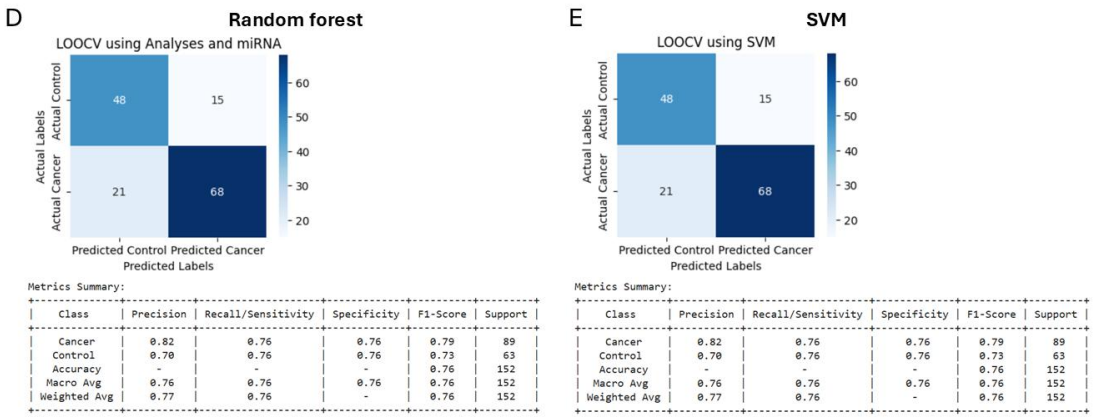


Figure 2.8 **Receiver-operating characteristic (ROC) curve analyses (A–C) and confusion matrices (D–F) for models trained on clinical and demographic data using Random Forest (A, D), SVM (B, E), and XGBoost (C, F)**

Integration of miRNA expression data with clinical variables into a single multi-modal model consistently outperformed both standalone configurations across all three classifiers, demonstrating the complementary diagnostic value of molecular and clinical data (Figure 2.9.)



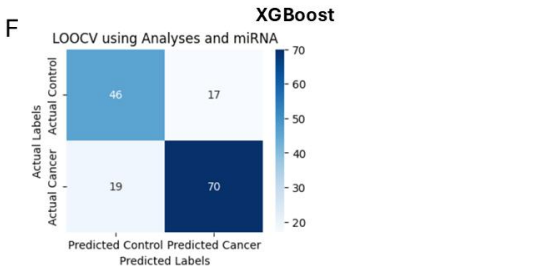


Metrics Summary:

Class	Precision	Recall/Sensitivity	Specificity	F1-Score	Support
Cancer	0.82	0.76	0.76	0.79	89
Control	0.70	0.76	0.76	0.73	63
Accuracy	-	-	-	0.76	152
Macro Avg	0.76	0.76	0.76	0.76	152
Weighted Avg	0.77	0.76	-	0.76	152

Metrics Summary:

Class	Precision	Recall/Sensitivity	Specificity	F1-Score	Support
Cancer	0.82	0.76	0.76	0.79	89
Control	0.70	0.76	0.76	0.73	63
Accuracy	-	-	-	0.76	152
Macro Avg	0.76	0.76	0.76	0.76	152
Weighted Avg	0.77	0.76	-	0.76	152



Metrics Summary:

Class	Precision	Recall/Sensitivity	Specificity	F1-Score	Support
Cancer	0.80	0.79	0.73	0.80	89
Control	0.71	0.73	0.73	0.72	63
Accuracy	-	-	-	0.76	152
Macro Avg	0.76	0.76	0.73	0.76	152
Weighted Avg	0.76	0.76	-	0.76	152

Figure 2.9 **Receiver-operating characteristic (ROC) curve analyses (A–C) and confusion matrices (D–F) for models trained on the combined miRNA and clinical dataset using Random Forest (A, D), SVM (B, E), and XGBoost (C, F)**

Feature importance was assessed for all three classifiers across the combined dataset (24 features: 11 miRNAs + 13 clinical variables). Consistent findings across all models identified the following top predictive features:

- Erythrocyturia (urinary erythrocytes on dipstick) – top-ranked clinical predictor across all three classifiers;
- Age (years) – consistently second-ranked clinical predictor;
- hsa-miR-20a-3p, hsa-miR-324-5p and hsa-miR-339-5p – top-ranked miRNA predictors, consistently identified across Random Forest, SVM and XGBoost;
- hsa-miR-28-5p, hsa-miR-429 and hsa-miR-455-3p – additional features contributing strongly to the tree-based models (Random Forest, XGBoost);
- Leukocyturia, sex, smoking history – lower but consistent clinical contributors.

Notably, smoking history – a well-established risk factor for BC – ranked relatively low across all models. This is likely attributable to the capture of current smoking status only, without inclusion of cumulative pack-years, which would better reflect the carcinogenic burden. UMAP dimensionality reduction confirmed clear separation between cancer and control groups when both data types were combined, visually corroborating the classifier performance.

Conclusions

1 The increase in bladder cancer incidence and mortality rates has been observed during the past 28 years, with the exception of cancer-specific mortality in the 40–59 age group, which showed a decreasing trend.

2 Five- and ten- year relative survival rates in Latvia have improved over time but remain lower than the median survival rates reported in other European countries.

3 A number of differentially expressed miRNAs involved in the epigenetic regulation of the *TP53* and *PTEN* genes were identified as putative diagnostic biomarkers.

4 The application of machine learning, with three complementary classifiers (Random Forest, SVM, XGBoost) evaluated across miRNA-only, clinical-only, and combined data configurations, demonstrated that the integration of clinical data and the identified miRNA biomarkers resulted in the best diagnostic performance, with the SVM classifier achieving an AUC of 0.85, sensitivity of 0.76, and specificity of 0.76.

