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Ocena biomarkerów nowej generacji w prognozowaniu przebiegu klinicznego raka pęcherza moczowego wysokiego ryzyka

ROZPRAWA DOKTORSKA
Cykl publikacji powiązanych tematycznie

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Składam serdeczne podziękowania
Promotorom niniejszej Rozprawy Doktorskiej,

Panu prof. dr. hab. Wojciechowi Krajewskiemu
oraz
Pani dr hab. Aleksandrze Pawlak,

za opiekę merytoryczną, wszechstronną pomoc, inspirację,
poświęcony czas, cierpliwość i wyrozumiałość.

Chciałabym również podziękować moim Rodzicom, najwspanialszej
Dziewczynie i Przyjaciołom za niegasnącą wiarę we mnie.

Spis treści

1. WYKAZ PRAC STANOWIĄCYCH ROZPRAWĘ DOKTORSKĄ

Publikacja 1 (P1):

Nowak, Ł., Krajewski, W., Małkiewicz, B., Szydełko, T., & Pawlak, A. (2022). Characteristics and Applications of Canine In Vitro Models of Bladder Cancer in Veterinary Medicine: An Up-to-Date Mini Review. Animals (Basel), 12(4), 516.

Współczynnik wpływu (IF): 3,231

Punktacja MEiN: 100

Publikacja 2 (P2):

Nowak, Ł., Krajewski, W., Poterek, A., Śliwa, A., & Zdrojowy, R. (2020). The prognostic value of programmed cell death protein ligand 1 in patients with non-muscle-invasive bladder cancer treated with bacille Calmette-Guérin immunotherapy: Current status. Arab journal of urology, 19(1), 67–70.

Współczynnik wpływu (IF): 0

Punktacja MEiN: 70

Publikacja 3 (P3):

Krajewski, W., **Nowak, Ł**., Moschini, M., Poletajew, S., Chorbińska, J., Necchi, A., Montorsi, F., Briganti, A., Sanchez-Salas, R., Shariat, S. F., Palou, J., Babjuk, M., Teoh, J. Y., Soria, F., Pradere, B., Ornaghi, P. I., Pawlak, A., Dembowski, J., & Zdrojowy, R. (2021). Impact of Adjuvant Chemotherapy on Survival of Patients with Advanced Residual Disease at Radical Cystectomy following Neoadjuvant Chemotherapy: Systematic Review and Meta-Analysis. Journal of clinical medicine, 10(4), 651.

Współczynnik wpływu (IF): 4,964

Punktacja MEiN: 140

Publikacja 4 (P4):

Nowak, Ł., Krajewski, W., Moschini, M., Chorbińska, J., Poletajew, S., Tukiendorf, A., Muilwijk, T., Joniau, S., Tafuri, A., Antonelli, A., Orlando, R., Di Trapani, E.,

Alvarez-Maestro, M., Simone, G., Zamboni, S., Simeone, C., Marconi, M. C., Mastroianni, R., Piszczek, R., Xylinas, E., European Association of Urology - Young Academic Urologists (EAU-YAU): Urothelial Carcinoma Working Group (2021). Assessment of the oncological outcomes of three different bacillus Calmette-Guérin strains in patients with high-grade T1 non-muscle-invasive bladder cancer. Arab journal of urology, 19(1), 78–85.

Współczynnik wpływu (IF): 0

Punktacja MEiN: 70

Publikacja 5 (P5):

Nowak, Ł., Krajewski, W., Dejnaka, E., Małkiewicz, B., Szydełko, T., & Pawlak, A. (2023). Ubiquitin-Specific Proteases as Potential Therapeutic Targets in Bladder Cancer-In Vitro Evaluation of Degrasyn and PR-619 Activity Using Human and Canine Models. Biomedicines, 11(3), 759.

Współczynnik wpływu (IF): 4,757

Punktacja MEiN: 100

Sumaryczny IF: 12,952

Sumaryczna punktacja MEiN: 480

2. WYKAZ SKRÓTÓW

AC	chemioterapia adjuwantowa (ang. *adjuvant chemotherapy*)
BC	rak pęcherza moczowego (ang. *bladder cancer*)
BCG	Bacillus Calmette-Guerin
CI	przedział ufności (ang. *confidence interval*)
CIS	rak śródnabłonkowy (ang. *carcinoma in situ*)
CUETO	Club Urológico Español de Tratamiento Oncológico
DSS	przeżycie do zgonu wywołanego badaną chorobą (ang. *disease-specific survival*)
EAU	Europejskie Towarzystwo Urologiczne (ang. *European Association of Urology*)
EORTC	Europejska Organizacja ds. Badań i Leczenia Raka (ang. *European Organisation for Research and Treatment of Cancer*)
HG	wysoki stopień złośliwości (ang. *high-grade*)
HR	współczynnik hazardu (ang. *hazard ratio*)
IF	współczynnik wpływu (ang. *impact factor*)
IPW	ważenie odwrotnego prawdopodobieństwa (ang. *inverse probability weighting*)
MEiN	Ministerstwo Edukacji i Nauki
MIBC	naciekający mięśniówkę rak pęcherza moczowego (ang. *muscle-invasive bladder cancer*)
NAC	chemioterapia neoadjuwantowa (ang. *neoadjuvant chemotherapy*)
NMIBC	nienaciekający mięśniówki rak pęcherza moczowego (ang. *non-muscle-invasive bladder cancer*)
OS	przeżycie całkowite (ang. *overall survival*)
PD-L1	ligand receptora programowanej śmierci 1 (ang. *programmed death ligand 1*)
PFS	przeżycie wole od progresji (ang. *progression-free survival*)
RC	cystektomia radykalna (ang. *radical cystectomy*)
RFS	przeżycie wolne od nawrotu (ang. *recurrence-free survival*)

TURBT	przezcewkowa resekcja guza pęcherza moczowego (ang. *transurethral resection of bladder tumor*)
USP	proteazy specyficzne dla ubikwityny (ang. *ubiquitin-specific proteases*)

3. STRESZCZENIE W JĘZYKU POLSKIM

Wprowadzenie

Na Rozprawę Doktorską składa się cykl 5 artykułów opublikowanych w międzynarodowych czasopismach naukowych indeksowanych w bazie MEDLINE, uwzględnionych na liście Journal Citation Reports (JCR) oraz znajdujących się w wykazie czasopism naukowych Ministerstwa Edukacji i Nauki (MEiN). Łączny współczynnik wpływu (*impact factor* – IF) artykułów wchodzących w skład rozprawy doktorskiej wynosi 12,952 a punktacja MEiN wynosi 480 punktów.

Rak pęcherza moczowego (*bladder cancer* – BC) jest drugim co do częstości występowania nowotworem wywodzącym się z układu moczowego. W momencie postawienia diagnozy 75% przypadków BC stanowią raki nienaciekające mięśniówki (*non-muscle-invasive bladder cancer* – NMIBC), natomiast pozostałe 25% stanowią raki naciekające mięśniówkę (*muscle-invasive bladder cancer* – MIBC). Niedoskonałość aktualnych modeli prognostycznych, opartych wyłącznie na parametrach kliniczno-patologicznych, warunkuje konieczność prowadzenia dodatkowych badań nad predyktorami wznowy oraz progresji pacjentów z NMIBC wysokiego ryzyka otrzymujących wlewki dopęcherzowe Bacillus Calmette-Guerin (BCG). Mimo pojawienia się w ostatnich kilkunastu latach nowych form leczenia MIBC w stadium zaawansowanym lub przerzutowym, pięcioletnie przeżycia chorych nie przekraczają 15 - 20%. Dlatego prowadzone obecnie badania skupiają się na poszukiwaniu nowych markerów molekularnych, mogących stanowić potencjalny cel terapeutyczny w MIBC. Jednym z obiecujących kierunków badań jest ocena wpływu zaburzeń ekspresji białek związanych z procesem deubikwitynylacji, proteaz specyficznych dla ubikwityny (*ubiquitin-specific* proteases – USP) na proces karcynogenezy oraz tworzenia przerzutów w różnych typach nowotworów. Procesy kontrolowane przez białka z rodziny USP mają kluczowe znaczenie dla prawidłowego funkcjonowania szlaków komórkowych, takich jak naprawa DNA czy apoptoza komórki. Z tego względu USP mogą stanowić potencjalne cele molekularne w terapii przeciwnowotworowej.

Onkologia porównawcza jest aktualnie szybko rozwijającą się dziedziną, mającą na celu integrację badań dotyczących naturalnie występujących nowotworów u zwierząt i ludzi. Spośród różnych gatunków zwierząt za najodpowiedniejszy model BC uważany jest pies, co wynika z podobieństw anatomicznych i fizjologicznych do BC człowieka. Bardzo złe rokowanie w kontekście przeżycia u psów z BC oraz możliwość wykorzystania modelu psiego

w onkologii porównawczej pozwalają na prowadzenie symultanicznych badań nad rozwojem nowych związków terapeutycznych mających potencjalne zastosowanie zarówno w medycynie człowieka, jak i medycynie weterynaryjnej. Ze względu na częste problemy natury etycznej dotyczące badań na modelach zwierzęcych *in vivo*, psie modele *in vitro* mogą stanowić cenne narzędzie badawcze, pozwalające na analizę nowych biomarkerów i środków terapeutycznych.

Cel badań

Celem badań wchodzących w skład Rozprawy Doktorskiej była analiza wybranych markerów molekularnych mających potencjalne znaczenie prognostyczne, jak i stanowiących potencjalny cel terapeutyczny w leczeniu BC oraz analiza wpływu wybranych form terapeutycznych na wyniki leczenia pacjentów z BC.

Celem realizacji założeń Programu „ProHum – Interdyscyplinarna Szkoła Doktorska – planowanie badań eksperymentalnych, tworzenie i optymalizacja zwierzęcych modeli doświadczalnych z umiejętnościami transferowania ich do badań klinicznych w medycynie człowieka" w wybranych badaniach wykorzystywano zarówno modele ludzkie, jak i zwierzęce.

Materiały i metody

W części teoretycznej Rozprawy Doktorskiej dokonano:

- **(P1)** Przeglądu systematycznego dostępnych danych literaturowych na temat psich modeli *in vitro* BC oraz ich potencjalnego zastosowania w kontekście badań nad nowymi biomarkerami oraz środkami terapeutycznymi.
- **(P2)** Przeglądu systematycznego dostępnych danych literaturowych na temat związku ekspresji ligandu receptora programowanej śmierci 1 (*programmed death ligand 1* – PD-L1) z parametrami onkologicznymi i odpowiedzią kliniczną na immunoterapię BCG u pacjentów z NMIBC wysokiego ryzyka.
- **(P3)** Przeglądu systematycznego i metaanalizy dostępnych danych literaturowych na temat wpływu chemioterapii adjuwantowej (*adjuvant chemotherapy* – AC) po cystektomii radykalnej (*radical cystectomy* – RC) poprzedzonej chemioterapią neoadjuwantową (neoadjuvant chemotherapy – NAC) na wyniki onkologiczne pacjentów z zaawansowaną chorobą rezydualną (obecność guza w stopniu zaawansowania T3-T4 i/lub potwierdzone histopatologicznie przerzuty do węzłów chłonnych).

W części badawczej Rozprawy Doktorskiej:

- **(P4)** Przeanalizowano retrospektywnie dane 590 pacjentów z potwierdzonym histopatologicznie pierwotnym NMIBC w stopniu zaawansowania T1 i wysokim stopniu złośliwości histopatologicznej. Oceniono wyniki onkologicznie w 3 podgrupach chorych otrzymujących różne szczepy BCG: Moreau, TICE oraz RIVM. Pierwszorzędowe punkty końcowe analizy stanowiły: przeżycie wolne od nawrotu (*recurrence-free survival* – RFS) oraz przeżycie wolne od progresji (*progression-free survival* – PFS).
- **(P5)** Oceniono ekspresję wybranych białek USP (USP5, USP9X, USP14) w modelowych liniach prawidłowego nabłonka urotelialnego człowieka (SV-HUC-1) oraz modelowych liniach BC człowieka (T24) i psa (K9TCC-PU-NK, RDSVS-TCC1). Określono potencjał działania przeciwnowotworowego selektywnego (degrasyn) oraz nieselektywnego (PR-619) inhibitora białek USP na komórki BC. Wpływ inhibitorów USP na proliferację komórek określony został za pomocą testu MTT. Nasilenie apoptozy po zastosowaniu inhibitorów USP oceniano przy użyciu testu z zastosowaniem Aneksyny V i jodku propidyny oraz poprzez analizę aktywacji kaspazy 3/7 za pomocą cytometrii przepływowej. Do oceny ekspresji wybranych białek z rodziny USP, białek związanych z uszkodzeniem DNA oraz kluczowych białek zaangażowanych w proces apoptozy wykorzystano metodę Western Blot.

Wyniki

Na podstawie danych pochodzących z 33 prac dokonano usystematyzowania dostępnej wiedzy na temat psich modeli *in vitro* BC. Ustalone linie komórkowe (hodowle dwuwymiarowe) są najczęściej wykorzystywanym materiałem do badań naukowych nad nowymi biomarkerami i lekami, jednak badania z użyciem ustalonych linii komórkowych nie odzwierciedlają w pełni warunków panujących *in vivo*, głównie z powodu braku interakcji komórek nowotworu z mikrośrodowiskiem guza. Wykorzystanie hodowli trójwymiarowych, charakteryzujących się budową sferoidalną imitującą warunki panujące *in vivo*, zwiększa translacyjność wyników prowadzonych badań **(P1)**.

Na podstawie danych pochodzących z 5 badań dokonano usystematyzowania dostępnej wiedzy na temat związku ekspresji PD-L1 z parametrami onkologicznymi i odpowiedzią kliniczną na immunoterapię BCG u pacjentów z NMIBC wysokiego ryzyka. Związek pozytywnej ekspresji PD-L1 z opornością na leczenie BCG u pacjentów z NMIBC wysokiego ryzyka jest

niejednoznaczny. W żadnej z dostępnych prac nie wykazano prognostycznego znaczenia pozytywnej ekspresji PD-L1 w kontekście parametrów RFS i PFS **(P2)**.

Zidentyfikowano 6 badań, które analizowały wpływ AC po RC poprzedzonej NAC na wyniki onkologiczne pacjentów z zaawansowaną chorobą rezydualną. W porównaniu do grupy kontrolnej (pacjenci poddani obserwacji), chorzy otrzymujący AC mieli istotnie wyższy wskaźnik OS. Stosowanie AC nie wpływało istotnie na OS w podgrupie pacjentów z potwierdzonymi histopatologicznie przerzutami do węzłów chłonnych **(P3)**.

Wykazano, że stosowanie szczepu RIVM wiązało się z istotnie wyższym prawdopodobieństwem nawrotu BC w porównaniu do szczepów Moreau i TICE. Analizowane szczepy nie różniły się istotnie w kontekście wskaźnika PFS. Analiza regresji proporcjonalnego hazardu Coxa z wykorzystaniem metody ważenia odwrotnego prawdopodobieństwa (*inverse probability weighting* – IPW) nie wykazała istotnie statystycznej różnicy w odniesieniu do RFS dla szczepów Moreau i TICE, podczas gdy stosowanie szczepu RIVM było związane z istotnie niższym RFS w porównaniu do szczepu Moreau oraz szczepu TICE. Nie stwierdzono istotnych różnic w PFS pomiędzy poszczególnymi szczepami **(P4)**.

Wykazano, że komórki modelowej linii BC człowieka (T24) charakteryzowały się wyższą ekspresją białka USP5 w porównaniu z nienowotworowymi komórkami urotelialnymi (SV-HUC-1). W teście MTT wykazano, że degrasyn hamował proliferację wszystkich badanych linii komórkowych w sposób zależny od stężenia i czasu. Analiza 24-godzinnego czasu inkubacji wykazała istotnie mniejszą aktywność antyproliferacyjną degrasynu wobec kontrolnej ludzkiej linii komórkowej SV-HUC-1 niż wobec modelowej, nowotworowej linii komórkowej T24. Przy ocenie wszystkich czasów inkubacji (24, 48 oraz 72 godziny) komórki BC człowieka były bardziej wrażliwe na antyproliferacyjne działanie degrasynu w porównaniu do psich linii komórkowych BC (K9TCC-PU-NK i RDSVS-TCC1). Stwierdzona aktywność antyproliferacyjna degrasynu była istotnie wyższa niż drugiego zastosowanego inhibitora, PR-619. Po 24 godzinach inkubacji komórek z degrasynem w rosnących stężeniach stwierdzono znaczny wzrost odsetka komórek wczesno- i późnoapoptotycznych w liniach T24 oraz RDSVS-TCC1. W przeciwieństwie do degrasynu, PR-619 nie wykazywał istotnego działania proapoptotycznego. Wyniki analizy mechanizmu proapoptotycznego działania degrasynu wykazały, że po 24-godzinnej inkubacji pod jego wpływem w komórkach BC człowieka i psa dochodzi do zmniejszenia poziomu ekspresji białek antyapoptotycznych Bcl-xl oraz Bcl-2. Nie zaobserwowano zmian w poziomie ekspresji Bcl-xl oraz Bcl-2 po inkubacji komórek BC ze wzrastającymi stężeniami PR-619. Po 24-godzinnej inkubacji

z degrasynem we wszystkich użytych linii komórkowych zaobserwowano zwiększenie ekspresji fosforylowanej formy białka H2AX (γH2AX) będącego markerem uszkodzenia DNA. 24-godzinna inkubacja z PR-619 nie wywoływała wyraźnego wzrostu ekspresji γH2AX **(P5)**.

Wnioski

1. Ustalone linie komórkowe psiego BC są wartościowym materiałem do badań naukowych nad nowymi biomarkerami i lekami, jednak nie odzwierciedlają one dokładnie warunków panujących *in vivo*. Tworzenie hodowli trójwymiarowych imitujących warunki *in vivo* jest niezwykle istotnym kierunkiem badań nad nowymi modelami BC, służącymi do badań eksperymentalnych.
2. Wartość pozytywnej ekspresji PD-L1 w przewidywaniu odpowiedzi na immunoterapię BCG oraz ryzyko nawrotu i progresji NMIBC wysokiego ryzyka jest wątpliwa. Aktualne dowody, oparte tylko na danych retrospektywnych, są wysoce heterogenne.
3. Podawanie AC pacjentom z zaawansowaną chorobą rezydualną chorobą po RC poprzedzonej NAC może mieć pozytywny wpływ na OS i DSS. Nie dotyczy to pacjentów ze stwierdzonymi przerzutami w węzłach chłonnych.
4. Stosowanie szczepu RIVM u pacjentów z NMIBC wysokiego ryzyka progresji wiąże się z istotnie niższym RFS w porównaniu do szczepów Moreau oraz TICE, co warunkuje ich preferencyjne stosowanie.
5. W komórkach BC człowieka białko USP5 jest nadekspresjonowane w porównaniu do komórek prawidłowych, co sugeruje możliwość wykorzystania selektywnych inhibitorów białka USP5 jako potencjalnych form terapii przeciwnowotworowej.
6. Degrasyn, selektywny inhibitor białek USP5, USP9X oraz USP14, wykazuje aktywność przeciwnowotworową w stosunku do komórek BC człowieka i psa. Działanie przeciwnowotworowe wywierane jest poprzez zahamowanie proliferacji komórkowej, indukcję apoptozy oraz indukcję uszkodzenia DNA.
7. Aktywność przeciwnowotworowa selektywnego inhibitora białek USP (degrasynu) jest wyższa w porównaniu do nieselektywnego inhibitora białek USP (PR-619).

4. STRESZCZENIE W JĘZYKU ANGIELSKIM

Introduction

The Doctoral Dissertation consists of a series of 5 articles published in international scientific journals indexed in the MEDLINE database and included in the Journal Citation Reports by Web of Science list, as well as in the list of scientific journals of the Ministry of Education and Science (MEiN). The total impact factor (IF) of the articles included in the doctoral dissertation is 12,952, and the MEiN score is 480 points.

Bladder cancer (BC) is the second most common malignancy of the urinary tract. At the time of diagnosis, 75% of BC cases are non-muscle-invasive bladder cancers (NMIBC), while the remaining 25% are muscle-invasive bladder cancers (MIBC). The current NMIBC prognostic models, based mainly on clinicopathological parameters, have some important limitations. Therefore, research on novel predictors of recurrence and progression in patients with high-risk NMIBC treated with Bacillus Calmette-Guerin (BCG) immunotherapy is highly needed. Despite the introduction of novel forms of advanced/metastatic MIBC treatment in recent years, the 5-year survival rate of patients does not exceed 15-20%. Therefore, current research is focused on new molecular markers that could be potential therapeutic targets in MIBC. One of the promising directions of research is the assessment of ubiquitin-specific proteases (USP) and the association of their expression with carcinogenesis and the formation of metastases in various types of cancer. Processes controlled by USP are crucial for various cellular pathways, such as DNA repair or cell apoptosis. For this reason, USP could potentially be molecular targets for cancer therapy.

Comparative oncology is currently a fast-growing research field that aims to integrate data from naturally occurring cancers in animals and humans. Among the various animal species, the dog is considered the most appropriate BC model due to its anatomical and physiological similarities to the human BC. Poor survival outcomes in dogs with BC implicate an urgent need for the development of novel treatment compounds, simultaneously with human medicine. Due to the frequent ethical problems associated with *in vivo* animal research, *in vitro* canine models can be a valuable research tool for the analysis of new biomarkers and therapeutic targets.

Aim

The aim of the study was to analyze selected molecular markers with potential prognostic and therapeutic significance in BC, as well as the impact of selected therapeutic forms on the survival of patients with BC.

In order to implement the assumptions of the project "ProHum - Interdisciplinary Doctoral School - planning experimental research, creating and optimizing animal experimental models with the ability to transfer them to clinical trials in human medicine", both human and animal models were used in particular studies.

Materials and methods

The theoretical part of the Doctoral Dissertation included:

- **(P1)** Systematic review of available literature data on *in vitro* models of canine BC and their potential applications in research on new biomarkers and therapeutic agents.
- **(P2)** Systematic review of available literature data on the association between the expression of programmed death ligand 1 (PD-L1) and oncological parameters and BCG-response in patients with high-risk NMIBC.
- **(P3)** Systematic review and meta-analysis of available literature data on the impact of adjuvant chemotherapy (AC) after radical cystectomy (RC) preceded by NAC on oncological outcomes in patients with advanced residual disease (presence of tumor at T3-T4 stage and/or histopathologically confirmed metastases to lymph nodes).

In the research part of the Doctoral Dissertation:

- **(P4)** Data from 590 patients with histopathologically confirmed primary T1 high-grade NMIBC were retrospectively analyzed. Oncological outcomes were assessed in 3 subgroups of patients receiving different BCG strains: Moreau, TICE and RIVM. The primary endpoints of the analysis were recurrence-free survival (RFS) and progression-free survival (PFS).
- **(P5)** Expression of selected USP proteins (USP5, USP9X, USP14) was assessed in human normal urothelium (SV-HUC-1) and cell lines of human (T24) and canine BC (K9TCC-PU-NK, RDSVS-TCC1). The anticancer activity of a selective (degrasyn) and a non-selective (PR-619) inhibitor of USP proteins against BC cells was determined. The effect of USP inhibitors on cell proliferation was determined using the MTT assay. The level of apoptosis after inhibition with USP inhibitors was assessed using the Annexin V and propidium iodide assay and analysis of the activation of caspase 3/7 by flow cytometry. The Western Blot method was used to assess the expression of selected proteins from the USP family, proteins associated with DNA damage, and key proteins involved in the process of apoptosis.

Results

Based on data from 33 articles, the available evidence regarding canine *in vitro* models of BC has been summarized. Established cell lines (two-dimensional cultures) are the most commonly used *in vitro* models of BC for research on new biomarkers and therapeutic agents. However, studies using established cell lines do not faithfully reflect conditions prevailing *in vivo*, mainly due to the lack of available interactions between tumor cells and tumor microenvironment. Use of three-dimensional cultures, with a spheroidal structure imitating the conditions prevailing *in vivo*, increases the translational value of the experiments **(P1)**.

Based on data from 5 studies, the available evidence regarding the association between PD-L1 expression and oncological outcomes and clinical response to BCG immunotherapy in patients with high-risk NMIBC was summarized. The association between positive PD-L1 expression and resistance to BCG treatment in patients with high-risk NMIBC is ambiguous. None of the available studies demonstrated the prognostic significance of positive PD-L1 expression in terms of RFS and PFS **(P2)**.

Six studies examined the effect of AC on oncological outcomes in patients with advanced residual disease after NAC followed by RC. Compared to the control group (observation), patients receiving AC had a significantly higher OS. The use of AC did not significantly affect OS in the subgroup of patients with histopathologically confirmed lymph node metastases **(P3)**.

It was demonstrated that the use of the RIVM strain could be associated with a significantly higher probability of BC recurrence compared to the Moreau and TICE strains. The analyzed strains did not differ significantly in terms of PFS. Cox proportional hazard regression analysis using the inverse probability weighting (IPW) method showed no statistically significant difference in RFS for the Moreau and TICE strains, while the use of the RIVM strain was associated with significantly lower RFS. There were no significant differences in PFS between analyzed strains **(P4)**.

Compared to control non-tumorigenic urothelial cells (SV-HUC-1), higher expression of USP5 protein was observed in human BC cell line (T24). MTT assay demonstrated that degrasyn inhibited the proliferation of all cell lines used in the present study in a concentration- and time-dependent manner. Relative to 24-hours incubation time, significantly less cytotoxicity of degrasyn on the human control SV-HUC-1 cell line compared to the T24 cell line was observed. Relative to all incubation times (24, 48 and 72 hours), human BC cells were more sensitive to the antiproliferative effect of degrasyn compared to canine BC cell lines (K9TCC-PU-NK and

RDSVS-TCC1). The antiproliferative activity of degrasyn was significantly higher than that of the second inhibitor used, PR-619. After 24-hours incubation with degrasyn at increasing concentrations, a significant increase in the number of early and late apoptotic cells was detected in the T24 and RDSVS-TCC1 cell lines. In contrast to degrasyn, PR-619 did not show a significant pro-apoptotic effect. After 24-hours incubation, the expression level of anti-apoptotic proteins Bcl-xl and Bcl-2 was reduced in human and canine BC cells. No changes in Bcl-xl and Bcl-2 expression levels were observed after incubation with increasing concentrations of PR-619. After 24-hour incubation with degrasyn, an increase in the expression of the phosphorylated form of the H2AX protein (γH2AX), which is a marker of DNA damage, was observed in all BC cell lines. 24-hour incubation with PR-619 did not induce a marked increase in γH2AX expression **(P5).**

Conclusions

1. Established canine BC cell lines are valuable tools for *in vitro* research on new biomarkers and therapeutic agents, but they do not accurately reflect the conditions prevailing *in vivo*. Creating three-dimensional cultures that mimic *in vivo* conditions is an extremely important direction of research on new models for *in vitro* experiments.
2. The value of positive PD-L1 expression in predicting the response to BCG immunotherapy and the risk of NMIBC recurrence and progression is questionable. The current evidence, based only on retrospective data, is highly heterogeneous.
3. OS and DSS may benefit from the administration of AC to patients with advanced residual disease following RC preceded by NAC. This does not apply to patients with lymph node metastases.
4. The use of the RIVM strain in patients with high-risk NMIBC is associated with a significantly lower RFS compared to the Moreau and TICE strains, which determines their preferential use.
5. USP5 protein is overexpressed in human BC cells compared to normal urothelial cells, suggesting the possibility of using selective USP5 protein inhibitors as potential forms of anticancer therapy.
6. Degrasyn, a selective inhibitor of USP5, USP9X, and USP14, shows anticancer activity against human and canine BC cells. The anticancer effect is exerted by inhibition of cell proliferation, induction of apoptosis, and induction of DNA damage.

7. The anticancer activity of selective USP inhibitor (degrasyn) is higher compared to the non-selective USP inhibitor (PR-619).

5. OMÓWIENIE ROZPRAWY DOKTORSKIEJ

5.1. Wstęp

Rak pęcherza moczowego (*bladder cancer* – BC) jest drugim co do częstości występowania nowotworem wywodzącym się z układu moczowego, czwartym pod względem częstości rozpoznawania nowotworem złośliwym u mężczyzn i ósmym wśród kobiet [1]. Według aktualnych danych Krajowego Rejestru Nowotworów w Polsce co roku odnotowuje się około 7500 nowych przypadków BC, z czego 4000 pacjentów rocznie umiera z powodu tego nowotworu [2].