5 The identified miRNA panel and the corresponding multimodal diagnostic approach require validation in larger cohorts and confirmation in biological studies before they can be considered for clinical use clinical use.

Proposals

1 Data collection within the Latvian Cancer Registry should continue to be systematically improved, with priority given to the complete recording of the TNM stage, tumour grade, treatment modalities, and comorbidities, so that future studies can provide a more detailed picture of survival and prognosis.

2 Although the clinical use of urinary miRNAs as cancer biomarkers is promising, several challenges remain. Many of them, although inherent to the field, could be overcome through closer collaboration within the scientific community – namely, by sharing datasets, using open-source platforms, applying standardised protocols for exosome isolation, RNA extraction and RT-qPCR, and making fuller use of emerging technologies.

3 Future studies should consider applying more advanced feature selection and dimensionality reduction methods to improve the interpretability and performance of the diagnostic model. Exploring the biological significance of the most influential features identified by the model could also provide insight into the molecular mechanisms underlying bladder cancer and may help to reveal new biomarkers for early diagnosis.

4 The urinary exosomal miRNA panel identified in this study – comprising miR-324-5p, miR-20a-3p, miR-429, miR-455-3p, miR-28-5p and miR-30b-3p – should be validated in an independent, prospectively recruited cohort, using the expression thresholds derived from the present work, to assess its reproducibility and clinical applicability.

5 The predicted interactions between the identified miRNAs and their target genes – particularly those targeting *TP53* and *PTEN* – should be confirmed using experimental approaches such as luciferase reporter assays, RNA immunoprecipitation or gene knockdown, in order to verify these regulatory relationships and gain a better understanding of the molecular mechanisms underlying bladder cancer.

6 The multi-modal diagnostic dataset should be expanded through multicentre collaboration, in order to reach a sample size sufficient for robust external validation of the model. The machine learning analysis should also be extended to longitudinal follow-up data, to evaluate whether the urinary exosomal miRNA expression profile can predict disease recurrence, progression and response to treatment.

List of publications, reports and patents on the topic of the Thesis

Publications:

1. Bitiņa-Barlote, Ē., Plonis, J., Cauce, V., Vjaters, E., Gardovskis, J., Miklaševičs, E., Nakazawa-Miklaševiča, M. 2021. Bladder cancer trends in Latvia during 1990-2017: incidence, mortality, and survival rates. Central European Journal of Urology 2021; 74(1): 14–23. doi: 10.5173/ceju.2021.0266.R2
2. Bitiņa-Barlote, Ē., Plonis, J., Andrejeva, M., Vjaters, E., Gardovskis, J., Daneberga, Z., Miklaševičs, E., Nakazawa-Miklaševiča, M. Profiles of urinal exosomal miRNAs derived from bladder cancer. Cent European J Urol. 2024; doi: 10.5173/ ceju.2023.279.R1
3. Bitiņa-Barlote, Ē., Bļizņuks, D., Siliņa, S., Šatcs, M., Vjaters, E., Lietuvietis, V., Nakazawa-Miklaševiča, M., Plonis, J., Miklaševičs, E., Daneberga, Z., Gardovskis, J. Liquid Biopsy Based Bladder Cancer Diagnostic by Machine Learning. Diagnostics. 2025; 15(4), 492. https://doi.org/10.3390/diagnostics15040492

Reports and theses at international congresses and conferences:

1. Bitina-Barlote E., Plonis J., Vjaters E., Gardovskis J., Daneberga Z., Miklaševičs E., Nakazawa-Miklaševiča M. Urinary exosomal miRNA profiles and target genes in bladder cancer: Pilot study. European Urology Open Science, Volume 52, Supplement 1, June 2023, Page S22. https://doi.org/10.1016/S2666-1683(23)00294-X.
2. Bitiņa-Barlote Ē., Plonis J., Vasiļonoks J.K., Vjaters E., Gardovskis J., Daneberga Z., Miklaševičs E., Nakazawa-Miklaševiča M. Urine exosomal miRNA profiles implicmate the key role of TP53 in bladder cancer development. European Urology Open Science, Volume 40, Supplement 1, June 2022, Pages S31-S32. https://doi.org/10.1016/S2666-1683(22)00653-X
3. Bitiņa-Barlote Ē, Plonis J., Andrejeva M., Vjaters E., Gardovskis J., Miklaševičs E., Nakazawa-Miklaševiča M. Urinary exosomal miRNAs as potential biomarker for bladder cancer diagnosis: pilot study. Rīga Stradiņš University International Conference on Medical and Health Care Sciences. "Knowledge for Use in Practice", Abstracts, Riga. Latvia, 2021, p 114.
4. Bitiņa-Barlote Ē., Plonis J., Cauce V., Vjaters E., Gardovskis J., Miklaševičs E., Nakazawa-Miklaševiča M. Prevalence trends and years of life lost due to bladder cancer among the inhabitants of Latvia over past 28 years. Medicina (Kaunas); Volume 56, Supplement 1, 2020, p 128.
5. Bitina-Barlote E. Plonis J. Cauce V. Jukonis J. Vjaters E. Gardovskis J. Miklaševičs E. Bladder cancer incidence and mortality trends in Latvia: 1996-2017. European Urology Supplements Volume 18, Issue 3, 2019. p e2501-e2502. DOI: https://doi.org/10.1016/S1569-9056(19)32197-9
6. Bitina-Barlote E. Plonis J. Cauce V. Jukonis J. Vjaters E. Gardovskis J. Miklaševičs E. Bladder cancer 5-year survival trends in Latvia: 1996-2017. European Urology Supplements Volume 18, Issue 3, 2019, p e2498-e2499. DOI: https://doi.org/10.1016/S1569-9056(19)32195-5

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