Najczęstszym typem histopatologicznym BC jest wywodzący się z nabłonka pokrywającego drogi moczowe (urotelium) rak urotelialny. Stwierdzany jest on u około 90 - 95% pacjentów [3,4]. W momencie postawienia diagnozy 75% przypadków BC stanowią raki pęcherza moczowego nienaciekające mięśniówki (*non-muscle-invasive bladder cancer* – NMIBC), natomiast pozostałe 25% stanowią raki pęcherza moczowego naciekające mięśniówkę (*muscle-invasive bladder cancer* – MIBC) [3,4]. Do NMIBC zalicza się zmiany ograniczone do błony śluzowej (stadium zaawansowania Ta i *carcinoma in situ* – CIS) oraz naciekające błonę podśluzową pęcherza (stadium zaawansowania T1) [3]. Do MIBC zalicza się raki naciekające lub przekraczające błonę mięśniową pęcherza (stadium zaawansowania T2 i więcej) [4].

Postępowanie terapeutyczne w BC zależy głównie od stopnia jego zaawansowania. Podstawą leczenia NMIBC jest doszczętna, przezcewkowa elektroresekcja guza pęcherza moczowego (*transurethral resection of bladder tumor* – TURBT). Po wykonaniu TURBT, w przypadku raków NMIBC o wysokim ryzku progresji, złotym standardem leczenia jest dopęchrzowa immunoterapia adjuwantowa z wykorzystaniem atenuowanych szczepów prątków Mycobacterium bovis (*Bacillus Calmette-Guerin* – BCG) [3]. Wiele badań przeprowadzonych w ostatnich kilkudziesięciu latach potwierdziło skuteczność immunoterapii BCG w profilaktyce nawrotów i progresji guzów NMIBC wysokiego ryzyka [5]. Jednak nawet do 40% pacjentów otrzymujących wlewki dopęcherzowe z BCG nie odpowiada na leczenie, a u ok. 20% pacjentów dochodzi do progresji guza do stadium MIBC [5]. Aktualnie do oceny ryzyka progresji i wznowy NMIBC u pacjentów poddanych immunoterapii BCG wykorzystywane są modele stworzone przez Club Urológico Español de Tratamiento Oncológico (CUETO) [6] oraz European Organisation for Research and Treatment of Cancer (EORTC 2016) [7]. Niedoskonałość wyżej wymienionych modeli, opartych wyłącznie na parametrach kliniczno-patologicznych, nakłada konieczność prowadzenia dodatkowych badań

nad predyktorami (np. markerami molekularnymi) wznowy, progresji oraz niepowodzenia immunoterapii BCG u pacjentów z NMIBC wysokiego ryzyka.

W immunoterapii dopęcherzowej BCG stosowanych jest kilkanaście różnych szczepów bakteryjnych wykazujących odrębności genetyczne między sobą [8]. Postuluje się, że różnice w odpowiedzi immunologicznej na terapię BCG pomiędzy pacjentami mogą być konsekwencją tychże odmienności, a co za tym idzie odrębności genetyczne szczepów bakteryjnych mogą odpowiadać za obserwowane różnice w wynikach onkologicznych pacjentów z NMIBC wysokiego ryzyka [8,9]. Międzynarodowe wytyczne Europejskiego Towarzystwa Urologicznego (*European Association of Urology* – EAU) nie zawierają konkretnych zaleceń dotyczących stosowania poszczególnych szczepów ze względu na niewielką ilość danych literaturowych porównujących zastosowanie szczepów BCG. Aktualnie na świecie obserwuje się rosnące niedobory w dostawach wlewek BCG [10]. Dlatego tak ważna staje się identyfikacja efektywności zastosowania poszczególnych szczepów BCG w zapobieganiu nawrotom oraz progresji NMIBC wysokiego ryzyka.

Standardową formą leczenia pacjentów, u których wykryto MIBC w stadium miejscowo zaawansowanym (T2 – T4a, N0 – Nx, M0) jest cystektomia radykalna (*radical cystectomy* – RC) wraz z wycięciem regionalnych węzłów chłonnych i następowym wytworzeniem alternatywnej drogi odprowadzenia moczu [4]. Zgodnie z aktualnymi danymi literaturowymi zastosowanie u tej grupy chorych chemioterapii neoadjuwantowej (*neoadjuvant chemotherapy* – NAC) przed RC wiąże się z 8% wzrostem odsetka 5–letniego przeżycia całkowitego (*overall survival* – OS) [4]. Zastosowanie NAC u części pacjentów prowadzi do obniżenia stopnia zaawansowania guza pierwotnego. Jednak nawet u 50% chorych, mimo leczenia adjuwantowego, obserwuje się obecność zaawansowanej choroby rezydualnej, definiowanej jako obecność w badaniu histopatologicznym guza w stopniu zaawansowania T3-T4 i/lub przerzutów w węzłach chłonnych [11]. Obecnie w takich przypadkach nie ma jednego standardu opieki i leczenia.

Mimo pojawienia się w ostatnich kilkunastu latach nowych form leczenia MIBC w stadium przerzutowym, głównie immunoterapii, pięcioletnie przeżycia chorych nie przekraczają 15 - 20% [4]. Dlatego prowadzone obecnie badania skupiają się na poszukiwaniu nowych czynników prognostycznych i metod terapeutycznych, np. badania markerów molekularnych. Uważa się, że markery mogą mieć zastosowanie w prognozowaniu przebiegu klinicznego u chorych z MIBC zarówno w stadium zaawansowanym, jak i przerzutowym, a także mogą stanowić potencjalny cel terapeutyczny.

Jednym z obiecujących kierunków badań jest ocena wpływu zaburzeń ekspresji białek związanych z procesem deubikwitynylacji, proteaz specyficznych dla ubikwityny (*ubiquitin-specific* proteases – USP) na proces karcynogenezy oraz tworzenia przerzutów w różnych typach nowotworów [12]. Procesy kontrolowane przez USP mają kluczowe znaczenie dla prawidłowego funkcjonowania szlaków komórkowych, takich jak naprawa DNA oraz apoptoza komórki [12,13]. Z tego względu USP mogą stanowić potencjalne cele molekularne w terapii przeciwnowotworowej. Dotychczasowe badania na temat roli i możliwości zastosowania inhibitorów USP w BC były niezwykle skąpe [14,15].

Onkologia porównawcza jest szybko rozwijającą się dziedziną, mającą na celu integrację badań dotyczących naturalnie występujących nowotworów u zwierząt i ludzi. Spośród różnych gatunków zwierząt za najodpowiedniejszy model BC uważany jest pies, co wynika z podobieństw anatomicznych i fizjologicznych [16]. Ponadto BC jest dość częstym nowotworem u psów (stanowiąc 2% wszystkich nowotworów) [16]. Jednak w przeciwieństwie do ludzi, większość BC u psów (90% przypadków) w momencie zdiagnozowania jest w stadium zaawansowanego lub przerzutowego BC. Dodatkowo guzy te często charakteryzują się agresywnym przebiegiem klinicznym i opornością na chemioterapię [16]. Bardzo złe rokowanie w kontekście przeżycia u psów z BC oraz możliwość wykorzystania modelu psiego w onkologii porównawczej pozwalają na prowadzenie symultanicznych badań nad rozwojem nowych związków terapeutycznych mających potencjalne zastosowanie zarówno w medycynie ludzkiej, jak i weterynaryjnej. Ze względu na częste problemy natury etycznej dotyczące badań na modelach zwierzęcych *in vivo,* psie modele *in vitro* mogą stanowić cenne narzędzie badawcze, pozwalające na analizę nowych biomarkerów i punktów terapeutycznych.

5.2. Cel badań

Celem badań Rozprawy Doktorskiej była analiza wybranych markerów molekularnych mających potencjalne znaczenie prognostyczne, jak i stanowiących potencjalny cel terapeutyczny w leczeniu BC oraz analiza wpływu wybranych form terapeutycznych na wyniki leczenia pacjentów z BC.

Celem realizacji założeń Programu „ProHum – Interdyscyplinarna Szkoła Doktorska – planowanie badań eksperymentalnych, tworzenie i optymalizacja zwierzęcych modeli doświadczalnych z umiejętnościami transferowania ich do badań klinicznych w medycynie człowieka" w wybranych badaniach wykorzystywano zarówno modele ludzkie, jak i zwierzęce.

Cele szczegółowe:

1. Usystematyzowanie aktualnej wiedzy na temat dostępnych modeli *in vitro* BC oraz ich zastosowania w badaniach nad nowymi markerami molekularnymi i środkami terapeutycznymi.
2. Usystematyzowanie aktualnej wiedzy na temat związku ekspresji PD-L1 z parametrami onkologicznymi i odpowiedzią kliniczną na immunoterapię BCG u pacjentów z NMIBC wysokiego ryzyka.
3. Usystematyzowanie aktualnej wiedzy na temat wpływu AC na wyniki onkologiczne pacjentów z zaawansowaną chorobą rezydualną po RC poprzedzonej NAC.
4. Analiza różnic skuteczności przeciwnowotworowej różnych szczepów bakteryjnych BCG używanych do immunoterapii dopęcherzowej raków NMIBC wysokiego ryzyka.
5. Ocena i porównanie ekspresji wybranych białek USP w modelowych liniach komórkowych ludzkiego nabłonka urotelialnego oraz ludzkiego i psiego BC.
6. Określenie potencjału i siły przeciwnowotworowego działania inhibitorów USP w stosunku do modelowych linii komórkowych BC człowieka oraz psa.
7. Porównanie siły działania przeciwnowotworowego selektywnego (degrasyn) oraz nieselektywnego (PR-619) inhibitora USP.

5.3. Materiały i metody

W niniejszym podrozdziale zaprezentowano zarys metodologii przeprowadzonych badań. Szczegółowy opis zawarty jest w załączonych publikacjach.

W Publikacji 1 dokonano przeglądu systematycznego dostępnych danych literaturowych na temat psich modeli *in vitro* BC oraz ich potencjalnego zastosowania w kontekście badań nad nowymi biomarkerami i środkami terapeutycznymi. Selekcji prac dokonano na podstawie analizy dwóch elektronicznych baz danych: PubMed oraz Scopus. Ostatnie wyszukiwanie przeprowadzono 28 stycznia 2022 roku. Zastosowano następującą kombinację słów kluczowych: (”bladder” OR ”transitional” OR ”urothelial”) AND (”cancer” OR ”carcinoma” OR ”neoplasm”) AND (”in vitro” OR ”model” OR ”cell*” OR ”culture”) AND (”dog” OR ”canine”).

W Publikacji 2 dokonano przeglądu systematycznego dostępnych danych literaturowych na temat związku ekspresji ligandu receptora programowanej śmierci 1 (*programmed death ligand 1* – PD-L1) z parametrami onkologicznymi i odpowiedzią kliniczną na immunoterapię BCG u pacjentów z NMIBC wysokiego ryzyka. Selekcji prac dokonano na podstawie analizy trzech

elektronicznych baz danych: PubMed, Scopus oraz Embase. Przegląd literatury przeprowadzono w maju 2020 roku. Zastosowano następującą kombinację słów kluczowych: (”bladder cancer”) AND (”non-muscle invasive” OR ”NMIBC”) AND (”PD-L1” OR ”PDL1”) AND (”BCG” OR ”immunotherapy”).

W Publikacji 3 dokonano przeglądu systematycznego i metaanalizy dostępnych danych literaturowych na temat wpływu chemioterapii adjuwantowej (*adjuvant chemotherapy* – AC) na wyniki onkologiczne pacjentów z zaawansowaną chorobą rezydualną (obecność guza w stopniu zaawansowania T3-T4 i/lub potwierdzone histopatologicznie przerzuty do węzłów chłonnych) po RC poprzedzonej NAC. Selekcji prac dokonano na podstawie analizy trzech elektronicznych baz danych: Pubmed, Embase oraz Cochrane Library. Ostatnie wyszukiwanie przeprowadzono 12 listopada 2020 roku. Zastosowano następującą kombinację słów kluczowych: (”adjuvant chemotherapy” OR ”AC”) AND (”neoadjuvant chemotherapy” OR ”NAC”) AND (”bladder cancer” OR ”muscle-invasive bladder cancer” OR ”MIBC”) AND (”residual disease” OR ”locally advanced disease”). W ramach przeprowadzonej metaanalizy, istotność statystyczną efektu łączonego dla współczynników hazardu (*hazard ratio* – HR) oraz 95% przedziałów ufności (*confidence interval* – CI) oceniano za pomocą testu Z. Wykorzystano model efektów losowych lub stałych w zależności od obserwowanej niejednorodności prac, ocenionej za pomocą testu Q Cochrana. Pierwszorzędowy punkt końcowy stanowiło przeżycie całkowite (*overall survival* – OS), natomiast drugorzędowy punkt końcowy stanowiło przeżycie do zgonu wywołanego badaną chorobą (*disease-specific survival* – DSS).

W Publikacji 4 przeanalizowano retrospektywnie dane 590 pacjentów z potwierdzonym histopatologicznie pierwotnym NMIBC w stopniu zaawansowania T1 i o wysokim stopniu złośliwości histopatologicznej (*high-grade* – HG). Wszyscy pacjenci włączeni do badania otrzymywali dopęcherzową immunoterapię BCG jako formę leczenia adjuwantowego. Stosowane były trzy różne szczepy BCG: Moreau, TICE oraz RIVM. Wybór rodzaju szczepu wynikał przede wszystkim z różnic w aktualnej podaży i dystrybucji leku. Pierwszorzędowe punkty końcowe badania stanowiły przeżycie wolne od nawrotu (*recurrence-free survival* – RFS) oraz przeżycie wolne od progresji (*progression-free survival* – PFS) w zależności od zastosowanego szczepu BCG. Podstawą analizy statystycznej było wykorzystanie modelu proporcjonalnego hazardu Coxa. Ze względu na różnice w wyjściowej charakterystyce socjodemograficznej pacjentów w analizie zastosowano dodatkowo metodę ważenia odwrotnego prawdopodobieństwa (*inverse probability weighting* – IPW).

W Publikacji 5 oceniono ekspresję wybranych białek USP (USP5, USP9X, USP14), jak i potencjał oraz siłę działania przeciwnowotworowego selektywnego (degrasyn) i nieselektywnego (PR-619) inhibitora USP na modelowe linie komórkowe prawidłowego nabłonka urotelialnego oraz modelowe linie komórkowe raka pęcherza moczowego. Do badań użyto następujących ustalonych linii komórkowych: SV-HUC-1 (prawidłowe komórki ludzkiego nabłonka urotelialnego); T24 (komórki ludzkiego raka urotelialnego w stopniu zaawansowania G3); K9TCC-PU-NK (komórki psiego raka urotelialnego w stopniu zaawansowania G3); RDSVS-TCC1 (komórki psiego raka urotelialnego w stopniu zaawansowania G3). Wpływ inhibitorów USP na proliferację komórek określony został za pomocą testu MTT. Nasilenie apoptozy po zastosowaniu inhibitorów USP oceniano przy użyciu testu z zastosowaniem Aneksyny V i jodku propidyny oraz poprzez analizę aktywacji kaspazy 3/7 za pomocą cytometrii przepływowej. Do oceny ekspresji wybranych białek z rodziny USP, białek związanych z uszkodzeniem DNA oraz kluczowych białek zaangażowanych w proces apoptozy wykorzystano metodę Western Blot.

5.4. Podsumowanie wyników

<u>Publikacja 1.</u> Nowak, Ł., Krajewski, W., Małkiewicz, B., Szydełko, T., & Pawlak, A. (2022). Characteristics and Applications of Canine In Vitro Models of Bladder Cancer in Veterinary Medicine: An Up-to-Date Mini Review. Animals (Basel), 12(4), 516.

W Publikacji 1 został szczegółowo opisany aktualny stan wiedzy na temat dostępnych psich modeli *in vitro* BC oraz ich zastosowaniach w badaniach nad nowymi markerami molekularnymi i środkami terapeutycznymi.

Na podstawie danych pochodzących z 33 włączonych prac dokonano usystematyzowania dostępnej wiedzy na temat psich modeli *in vitro* BC. Podsumowano charakterystykę histopatologiczną i molekularną 19 ustalonych linii komórkowych (Tabela 1 oraz Tabela 2 w załączonym manuskrypcie) oraz omówiono wady i zalety hodowli dwu- i trójwymiarowych. Większość dostępnych ustalonych linii komórkowych BC psa charakteryzuje się pochodzeniem z pierwotnie inwazyjnych guzów pęcherza o wysokim stopniem złośliwości (G3). Ustalone linie komórkowe (hodowle dwuwymiarowe) są najczęściej wykorzystywanym materiałem do badań naukowych nad nowymi biomarkerami i lekami, przede wszystkim ze względu na swój relatywnie niski koszt oraz możliwość prowadzenia eksperymentów w ściśle kontrolowanych warunkach laboratoryjnych. Jednakże badania z użyciem ustalonych linii komórkowych nie odzwierciedlają dokładnie warunków panujących *in vivo* głównie z powodu braku dostępnych

interakcji komórek nowotworu z mikrośrodowiskiem guza. Nieograniczony dostęp ustalonych linii komórkowych do tlenu i składników odżywczych, w przeciwieństwie do warunków panujących *in vivo*, może ponadto indukować nagromadzenie zmian genetycznych, które nie występują w guzach pierwotnych. Z powodu wyżej wymienionych ograniczeń, mimo szerokiego zastosowania ustalonych linii komórkowych BC psa w badaniach nad nowymi biomarkerami i terapiami molekularnymi, wiele wyników uzyskanych w takich eksperymentach nie przekłada się następnie na zastosowanie kliniczne. W literaturze opisane zostały przykłady komórkowych hodowli trójwymiarowych BC psa. Są to organoidy powstałe z wyselekcjonowanych komórek urotelialnych izolowanych z moczu pacjentów z BC. Komórki po wstępnej selekcji umieszczane są na rusztowaniach tkankowych. Powstałe hodowle mają budowę sferoidalną imitującą warunki panujące *in vivo*, co mimo wysokich kosztów prowadzenia hodowli zwiększa translacyjność uzyskanych wyników.

Publikacja 2. Nowak, Ł., Krajewski, W., Poterek, A., Śliwa, A., & Zdrojowy, R. (2020). The prognostic value of programmed cell death protein ligand 1 in patients with non-muscle-invasive bladder cancer treated with bacille Calmette-Guérin immunotherapy: Current status. Arab journal of urology, 19(1), 67–70.

W Publikacji 2 został szczegółowo opisany aktualny stan wiedzy na temat związku ekspresji PD-L1 z parametrami onkologicznymi i odpowiedzią kliniczną na immunoterapię BCG u pacjentów z NMIBC wysokiego ryzyka.

Zidentyfikowano 5 badań analizujących związek ekspresji PD-L1 z parametrami onkologicznymi i odpowiedzią kliniczną na immunoterapię BCG u pacjentów z NMIBC wysokiego ryzyka (Tabela 1 w załączonym manuskrypcie). Dostępne dane literaturowe na temat związku pozytywnej ekspresji PD-L1 i oporności na BCG terapię były wysoce niejednorodne. Jedynie w 2 z 5 dostępnych prac wykazano związek pozytywnej ekspresji PD-L1 z opornością na leczenie BCG u pacjentów z NMIBC wysokiego ryzyka. W żadnej z dostępnych prac nie wykazano prognostycznego znaczenia pozytywnej ekspresji PD-L1 w kontekście parametrów RFS i PFS.

Publikacja 3. Krajewski, W., Nowak, Ł., Moschini, M., Poletajew, S., Chorbińska, J., Necchi, A., Montorsi, F., Briganti, A., Sanchez-Salas, R., Shariat, S. F., Palou, J., Babjuk, M., Teoh, J. Y., Soria, F., Pradere, B., Ornaghi, P. I., Pawlak, A., Dembowski, J., &

Zdrojowy, R. (2021). Impact of Adjuvant Chemotherapy on Survival of Patients with Advanced Residual Disease at Radical Cystectomy following Neoadjuvant Chemotherapy: Systematic Review and Meta-Analysis. Journal of clinical medicine, 10(4), 651.

W Publikacji 3 został szczegółowo opisany aktualny stan wiedzy na temat wpływu AC na wyniki onkologiczne pacjentów z zaawansowaną chorobą rezydualną po RC poprzedzonej NAC. Przegląd systematyczny został uzupełniony metaanalizą.

Zidentyfikowano 6 badań, które analizowały wpływ AC po RC poprzedzonej NAC na wyniki onkologiczne pacjentów z zaawansowaną chorobą rezydualną (obecność guza w stopniu zaawansowania T3-T4 i/lub potwierdzone histopatologicznie przerzuty do węzłów chłonnych). Spośród 3096 pacjentów, 2355 (76,1%) stanowiło grupę kontrolną (pacjenci poddani obserwacji po RC), natomiast 741 (23,9%) pacjentów otrzymało AC. Wszystkie uwzględnione badania miały retrospektywny charakter. Mediana okresu obserwacji wahała się od 30 do 50 miesięcy. W porównaniu do grupy kontrolnej pacjenci otrzymujący AC mieli istotnie statystycznie wyższy wskaźnik OS (HR 0,84; 95% CI 0,75-0,94; p = 0,002) oraz DSS (HR 0,56; 95% CI 0,32-0,99; p = 0,05). Dodatkowa analiza podgrupy pacjentów z potwierdzonymi histopatologicznie przerzutami do węzłów chłonnych wykazała, że stosowanie AC nie wpływało istotnie na OS (HR 0,89; 95% CI 0,58-1,35; p = 0,58).

<u>Publikacja 4.</u> Nowak, Ł., Krajewski, W., Moschini, M., Chorbińska, J., Poletajew, S., Tukiendorf, A., Muilwijk, T., Joniau, S., Tafuri, A., Antonelli, A., Orlando, R., Di Trapani, E., Alvarez-Maestro, M., Simone, G., Zamboni, S., Simeone, C., Marconi, M. C., Mastroianni, R., Piszczek, R., Xylinas, E., European Association of Urology - Young Academic Urologists (EAU-YAU): Urothelial Carcinoma Working Group (2021). Assessment of the oncological outcomes of three different bacillus Calmette-Guérin strains in patients with high-grade T1 non-muscle-invasive bladder cancer. Arab journal of urology, 19(1), 78–85.

Publikacja 4 jest pracą badawczą analizującą kliniczne różnice w skuteczności przeciwnowotworowej różnych szczepów bakteryjnych BCG używanych do immunoterapii dopęcherzowej raków NMIBC wysokiego ryzyka.

Spośród 590 pacjentów poddanych analizie, 138 (23,4%) chorych otrzymywało szczep Moreau, natomiast 272 (46,1%) i 180 (30,5%) pacjentów było leczonych szczepami TICE i RIVM.

5-letni wskaźnik RFS wynosił 70,5% u pacjentów leczonych szczepem Moreau, 66,7% u pacjentów leczonych szczepem TICE, oraz 55,2% u pacjentów leczonych szczepem RIVM (p = 0,016). Nie wykazano statystycznie istotnej różnicy w RFS pomiędzy szczepami Moreau oraz TICE. Stosowanie szczepu RIVM wiązało się z istotnie wyższym prawdopodobieństwem nawrotu guza w porównaniu do dwóch pozostałych szczepów BCG. 5-letni wskaźnik PFS wynosił 84%, 85% i 77,8%: odpowiednio w grupach Moreau, TICE i RIVM. Analizowane szczepy nie różniły się istotnie w kontekście 5-letniego wskaźnika PFS (p = 0,108). Analiza regresji proporcjonalnego hazardu Coxa z wykorzystaniem metody IPW nie wykazała istotnie statystycznej różnicy w RFS dla szczepów Moreau i TICE (p = 0,69), podczas gdy stosowanie szczepu RIVM było związane z istotnie niższym RFS w porównaniu do szczepu Moreau (HR 1,69; 95% CI 1,03-2,78; p = 0,034) oraz szczepu TICE (HR 1,87; 95% CI 1,25-2,81, p = 0,002). Nie stwierdzono istotnych różnic w PFS pomiędzy poszczególnymi szczepami.

<u>Publikacja 5.</u> Nowak, Ł., Krajewski, W., Dejnaka, E., Małkiewicz, B., Szydełko, T., & Pawlak, A. (2023). Ubiquitin-Specific Proteases as Potential Therapeutic Targets in Bladder Cancer-In Vitro Evaluation of Degrasyn and PR-619 Activity Using Human and Canine Models. Biomedicines, 11(3), 759.

Publikacja 5 jest pracą badawczą oceniającą ekspresję wybranych białek USP (USP5, USP9X, USP14) w komórkach BC człowieka i psa oraz analizującą potencjał oraz siłę działania przeciwnowotworowego selektywnego (degrasyn) i nieselektywnego (PR-619) inhibitora USP na komórki BC.

W porównaniu z nienowotworowymi ludzkimi komórkami urotelialnymi (SV-HUC-1), komórki modelowej ludzkiej linii BC (T24) charakteryzowały się wyższą ekspresją białka USP5. Stwierdzono ponadto porównywalny poziom ekspresji białek USP9X i USP14. W teście MTT wykazano, że degrasyn (selektywny inhibitor USP5, USP9X oraz USP14) hamował proliferację wszystkich badanych linii komórkowych w sposób zależny od stężenia i czasu. Analiza 24-godzinnego czasu inkubacji wykazała istotnie mniejszą aktywność antyproliferacyjną degrasynu wobec kontrolnej ludzkiej linii komórkowej SV-HUC-1 niż wobec modelowej, nowotworowej linii komórkowej T24 (p < 0,05). Przy ocenie wszystkich czasów inkubacji (24, 48 oraz 72 godziny) komórki BC człowieka były bardziej wrażliwe na antyproliferacyjne działanie degrasynu w porównaniu do psich linii komórkowych (K9TCC-PU-NK i RDSVS-TCC1). Stwierdzona aktywność antyproliferacyjna degrasynu była istotnie wyższa niż drugiego zastosowanego inhibitora, PR-619.

Po potwierdzeniu działania antyproliferacyjnego degrasynu zbadano, czy hamowanie proliferacji komórek miało związek z indukcją procesu apoptozy w komórkach. Po 24 godzinach inkubacji komórek z degrasynem w rosnących stężeniach stwierdzono znaczny wzrost odsetka komórek wczesno- i późnoapoptotycznych w liniach T24 oraz RDSVS-TCC1. W przeciwieństwie do degrasynu, PR-619 nie wykazywał istotnego działania proapoptotycznego. W badaniu cytometrycznym stwierdzono dodatkowo, że zastosowanie degrasynu powoduje aktywację efektorowej kaspazy 3/7. Wyniki analizy mechanizmu proapoptotycznego działania degrasynu wykazały, że po 24-godzinnej inkubacji pod jego wpływem w komórkach BC zarówno człowieka, jak i psa, dochodzi do zmniejszenia poziomu ekspresji białek antyapoptotycznych Bcl-xl oraz Bcl-2. W analizowanych liniach komórkowych nie zauważono wyraźnie widocznych zmian w poziomie ekspresji Bcl-xl oraz Bcl-2 po inkubacji komórek BC ze wzrastającymi stężeniami PR-619. Po 24-godzinnej inkubacji z degrasynem we wszystkich użytych linii komórkowych zaobserwowano zwiększenie ekspresji fosforylowanej formy białka H2AX (γH2AX) będącego markerem uszkodzenia DNA. 24-godzinna inkubacja z PR-619 nie wywoływała wyraźnego wzrostu ekspresji γH2AX.

5.5. Etyka badań

Przedstawione badania nie stanowiły eksperymentu medycznego w rozumieniu art. 21 ust. 1 ustawy z dnia 5 grudnia 1996 r. i zawodach lekarza i lekarza dentysty (Dz. U. z 2018 r. poz 617) i nie wymagały uzyskania opinii Komisji Bioetycznej, o której mowa w art. 29 ust. 1 ww. ustawy.

5.6. Wnioski

1. Ustalone linie komórkowe BC psa są wartościowym materiałem do badań naukowych nad nowymi biomarkerami i lekami, jednak nie odzwierciedlają one dokładnie warunków panujących *in vivo*. Tworzenie hodowli trójwymiarowych imitujących warunki *in vivo* jest niezwykle istotnym kierunkiem badań nad nowymi modelami BC, służącymi do badań eksperymentalnych.
2. Wartość pozytywnej ekspresji PD-L1 w przewidywaniu odpowiedzi na immunoterapię BCG oraz ryzyko nawrotu i progresji NMIBC wysokiego ryzyka jest wątpliwa. Aktualne dowody, oparte tylko na danych retrospektywnych, są wysoce heterogenne.

3. Podawanie AC pacjentom z zaawansowaną chorobą rezydualną chorobą po RC poprzedzonej NAC może mieć pozytywny wpływ na OS i DSS. Nie dotyczy to pacjentów ze stwierdzonymi przerzutami w węzłach chłonnych.
4. Stosowanie szczepu RIVM u pacjentów z NMIBC wysokiego ryzyka progresji wiąże się z istotnie niższym RFS w porównaniu do szczepów Moreau oraz TICE, co warunkuje ich preferencyjne stosowanie.
5. W komórkach BC człowieka białko USP5 jest nadekspresjonowane w porównaniu do komórek prawidłowych,, co sugeruje możliwość wykorzystania selektywnych inhibitorów białka USP5 jako potencjalnych form terapii przeciwnowotworowej.
6. Degrasyn, selektywny inhibitor białek USP5, USP9X oraz USP14 wykazuje aktywność przeciwnowotworową w stosunku do komórek BC człowieka i psa. Działanie przeciwnowotworowe wywierane jest poprzez zahamowanie proliferacji komórkowej, indukcję apoptozy oraz indukcję uszkodzenia DNA.
7. Aktywność przeciwnowotworowa selektywnego inhibitora białek USP (degrasynu) jest wyższa w porównaniu do nieselektywnego inhibitora białek USP (PR-619).

6. ARTYKUŁ PIERWSZY:

Characteristics and Applications of Canine In Vitro Models of Bladder Cancer in Veterinary Medicine: An Up-to-Date Mini Review

animals

MDPI

Review

Characteristics and Applications of Canine In Vitro Models of Bladder Cancer in Veterinary Medicine: An Up-to-Date Mini Review

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Simple Summary: Bladder cancer (BC) in dogs is often lethal at the time of diagnosis. Therefore, there is a constant need for novel research on improvements of its characterization and treatment. Due to high cost and limited number of available dog patients, in vitro models of canine BC have been increasingly used for the last 25 years. In the present article, we present existing in vitro models of canine BC, including available simple (two-dimensional) and more complex (three-dimensional) models.

Abstract: Bladder cancer (BC) constitutes approximately 2% of all spontaneously occurring cancers in dogs. It is characterized by a devastating clinical course in most cases, which emphasizes a constant need for the development of novel methods of disease characterization and treatment. Over the past years, advances in cell engineering have resulted in the development of various canine in vitro models of BC, emerging as complements for in vivo research. In this article, we aimed to review the available data on existing in vitro models of canine BC, focusing primarily on their characteristics, applications in veterinary medicine, as well as advantages and disadvantages. The most commonly used in vitro models of canine BC comprise immortalized cell lines grown as adherent monolayers. They provide an unlimited supply of research material, however, they do not faithfully reflect the conditions prevailing in vivo, since the spatial cellular interactions are lost. The importance of the three-dimensional (3D) features of solid tumors in relation to carcinogenesis or drug response process has resulted in the development of the first canine 3D models of BC available for in vitro research. So far, results obtained with in vitro and in vivo research should be interpreted together. With the constantly growing complexity of in vitro models of BC cancer, animal-based research might be reduced in the future.

Keywords: canine; bladder; urothelial cancer; in vitro model

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1. Introduction

Bladder cancer (BC) constitutes approximately 2% of all spontaneously occurring cancers in dogs [1]. With estimates that 4–6 million pet dogs develop cancer in the United States annually, this equates to more than 60,000 cases of BC in dogs each year [2]. More than 95% of canine BCs are urothelial carcinomas (UCs), also known as transitional cell carcinomas (TCCs). Most canine BCs are characterized by adverse histopathological features at the time of diagnosis, such as muscle infiltration and high cellular grade [1–3]. Distant metastases are initially found in about 20% of newly diagnosed cases, leaving most dogs incurable [1–3]. The devastating clinical course and poor survival outcomes in considerable number of dogs with BC emphasize a constant need for development of

novel research tools used for disease characterization and treatment, primarily resulting in survival outcomes improvements.

Though obvious benefits that could be gained from the research of canine BC in vivo, there are some inevitable limitations (e.g., high cost, long duration, and insufficient numbers of pet dogs to test even a fraction of the new drugs, especially when considering various possible drug combinations), making extensive in vivo research difficult [1,2]. Current strategies to overcome these challenges include increased utilization of in vitro models. Their resemblance to the primary tumors in the context of molecular behavior and genomic landscape has been increasingly evaluated in the past few years. Moreover, advances in cell engineering have resulted in the development of novel complex in vitro models of canine BC, emerging as unique complements for in vivo research.

In the present article, we aimed to review the available data on existing in vitro models of canine BC as a tool in veterinary research, focusing primarily on their characteristics, potential applications in veterinary medicine, and advantages and disadvantages. To the best of our knowledge, this is the first review summarizing the current evidence regarding this topic.

2. Evidence Acquisition

We conducted a literature search using two electronic databases, namely, Pubmed and Scopus. The most recent search was performed on 28 January 2022. Screening of the literature was conducted using the following search string: ("bladder" OR "transitional" OR "urothelial") AND ("cancer" OR "carcinoma" OR "neoplasm") AND ("in vitro" OR "model" OR "cell*" OR "culture") AND ("dog" OR "canine"). Auto-alerts in Medline were run, as well as reference lists of original articles and review articles for further eligible data. We exclusively included data regarding in vitro models of canine bladder TCC. Only papers in English were considered eligible without restrictions on publication year. The flow diagram of the study selection process is presented in Figure 1.

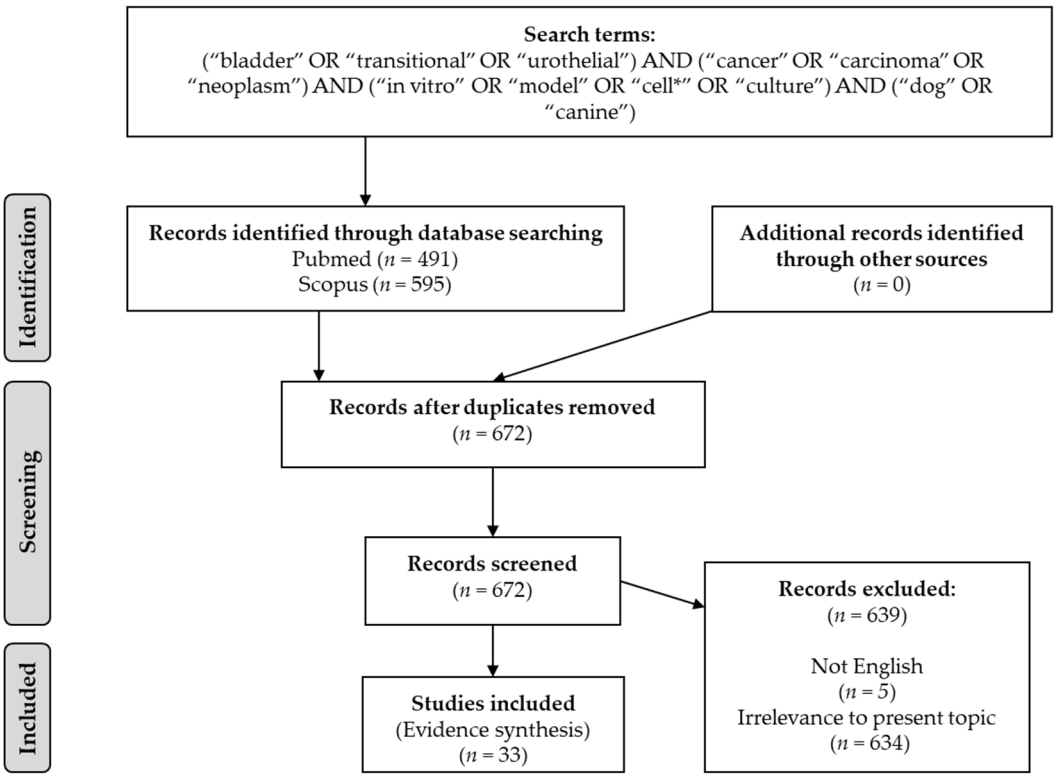

Figure 1. Flow diagram of study selection process.

3. Evidence Synthesis

3.1. Two Dimensional (2D) Models

The most commonly used two dimensional (2D) models of canine BC comprise immortalized cell lines and primary cell cultures grown as adherent monolayers in appropriate media. The first immortalized canine BC cell line, called K9TCC, was established by Knapp et al. in 1995 [4]. Since then, several novel canine BC cell lines have been developed and described in the literature [5–10]. Almost all of these cell lines were established from invasive and metastatic tumors, benefiting the investigation of late tumor progression and metastatic lesions. Their baseline characteristics were presented in Table 1.

Table 1. Baseline characteristics of the available canine bladder cancer cell lines.

Cell Line Name	First Report Date	Development	Characteristics of Primary Tumor				Doubling Time	Reference (First Report)
			Breed of Origin	Age at Sampling	Gender	Pathological Data		
K9TCC	1995	Cultured cells from bladder tumor biopsy samples	Mixed breed	NR	Female	Invasive TCC	24 h	[4]
K9TCC-PU-AxA	2009	Cultured cells from bladder tumor biopsy samples	NR	NR	Female	Invasive TCC G3	23.5 h	[5]
K9TCC-PU-AxC	2009	Cultured cells from bladder tumor biopsy samples	NR	NR	Female	Invasive TCC G3	36.2 h	[5]
K9TCC-PU-In	2009	Cultured cells from bladder tumor biopsy samples	German Shepherd	NR	Female	Invasive TCC G3	41.2 h	[5]
K9TCC-PU-Mx	2009	Cultured cells from bladder tumor biopsy samples	German Shepherd	NR	Female	Invasive TCC G3	23.5 h	[5]
K9TCC-PU-Nk	2009	Cultured cells from bladder tumor biopsy samples	NR	NR	Female	Invasive TCC G3	58.4 h	[5]
K9TCC-PU-Pu	2009	Cultured cells from bladder tumor biopsy samples	NR	NR	Female	Invasive TCC G3	51.8 h	[5]
K9TCC-PU-Sh	2009	Cultured cells from bladder tumor biopsy samples	Collie	NR	Female	Invasive TCC G3	29.1 h	[5]
Bliley	2012	NR	Shetland Sheepdog	NR	Female	TCC	20 h	[6]
K9TCC#1Lille	2014	Cultured cells from bladder tumor biopsy samples	Pointer	16 years	Female	Invasive TCC	47.4 h	[7]
K9TCC#2Dakota	2014	Cultured cells from bladder tumor biopsy samples	Bichon Fries	13 years	Female	Invasive TCC	31.96 h	[7]
K9TCC#4Molly	2014	Cultured cells from bladder tumor biopsy samples	Maltese	10 years	Female	Invasive TCC	44.69 h	[7]
K9TCC#5Lilly	2014	Cultured cells from bladder tumor biopsy samples	Mixed breed	13 years	Female	Invasive TCC	48.3 h	[7]
LCTCC	2015	Cultured cells from bladder tumor biopsy samples	NR	NR	NR	TCC	NR	[8]
MCTCC	2015	Cultured cells from bladder tumor biopsy samples	NR	NR	NR	TCC	NR	[8]
MegTCC	2015	Cultured cells from bladder tumor biopsy samples	NR	NR	NR	TCC	NR	[8]

Table 1. *Cont.*

Cell Line Name	First Report Date	Development	Characteristics of Primary Tumor				Doubling Time	Reference (First Report)
			Breed of Origin	Age at Sampling	Gender	Pathological Data		
MonoTCC	2015	Cultured cells from bladder tumor biopsy samples	NR	NR	NR	TCC	NR	[8]
K9TCC-PU-An	2015	Cultured cells from bladder tumor biopsy samples	Scottish Terrier	NR	Female	Invasive TCC	NR	[9]
TihoDUrtTCC1506	2020	Cultured cells from bladder tumor biopsy samples	Labrador Retriever	10 years	Female	Invasive TCC	19.9 h	[10]

Abbreviations: G = grade; NR = not reported; TCC = transitional cell carcinoma.

The majority of canine BC cell lines express specific cancer-related markers, resembling those presented by the primary tumors in vivo. Due to differences in the development process (mainly related to primary tumor characteristics), the available canine TCC cell lines differ in terms of expressed biomarkers (Table 2). This allows for the selection of appropriate cell lines adapted to the research purposes, such as investigation of specific proteins and their potential impact on carcinogenesis or treatment response. Characterization of 8 canine BC cell lines (K9TCC, K9TCC-PU-AxA, K9TCC-PU-AxC, K9TCC-PU-Sh, K9TCC-PU-Mx, K9TCC-PU, K9TCC-PU-Nk, and K9TCC-PU-Pu) was provided by Dhawan et al. [5]. All cell lines revealed high expression of E-cadherin and cytokeratin. High cox-2 protein expression was present in all cell lines. The K9TCCAxA, K9TCC-PU-AxC, and K9TCC-PU-In cell lines were also characterized by high expression of p53 protein, whereas K9TCC, K9TCC-PU-Mx, K9TCC-PU-Nk, K9TCC-PU-Sh, and K9TCC-PU-Pu had low expression of p53 protein. Another available canine BC cell lines (K9TCC#1Lillie, K9TCC#2Dakota, K9TCC#4Molly, K9TCC#5Lilly) were characterized by Rathore et al. [7]. All cell lines highly or moderately expressed the cytokeratin. Cell proliferation marker Ki-67 was highly expressed in three of these cell lines, except K9TCC#4 Molly. Expression of kinase-tyrosine receptors (EGFR, PDGDR) differed between cell lines. PDGFR was more expressed in K9TCC#1Lillie, K9TCC#2Dakota, and K9TCC#4Molly than in the K9TCC#5Lilly. EGFR was moderately expressed in all tested K9TCC, whereas VEGFR seemed to be not expressed. Moreover, Cox-2 was highly expressed in all cell lines [7].

To date, only a few studies have provided data regarding high-throughput molecular characterization of existing canine BC cell lines. Initial molecular characterization (including genotypic data) of several canine BC cell lines was performed by the Flint Animal Cancer Center (FACC) [11]. Subsequently, Das et al. conducted whole exome sequence analyses on 33 canine cancer cell lines, including canine BC cell lines [12]. Authors provided a wide database of somatic mutations that can be explored for their role in the development and progression of canine BC [12]. Further investigations of the cellular biology through molecular characterizations of canine BC cell lines may provide valuable information regarding cancer biology and play a crucial role in predicting the variable treatment responses. Thus, in vitro analysis of drug sensitivity in a background of known protein coding somatic mutations could be used to correlate drug sensitivity to the observed genomic profile in further research.

Table 2. Histological and molecular characterization of available canine bladder cancer cell lines.

Cell Line Name	Expression of Cancer-Related Markers									Available Molecular Data	Reference
	Uroplakin	Cytokeratin	E-Cadherin	Vimentin	Ki67	PDGFR	EGFR	COX-2	p53		
K9TCC	NR	High	High	Moderate	NR	NR	NR	High	Low	Array-based CGH, CNV analysis, transcriptome analysis	[4,5,9]
K9TCC-PU-AxA	NR	High	High	Moderate	NR	NR	NR	High	High	NR	[5]
K9TCC-PU-AxC	NR	High	High	High	NR	NR	NR	High	High	NR	[5]
K9TCC-PU-In	NR	High	High	Moderate	NR	NR	NR	High	High	Array-based CGH, CNV analysis	[5,9]
K9TCC-PU-Mx	NR	High	High	Low	NR	NR	NR	High	Low	Array-based CGH, CNV analysis	[5,9]
K9TCC-PU-Nk	NR	High	High	Moderate	NR	NR	NR	High	Low	NR	[5]
K9TCC-PU-Pu	NR	High	High	Moderate	NR	NR	NR	High	Low	NR	[5]
K9TCC-PU-Sh	NR	High	High	Moderate	NR	NR	NR	High	Low	Array-based CGH, CNV analysis	[5,9]
Bliley	NR	NR	NR	NR	NR	NR	NR	NR	NR	Deep exome analysis, transcriptome analysis	[6,11,12]
K9TCC#1Lilly	High	High	NR	Low	High	High	Moderate	High	NR	NR	[7]
K9TCC#2Dakota	High	High	NR	Low	High	High	Moderate	High	NR	NR	[7]
K9TCC#4Molly	Low	Moderate	NR	Low	Moderate	High	Moderate	High	NR	NR	[7]
K9TCC#5Lilly	Moderate	Moderate	NR	Low	High	Moderate	Moderate	High	NR	NR	[7]
TihoDUrtTCC1506	Low	High	High	Low	NR	NR	NR	High	Moderate	NR	[10]

Abbreviations: CGG = comparative genomic hybridization; CNV = copy number variations; COX = cyclooxygenase; EGFR = epidermal growth factor receptor; NR = not reported; PDGFR = platelet-derived growth factor receptor.

In vitro studies using BC cell lines play a significant role in the novel drug discovery and development process, providing crucial data on drug effects in the early preclinical stages. Such information is of paramount importance in the decision-making process for drugs moving forward into more expensive and time-consuming in vivo clinical trials. Initial studies using canine TCC cell lines were extensively focused on non-selective cyclooxygenase inhibitors (Cox inhibitors, non-steroidal anti-inflammatory drugs—NSAIDs) and various chemotherapeutic agents [4]. Following the clinical trials with pet dogs, therapy with NSAIDs, with or without the addition of chemotherapeutics became the standard of care for canine invasive and metastatic TCC [13,14]. However, the overall median survival time for dogs that respond to NSAIDs and chemotherapy was still relatively short (up to a few months), which led to the search for novel therapeutic agents. In the past years, multiple studies including canine BC cell lines were conducted in order to evaluate the activity of novel anticancer agents (Table 3) [15–25]. Although many of them were not transferred to in vivo studies, novel therapeutic agents that could improve survival of dog pets with bladder TCC were also found. One of the most promising directions was molecular-targeted therapy using receptor tyrosine kinase inhibitors. As an example, Sakai et al. demonstrated that lapatinib (tyrosine kinase inhibitor of HER2 and EGFR) could inhibit canine BC cell growth in vitro [20]. Subsequently, Maeda et al. showed that compared to the dogs treated with piroxicam alone, those administered the lapatinib had a significantly greater reduction in the size of the primary bladder tumor and improved overall and progression-free survival [26].

Table 3. Examples of in vitro studies using canine bladder cancer cell lines to assess the efficacy of therapeutic agents.

Author	Therapeutic Agent	Cell Lines Used	Main Results	Reference
Knapp et al.	Piroxicam (COX-2 inhibitor)	K9TCC	Piroxicam had no direct cytotoxicity against canine BC cells Piroxicam increased cytotoxicity of chemotherapeutic agents	[4]
Galvao et al.	Gemcitabine + carboplatin (chemotherapeutic drugs)	K9TCC-PU: -AxA, -AxC, -Pu, -Sh	The combination of gemcitabine and carboplatin had synergistic antitumor effects on canine BC cells	[15]
Rathore et al.	AD198 (derivate of doxorubicin, chemotherapeutic drug)	K9TCC#Lillie, K9TCC#2Dakota, K9TCC#4Molly	AD198 inhibited cell viability of canine BC cells more efficiently as compared to doxorubicin at the same concentration	[16]
Gustafson et al.	Cyclopamine GANT6 (hedgehog signaling pathways inhibitors)	K9TCC, K9TCC-PU-Sh	Cyclopamine and GANT6 led to significantly decreased canine BC cells proliferation but had a smaller effect on apoptosis	[17]
Grayton et al.	KPT-185 KPT-335 (selective inhibitors of nuclear export)	Bliley	Canine BC cells were resistant to both drugs	[18]
Bourn et al.	Axitinib Masitinib (receptor tyrosine kinase inhibitors)	K9TCC#1Lillie, K9TCC#5Lilly	Axitinib and masitinib inhibited cell viability and increased apoptosis in a dose-dependent manner in tested canine BC cell lines	[19]
Sakai et al.	Lapatinib (tyrosine kinase inhibitor of HER2 and EGFR)	LCTCC, MCTCC	Lapatinib inhibited canine BC cell growth in a dose-dependent manner	[20]
Cronise et al.	Vemurafenib (BRAF inhibitor)	Bliley	BRAF mutant BC cell lines were insensitive to vemurafenib	[21]
Hurst et al.	Mavacoxib (selective COX-2 inhibitor)	K9TCC, K9TCC-PU: -AxA, - In, -Sh	Mavacoxib reduced cell viability in a dose-dependent manner in all tested canine BC cell lines	[22]
Byer et al.	Taurolidine (inhibitor of angiogenesis)	Bliley	Taurolidine showed significant effects on canine BC cell viability	[23]
Klose et al.	Metformin (biguanide antihyperglycemic agent)	TCC1506	Metformin inhibited the metabolic activity and cell proliferation of the canine BC cells	[24]
Korec et al.	Toceranib (multi-target receptor tyrosine kinase inhibitor)	K9TCC-PU-AxA, -AxC, -Nk, -Pu, -Sh	Toceranib at physiologically relevant concentrations has no direct anti-proliferative effect on canine BC cells	[25]

Abbreviations: BC = bladder cancer; COX = cyclooxygenase; EGFR = epidermal growth factor receptor; HER2 = human epidermal growth factor receptor 2.

Canine BC cell lines can be also used for investigation of other forms of anti-cancer therapy. As an example, Parfitt et al. investigated and characterized the radiosensitivity and capacity for cellular damage repair of canine BC cell lines [27]. Authors found that canine BC cell lines were moderately radioresistant and exhibited a high repair capacity. They concluded that larger radiation doses may be optimal for the treatment of naturally occurring BC in dogs [27]. In another study conducted by Maeda et al., significant differences in radiosensitivity between particular canine TCC cell lines (K9TCC and Bliley) were demonstrated. Bliley cell line was classified as radioresistant and K9TCC as radiosensi-

tive, which might be used in further investigations on predicting individual response to radiation therapy in dogs with BC [28].

Conventional 2D cultures have several advantages supporting their important role in preclinical research. Research using cell lines is significantly less expensive than in vivo animal studies and provide an unlimited supply of material, which is widely available and easy to propagate under completely controlled and reproducible environmental conditions [29]. Nevertheless, 2D cell lines do not faithfully reflect the conditions prevailing in vivo since proper tissue structure and interactions with tumor microenvironment (TME), extracellular matrix (ECM), and host immune cells (ICs) are lost [30]. Moreover, the use of 2D cultures is usually restricted to one cell type, while tumors in vivo are frequently heterogenous in terms of the forming cell populations, being composed either by neoplastic cells or by stromal and ICs [29]. During each passage, cultured cells could experience genetic alterations due to selective pressure, which may lead to substantial changes in their phenotype. Unlimited access to oxygen and nutrients, unlike in vivo, can also induce accumulation of genetic changes that are not found in the primary lesions [29,30].

3.2. Three Dimensional (3D) Models

The importance of the three-dimensional (3D) features of solid tumors in relation to carcinogenesis or drug response process has prompted efforts to develop in vitro models mimicking in vivo tumor growth more precisely. Examples of 3D culture systems include multicellular aggregates grown as spheroids, scaffold-based models grown within polymer networks, and organoids defined as stem cell-containing self-organizing structures possessing multiple features of the original tumor [31,32]. Despite the increasing number of human studies using 3D models of BC, reports on canine models are still scarce.

The first 3D models of canine BC were established by Elbadawy et al. [33]. They generated four BC organoids using cells from urine samples collected from dogs with urothelial BC. Collected cells were mixed with natural polymer (Matrigel) and cultured with stem cell-stimulated medium. Established organoids had a spheroidal structure and a similar histology to naturally occurring BC in dogs. They were characterized by expression of urothelial cell markers and resembled the cellular architecture of invasive type of canine BC. Initial molecular characterization of established canine BC organoids has been performed and several novel genes were found to be specifically upregulated, being potential targets for novel therapies. Expression of several basal cell markers was found to be upregulated in generated organoids, suggesting that the cell origin of dog BC might be basal, which corresponds with poor response to chemotherapy in advanced stages. In a cell viability assay, the response to treatment with a range of anticancer drugs (e.g., cisplatin, vinblastine, gemcitabine, or piroxicam) was markedly different in each BC organoid, which forms the basis for further extensive research [33]. In addition, authors provided data on novel therapeutic agents, trametinib and verteporfin, which significantly inhibited the BC organoid viability. Additionally, trametinib induced basal to luminal differentiation of BC organoids, enhancing the sensitivity of cancer cells to carboplatin [34]. In another available study, the same research team demonstrated feasibility of performing 2D culture conditions using patient-derived 3D organoid cells without losing their characteristics, such as marker expression or stemness, creating a "2.5D" organoid canine BC model [35].

3D cell culture approaches hold great potential and offer complex systems for various purposes, such as disease modeling and investigation of anticancer drug efficacy. The similarities in the drug responsiveness among the 3D in vitro models and the in vivo models might largely be due to their similarities in enhanced cellular interactions via adhesion and secretion of soluble factors of tumors [31,36]. These new findings support the notion that cancer drugs which are currently being tested need to be screened using more complex tissue-like systems, rather than by using conventional 2D cultures that do not fully manifest features of in vivo tumors. However, 3D models are significantly more expensive than conventional 2D cultures, mainly due to the high cost of processing. In

addition, current 3D models of BC are limited by a relatively narrow range of physical properties [31,36].

4. Conclusions

Although significant advances have been made over the past years, modeling the complexity of canine BC in in vitro models has not been completely successful. There are still various challenges, including the need for extensive molecular characterization of existing cell lines or creation of new reliable 3D models, incorporating multicellular cultures and diverse cellular microenvironments. So far, results obtained by in vitro and in vivo research should be interpreted together. With the constantly growing complexity of in vitro models of BC cancer, animal-based research might be reduced in the future

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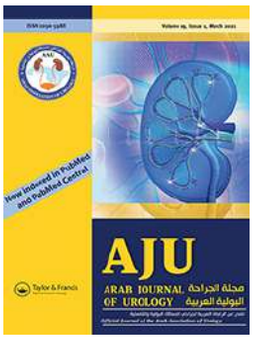
7. ARTYKUŁ DRUGI:

The prognostic value of programmed cell death protein ligand 1 in patients with non-muscle invasive bladder cancer treated with bacilli Calmette–Guérin immunotherapy: Current status



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MANAGEMENT OF NMIBC: MINI-REVIEW

The prognostic value of programmed cell death protein ligand 1 in patients with non-muscle-invasive bladder cancer treated with bacille Calmette–Guérin immunotherapy: Current status

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ABSTRACT

Objective: To summarise the current evidence of the significance and prognostic value of programmed cell death protein ligand 1 (PD-L1) expression in patients with non-muscle-invasive bladder cancer (NMIBC) treated with bacille Calmette–Guérin (BCG) immunotherapy.

Methods: A search was conducted in May 2020 of three electronic databases; MEDLINE, Scopus, and EMBASE. In this review we included results from original studies investigating the relationship between the PD-L1 expression and BCG response in patients with NMIBC.

Results: Only five relevant articles were identified in the literature to date. Some studies showed an association between increased PD-L1 expression and BCG unresponsiveness; however, other authors provided contradictory results and suggested that PD-L1 evaluation could not be used for reliable prediction of BCG response.

Conclusions: The value of PD-L1 evaluation in predicting BCG response is debatable. Current evidence, based only on retrospective analyses, is inconsistent. Comparability of the results is diminished by the methodological limitations of immunohistochemistry assessment. Further multicentre, randomised trials are needed to make definitive conclusions.

Abbreviations: ICs: immune cells; IHC: immunohistochemical staining; (N)MIBC: (non-) muscle-invasive bladder cancer; PD-L1: programmed cell death protein ligand 1; PD-1: programmed cell death protein 1; RC: radical cystectomy; TCs: tumour cells.

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Introduction

In the management of high-risk non-muscle-invasive bladder cancer (NMIBC), adjuvant intravesical BCG immunotherapy has been considered the 'gold standard' for several decades [1]. Nevertheless, the clinical utility and oncological outcomes of BCG treatment are impaired by the fact that eventually 30–40% of patients experience tumour recurrence and 15–20% of patients progress to muscle-invasive disease (muscle-invasive bladder cancer [MIBC]) [2]. Radical cystectomy (RC) remains the preferred treatment in patients who fail to respond to BCG, with limited data for bladder-sparing approaches such as tri-modality treatment [2]. However, performing RC is potentially related to severe complications and considerable mortality. Thus, it can be considered as overtreatment in some individuals [1,2]. Early prediction of patients who might not benefit from BCG and demand immediate radical treatment is crucial, but precise identification of such patients has been challenging to date.

The ability of the programmed cell death protein ligand 1 (PD-L1) to predict response to therapy has been investigated in various solid tumour types [3]. PD-L1 is a cell surface co-stimulatory glycoprotein, which can be expressed on tumour cells (TCs) and immune cells (ICs) infiltrating the tumour microenvironment. In the setting of cancer, the excessive activation of PD-L1 pathway can lead to T-cell dysfunction and exhaustion, resulting in decreased cytotoxic activity and ineffective targeting of TCs [3]. In clinical practice, PD-L1 expression is measured predominantly by immunohistochemical staining (IHC). PD-L1 levels can be evaluated separately in TCs and tumour-infiltrating ICs, or simultaneously in both cell populations [3].

In bladder cancer, the prognostic value of PD-L1 has been investigated primarily in relation to MIBC. It was shown that high PD-L1 expression could be associated with worse clinical and oncological outcomes [4]. In patients with NMIBC, PD-L1 has been suggested as a potential predictor of BCG response. Therefore, in the present review we wanted to briefly summarise the current evidence of the prognostic value of PD-L1 in patients with NMIBC treated with BCG.

Methods

A search was conducted in May 2020 of the three online databases; the Medical Literature Analysis and Retrieval System Online (MEDLINE), Scopus and the Excerpta Medica dataBASE (EMBASE), according to Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement [5]. The Medical

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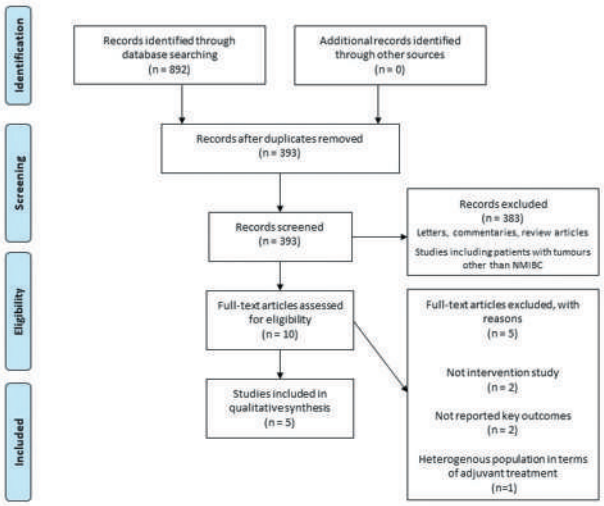

Figure 1. Flow diagram of study selection.

Subject Heading (MeSH) terms and/or keywords and/or free words were: 'bladder cancer', 'non-muscle invasive', 'NMIBC', 'PD-L1', 'PDL1', 'BCG', and 'immunotherapy'. Boolean operators (NOT, AND, OR) were used in succession to narrow and broaden the search. Auto-alerts in MEDLINE were also run, as well as reference lists of original articles and review articles for further eligible data. The search included articles without language limitations and evidence was limited to human data.

In total, 892 publications were initially identified through database searching. The flow diagram of the study selection process with subsequent exclusions (with reasons) is presented in Figure 1. In the present review, we included results from original comparative studies investigating the relationship between the PD-L1 expression and BCG response in patients with NMIBC.

Results

Only relevant five articles were identified in the literature to date. The characteristic and primary findings of available studies are summarised in Table 1 [6–10]. In an analysis of 22 patients with high-risk NMIBC, Martínez et al. [6] found that pre-treatment PD-L1 expression did not differ significantly between patients classified as BCG 'responders' and 'non-responders', defined as patients without a recurrence or progression for ≥30 months after BCG treatment initiation. Positive PD-L1 expression was observed in nine of the 12 BCG non-responders and seven of the 10 responders. Delcourt et al. [7] investigated the association between early recurrence (the occurrence of refractory tumour as defined by International Bladder Cancer Group [IBCG] and American Society of Clinical Oncology Genitourinary Group [ASCO GU] definitions) and the PD-L1 expression in a large cohort of 186 patients with high-risk NMIBC. The rate of early recurrence among patients with positive and negative pre-treatment PD-L1 expressions on TCs was 20% (seven of 35) and 20.5% (31/151), respectively (not significant). Similarly, no significant association was found when tumour infiltrating ICs were analysed. Additionally, the authors showed that the PD-L1 expression was significantly increased after BCG installations compared to the pre-treatment level. In another paper, including patients with high-risk NMIBC, Kates et al. [8] reported a significantly increased PD-L1 expression among pre-treatment samples collected from 32 BCG 'non-responders' (BCG unresponsive, relapsing progressors) compared to 31 'responders'. The positive PD-L1 expression was observed in 25–28% and 0–4%, respectively. PD-L1 expression, evaluated after BCG treatment, did not change significantly. Aydin et al. [9] analysed the pre-treatment PD-L1 expression in 117 patients with high-grade NMIBC. The authors found a significant association between positive PD-L1 expression on tumour infiltrating ICs and refractory recurrence (defined according to criteria specified in European Association of Urology [EAU] guidelines). However, no correlation was found regarding relapsing recurrence or progression. Also, PD-L1 expression was not a significant predictor of recurrence-free survival (RFS) or progression-free survival (PFS) in multivariate Cox regression analysis. Post-treatment PD-L1 expression levels on ICs were significantly decreased in patients who had refractory recurrence. In the most recent study, Pierconti et al. [10] compared PD-L1 expression between BCG unresponsive patients and those who responded to BCG therapy. Only patients with primary carcinoma *in situ* (CIS) were included in this study. The authors showed that the PD-L1 expression on both TCs and tumour infiltrating ICs was significantly higher in BCG unresponsive patients.

Discussion

The value of PD-L1 as a predictive biomarker for BCG response is questionable. Although some studies have shown a positive correlation between increased PD-L1 expression and BCG unresponsiveness, results could be biased because of the small patient cohorts [8,10]. Studies with a large number of participants provided contradictory conclusions and suggested that evaluating PD-L1 expression could not be used for the prediction of BCG response [7,9]. The high level of inconsistency among the available studies may result from fact that PD-L1 expression was evaluated by IHC and multiple assays with different antibody clones were used. Moreover, the intra-tumoral heterogeneity of PD-L1 expression and the possibility of continuous changes in PD-L1 expression due to the animated nature of the tumour microenvironment could also distort the results and limit the interchangeability and comparability of particular studies. To

Table 1. Characteristic and primary findings of the presented studies.

Reference	Design	No. of patients	Tumours (NMIBC)	BCG course	Analysed cell population	Assay*	PD-L1 positivity threshold, %	Pre-treatment positive PD-L1 expression, %	Findings
Martínez et al., [6]	R	22	T1 high-grade	Induction and maintenance	TCs, tumour infiltrating ICs	SP142	≥1	TCs: 9 ICs: 77	No significant difference in the expression pattern of PD-L1 was identified between the BCG responders and BCG non-responders[a].
Delcourt et al., [7]	R	186	High-risk	Induction	TCs, tumour infiltrating ICs	E1L3 N	≥1	TCs: 18.1 ICs: 58.1	Early recurrence[b] was not significantly more frequent in the PD-L1-positive patients vs the PD-L1-negative patients. PD-L1 expression was significantly increased on ICs after BCG therapy.
Kates et al., [8]	R	63	High-risk	Induction	TCs, tumour infiltrating ICs	SP142, 22C3	≥5	CPS: SP-142: 25 22C3: 28	PD-L1 was significantly increased in the pre-treatment samples collected from BCG non-responders[b] vs BCG responders. BCG treatment did not increase PD-L1 expression.
Aydin et al. [9]	R	117	High grade	At least induction	TCs, tumour infiltrating ICs	SP142	≥1	TCs: 8.5 ICs: 77	Positive pre-treatment PD-L1 expression (on ICs) was significantly associated with refractory tumour recurrence[c] but not with relapsing recurrence[c] and progression[c]. RFS and PFS did not correlate with positive PD-L1 expression. Post-treatment PD-L1 expression levels on ICs were significantly decreased in patients who had refractory recurrence.
Pierconti et al., [10]	R	60	Primary CIS	At least induction	TCs, tumour infiltrating ICs	SP263, 22C3, SP142	Low (0–5) High (>5)	TCs: 16.7 ICs: 38.3	PD-L1 expression on TCs and on ICs was higher in BCG-unresponsive[c] patients vs BCG responders, but only the PD-L1 22C3 expression in TCs was associated with recurrence of disease.

CPS, combined positivity score; R, retrospective.
* In all studies PD-L1 expression was evaluated by IHC.
[a]Defined as patients without a recurrence or progression based on follow-up cystoscopy and urinary cytology ≥30 months after BCG treatment initiation.
[b]Defined by IBCG and GU ASCO definitions
[c]Defined according to criteria specified in EAU Guidelines,

overcome limitations of IHC method, the measurement of PD-L1 mRNA level by quantitative reverse transcriptase-polymerase chain reaction was suggested as an alternative. Recently, one study has assessed the prognostic value of the PD-L1 RNA expression in patients with NMIBC (with T1 tumours); however, a heterogeneous population in terms of adjuvant treatment regimen (BCG and mitomycin C) was analysed. The results indicated that high PD-L1 mRNA expression was associated with significantly improved RFS, PFS and cancer-specific survival [11]. Among the included studies, only Aydin et al. [9] provided data for RFS and PFS, and positive PD-L1 expression was not correlated with any survival parameter. This raises doubts as to whether increased PD-L1 expression reflects poor overall prognosis in patients with NMIBC treated with BCG.

Even though PD-L1 does not appear to have utility as a prognostic biomarker for BCG response and overall disease outcomes in patients with NMIB, it might still emerge as a predictive biomarker for checkpoint inhibitors response (PD-L1/programmed cell death protein 1 [PD-1] inhibitors), as this therapeutic approach represent a considerable opportunity in high-risk, BCG unresponsive NMIBC. To date, these conclusions have been based only on publications including patients with MIBC. Increased PD-L1 expression was reported to be predominantly associated with improved objective responses to PD-L1/PD-1 inhibitors [11]. As it was reported in some of the presented studies, an increase in PD-L1 expression after BCG treatment may occur [6]. Theoretically, such patients could significantly benefit from treatment with checkpoint inhibitors. On the other hand, the decrease in post-treatment PD-L1 expression was also observed and, in such patients, anti-PD-L1/PD-1 therapy following BCG failure might be limited [8]. Notwithstanding, it should be emphasised that patients with negative PD-L1 expression could also benefit from checkpoint immunotherapy [12]. Therefore, further clinical trials should be conducted to precisely elucidate the utility of PD-L1 expression as a predictor of checkpoint inhibitors response in patients with failure of BCG therapy.

Conclusions

The value of PD-L1 expression in predicting BCG response is questionable. Current evidence, based only on retrospective analyses, is inconsistent. The comparability of the results is diminished by the methodological limitations of IHC assessment. Further multicentre, randomised trials are needed to make definitive conclusions.

Disclosure statement

No potential conflict of interest was reported by the authors.

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8. ARTYKUŁ TRZECI:

Impact of Adjuvant Chemotherapy on Survival of Patients with Advanced Residual Disease at Radical Cystectomy following Neoadjuvant Chemotherapy: Systematic Review and Meta-Analysis

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Review

Impact of Adjuvant Chemotherapy on Survival of Patients with Advanced Residual Disease at Radical Cystectomy following Neoadjuvant Chemotherapy: Systematic Review and Meta-Analysis

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Abstract: Background: Cisplatin-based neoadjuvant chemotherapy (NAC) followed by radical cystectomy (RC) with pelvic lymph-node dissection is the standard treatment for cT2-4a cN0 cM0 muscle-invasive bladder cancer (MIBC). Despite the significant improvement of primary-tumor downstaging with NAC, up to 50% of patients are eventually found to have advanced residual disease (pT3–T4 and/or histopathologically confirmed nodal metastases (pN+)) at RC. Currently, there is no established standard of care in such cases. The aim of this systematic review and meta-analysis was to assess differences in survival rates between patients with pT3–T4 and/or pN+

MIBC who received NAC and surgery followed by adjuvant chemotherapy (AC), and patients without AC. Materials and Methods: A systematic search was conducted in accordance with the PRISMA statement using the Medline, Embase, and Cochrane Library databases. The last search was performed on 12 November 2020. The primary end point was overall survival (OS) and the secondary end point was disease-specific survival (DSS). Results: We identified 2124 articles, of which 6 were selected for qualitative and quantitative analyses. Of a total of 3096 participants in the included articles, 2355 (76.1%) were in the surveillance group and 741 (23.9%) received AC. The use of AC was associated with significantly better OS (hazard ratio (HR) 0.84, 95% confidence interval (CI) 0.75–0.94; $p = 0.002$) and DSS (HR 0.56, 95% CI 0.32–0.99; $p = 0.05$). Contrary to the main analysis, in the subgroup analysis including only patients with pN+, AC was not significantly associated with better OS compared to the surveillance group (HR 0.89, 95% CI 0.58–1.35; $p = 0.58$). Conclusions: The administration of AC in patients with MIBC and pT3–T4 residual disease after NAC might have a positive impact on OS and DSS. However, this may not apply to N+ patients.

Keywords: adjuvant chemotherapy; muscle-invasive bladder cancer; neoadjuvant chemotherapy

1. Introduction

According to European Association of Urology (EAU) guidelines, cisplatin-based neoadjuvant chemotherapy (NAC) followed by radical cystectomy (RC) with bilateral pelvic lymph-node dissection is the standard treatment of cT2-4a cN0 cM0 muscle-invasive bladder cancer (MIBC) [1]. NAC administration is consequently associated with an 8% absolute improvement in five-year overall survival (OS) [2]. However, despite the significant effect of primary-tumor downstaging with NAC, up to 50% of patients are eventually found to have advanced residual disease (pT3–T4 and/or histopathologically confirmed nodal metastases (pN+)) at RC [3,4]. Currently, there is no established standard of care in such cases.

Although several studies and meta-analyses investigated whether post-RC adjuvant chemotherapy (AC) in chemotherapy-naïve patients with pT3–T4 and/or pN+ disease can improve oncological outcomes [5,6], data regarding the role of AC after NAC and RC are scarce.

The aim of this systematic review and meta-analysis was to assess differences in survival rate between patients with pT3–T4 and/or pN+ MIBC who received NAC and surgery followed by AC, and patients without AC.

2. Materials and Methods

2.1. Search Strategy

The systematic review and meta-analysis were performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement and the Cochrane Handbook for Systematic Reviews of Interventions [7,8]. Study protocol was established in priori and was registered with PROSPERO (CRD42021226369).

Two review authors (L.N. and W.K.) independently conducted a systematic search through three electronic databases: Medline, Embase, and Cochrane Library. The last search was performed on 12 November 2020. The following terms and/or keywords were used: ("adjuvant chemotherapy" OR "AC") AND ("neoadjuvant chemotherapy" OR "NAC") AND ("bladder cancer" OR "muscle-invasive bladder cancer" OR "MIBC") AND ("residual disease" OR "locally advanced disease"). No specific time or language restrictions were applied. A cross-reference search was also performed on articles selected for full-text review. Additional articles were screened from articles published ahead of print in various urological journals.

2.2. Inclusion Criteria

Studies were included if they met all of the following criteria: (1) comparing patients with pT3–T4 and/or pN+ disease at RC who received NAC and surgery followed by AC, with those without AC; (2) reporting at least one survival outcome of interest, including overall survival (OS) and disease-specific survival (DSS); (3) reported median follow-up of a minimum of 12 months; and (4) retrospective or prospective design. Reviews, case reports, letters, or commentaries were excluded.

The primary end point of this systematic review and meta-analysis was OS, and the secondary end point was DSS.

2.3. Data Extraction

After removal of duplicates, two review authors (W.K. and L.N.) independently screened the titles and abstracts of the retrieved records using a standardized item form. All potentially eligible studies were evaluated as full text if available. Any disagreements were subsequently resolved by consultation with the other authors.

The following data were initially extracted: first author, year of publication, journal, study region, study design, study duration, number of patients in AC and surveillance group, and median follow-up. Further, the following clinicopathological data were retrieved: age, gender, Charlson comorbidity index (CCI), NAC regimen, number of NAC cycles, pathological stage, positive nodal status, surgical margin status, AC regimen, and number of AC cycles.

Subsequently, we extracted the outcome measurements of OS and DSS, including the hazard ratios (HRs) and 95% confidence intervals (CIs). OS was primarily defined as time from the date of RC to death from any case or to the time of the last known follow-up. DSS was primarily defined as the time from RC to death from cancer/MIBC or to the time of the last known follow-up.

For articles that lacked some data, the corresponding authors were contacted, requesting additional information from their research, but no additional data were received.

2.4. Quality and Risk-of-Bias Assessment

The quality of the selected studies was assessed independently by two review authors (W.K. and L.N.). The evaluation of the methodological quality of eligible studies was performed according to the Newcastle–Ottawa Scale (NOS) [9].

Risk-of-bias (RoB) was determined using the pragmatic approach for the evaluation of nonrandomized studies by examining the adjustments for confounders according to the Cochrane Handbook for Systematic Reviews of Interventions [8]. To this end, the articles were reviewed on the basis of the adjustment for the effects of age, gender, CCI, pathological stage, positive surgical margin, and type of NAC regimen. RoB and confounder evaluation in each study were independently assessed by two authors (W.K. and M.M.), and disagreements were resolved by consultation with the other authors. To assess the publication bias of the selected studies, funnel plots were generated and evaluated for asymmetry.

2.5. Statistical Analysis

Statistical analysis was conducted using Review Manager 5.3 (The Nordic Cochrane Center, The Cochrane Collaboration, Copenhagen, Denmark) and the R platform (R project, Vienna, Austria). If available, the reported HRs and 95% CIs were included in the meta-analysis. We preferred to collect multivariate-analysis data; otherwise (if not reported), data from univariate analyses were extracted. The statistical significance of the pooled HRs was evaluated by the Z test. The heterogeneity I^2 index was calculated in order to indicate the proportion of inconsistency between studies that could not be attributed to chance. Significant heterogeneity was indicated by a ratio >50% for I^2 or a p value $\leq$0.10 in the Cochrane Q test, in which case a random-effect model was used. When no significant

heterogeneity was observed among the studies, a fixed-effect model was used. For all other tests, $p \leq 0.05$ was considered a statistically significant difference.

3. Results

3.1. Study Identification and Quality Assessment

Our search strategy initially identified 2124 articles (2098 from online databases and 26 from additional sources). A flow diagram of the study selection and subsequent exclusions (with reasons) is presented in Figure 1. Eventually, we identified six studies for qualitative and quantitative analyses [10–15]. Table 1 summarizes the baseline characteristics of the eligible studies. Of the 3096 total participants in the selected articles, 2355 (76.1%) and 741 (23.9%) were in the surveillance and AC groups, respectively. All included studies had a retrospective design. The reported median follow-up ranged from 30 to 50 months. The assessment of the quality scores for the selected trials based on NOS ranged from 5 to 7, which was considered adequate for the subsequent systematic review and meta-analysis.

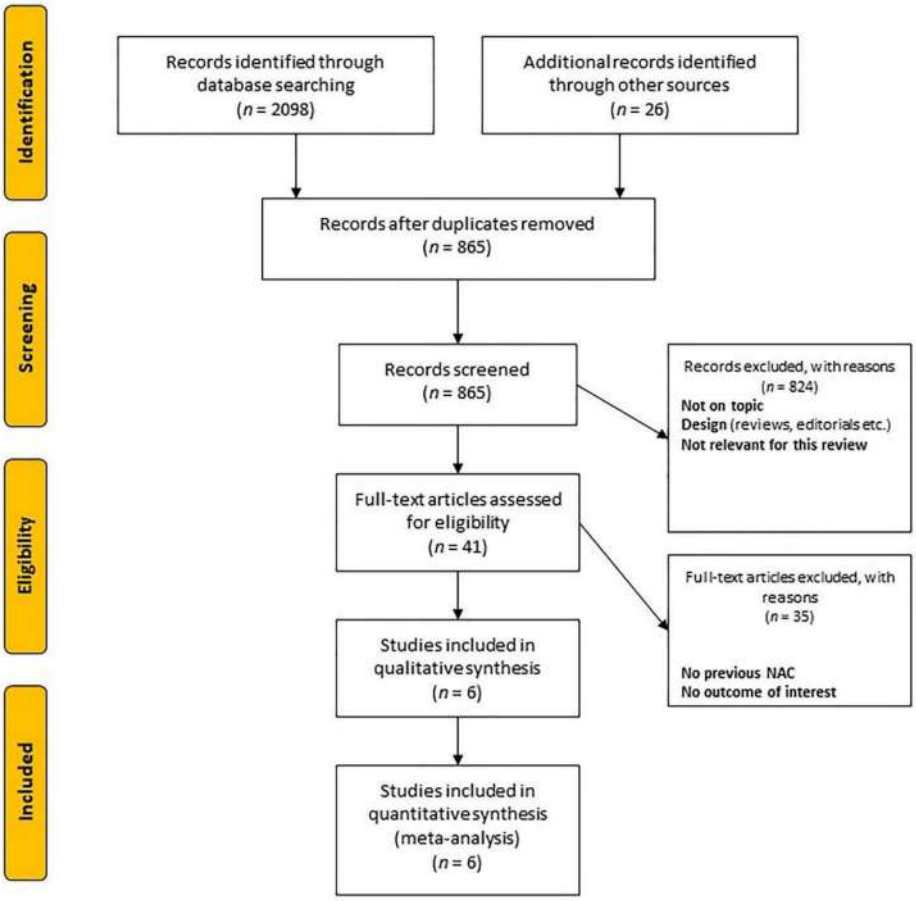


Figure 1. Flow diagram of the study selection.

Table 1. Baseline characteristics of the included studies.

Study	Country	Design	Duration	Number of Patients AC/Surveillance	Follow Up Median (Months)	Reported Outcomes of Interest	NOS
[10]	Canada	Retrospective	1993–2003	11/24	50	OS, DSS	5
[11]	United States	Retrospective	1991–2013	23/106	30	OS	7
[12]	United States	Retrospective	2006–2012	326/1033	44.4	OS	6
[13]	United States	Retrospective	2006–2012	184/604	45.7	OS	6
[14]	United States	Retrospective	2004–2013	168/537	44	OS	6
[15]	United States	Retrospective	2001–2013	29/51	NR	DSS	6

AC, adjuvant chemotherapy; DSS, disease-specific survival; NOS, Newcastle–Ottawa Scale; NR, not reported; OS, overall survival.

3.2. Clinical Characteristics and Pathological Data

Table 2 summarizes the clinicopathological characteristics of patients in the AC and surveillance groups. Generally, no statistically significant differences in the baseline clinical parameters (age, gender, and CCI) were observed in the specific studies. Patients receiving AC were significantly younger in two studies [10,11]. Detailed data regarding the NAC regimen and number of administered NAC cycles were reported in three out of the six articles [10,11,15]. The majority of patients in both the AC and surveillance groups received cisplatin-based NAC regimens. The median number of NAC cycles ranged from 3 to 5. One study only reported data for patients with pN+ [10]. In the five other trials, rates of pN+ in the AC and surveillance groups ranged from 45.5% to 83% and 45.8% to 50%, respectively [11–14]. The surgical margin status was reported in three of the included articles, and positive rates ranged from 17% to 24% and 16% to 34% in the AC and surveillance groups, respectively [11,13,15]. Only one study provided data regarding the proportion of patients with pure variant histology (VH)—21.7% of patients had pure VH in both groups [11]. Detailed data regarding the AC regimen and the number of administered AC cycles were reported in three of the six studies [10,11,15]. In two trials, the majority of patients received cisplatin-based AC regimens [10,11], while in one study, a carboplatin-based AC regimen was predominantly used [15]. The median number of AC cycles ranged from four to five.

Table 2. Clinicopathological characteristics of the patients in the included studies.

Study	Group	No. of Patients	Age (Median, IQR)	Gender, Male (%)	CCI, (%)	NAC Regimen, (%)	No. of NAC Cycles (Median, IQR)	Clinical Stage (%)	Pathological Stage (%)	Positive Nodal Status (pN+) (%)	Positive Surgical Margins, (%)	VH, (%)	AC Regimen (%)	No. of AC Cycles (Median, IQR)
[10]	AC	11	52	NA	NR	Platinum-based: 81% Other/unknown: 19%	5	NA	NA	100%	NA	NA	Platinum-based: 73% Other/unknown: 27%	NR
	Surveillance	24	64										-	-
[11]	AC	23	61 (51–68)	74%	0: 39% 1–2: 35% >2: 26%	Cisplatin-based: 64% Carboplatin-based: 21% Other/unknown: 13%	3 (3–4)	>cT2: 22% cN+: 17%	pT3a–T4a: 17% pT4b and/or pN+: 83%	NA	17%	21.7%	Cisplatin-based: 47% Carboplatin-based: 22% Other/unknown: 30%	4 (3–4)
	Surveillance	106	66 (59–71)	71%	0: 48% 1–2: 25% >2: 25%	Cisplatin-based: 70% Carboplatin-based: 15% Other/unknown: 15%	3 (3–4)	>cT2: 39% cN+: 16%	pT3a–pT4a: 53% pT4b and/or pN+: 47%	NA	16%	21.7%	-	-
[12]	AC	326	<60 years: 31.8% ≥60 years: 66.8%	73.3%	0: 74.2% 1: 19.6% ≥2: 6.1%	NR	NR	NR	pT3N0: 25.9% pT4N0: 13.7% pTanyN+: 60.4%	60.4%	NR	NR	NR	NR
	Surveillance	1033	<60 years: 32.1% ≥60 years: 67.9%	74.7%	0: 74.4% 1: 20.1% ≥2: 5.4%	NR	NR	NR	pT3N0: 34.8% pT4N0: 11.3% pTanyN+: 53.9%	53.9%	NR	NR	-	-
[13] *	AC	184	mean (SD) 65.7 (10.0)	75.8%	0: 75% 1: 20.3% ≥2: 4.7%	NR	NR	NR	pT3N0: 40.8% pT4N0: 13.7% pTanyN+: 45.5%	45.5%	17.1%	NR	NR	NR
	Surveillance	604	mean (SD) 65.3 (9.3)	76.6%	0: 74.5% 1: 21% ≥2: 5.5%	NR	NR	NR	pT3N0: 39.9% pT4N0: 14.3% pTanyN+: 45.8%	45.8%	16.8%	NR	-	-
[14]	AC	168	<60 years: 38% ≥60 years: 62%	67%	0: 76% 1: 19% ≥2: 5%	NR	NR	NR	≥pT3: 87%	57%	NR	NR	NR	NR
	Surveillance	537	<60 years: 33% ≥60 years: 68%	66%	0: 74% 1: 20% ≥2: 6%	NR	NR	NR	≥pT3: 90%	45%	NR	NR	-	-
[15]	AC	29	66 (56–76)	83%	median (IQR) 3 (2–4)	Cisplatin-based: 79% Carboplatin-based: 21% Other/unknown: 0%	3 (3–4)	>cT2: 48% cN+: 20%	pT3–T4: 76%	83%	24%	NR	Cisplatin-based: 27.6% Carboplatin-based: 55.2% Other/unknown: 17.2%	5 (3–8) 4 (2–6) 4 (4–6)
	Surveillance	51	68 (63–75)	69%	median (IQR) 3 (2–3)	Cisplatin-based: 74% Carboplatin-based: 26% Other/unknown: 0%	3 (3–4)	>cT2: 57% cN+: 29%	pT3–T4: 88%	50%	34%	NR	-	-

* IPTW-weighted study population; abbreviations: AC, adjuvant chemotherapy; CCI, Charlson comorbidity index; cN+, clinically suspected nodal metastases; IQR, interquartile range; IPTW, inverse probability of treatment weighting; NA, not applicable; NAC, neoadjuvant chemotherapy; No., number of; NR, not reported; SD, standard deviation; VH, variant histology.

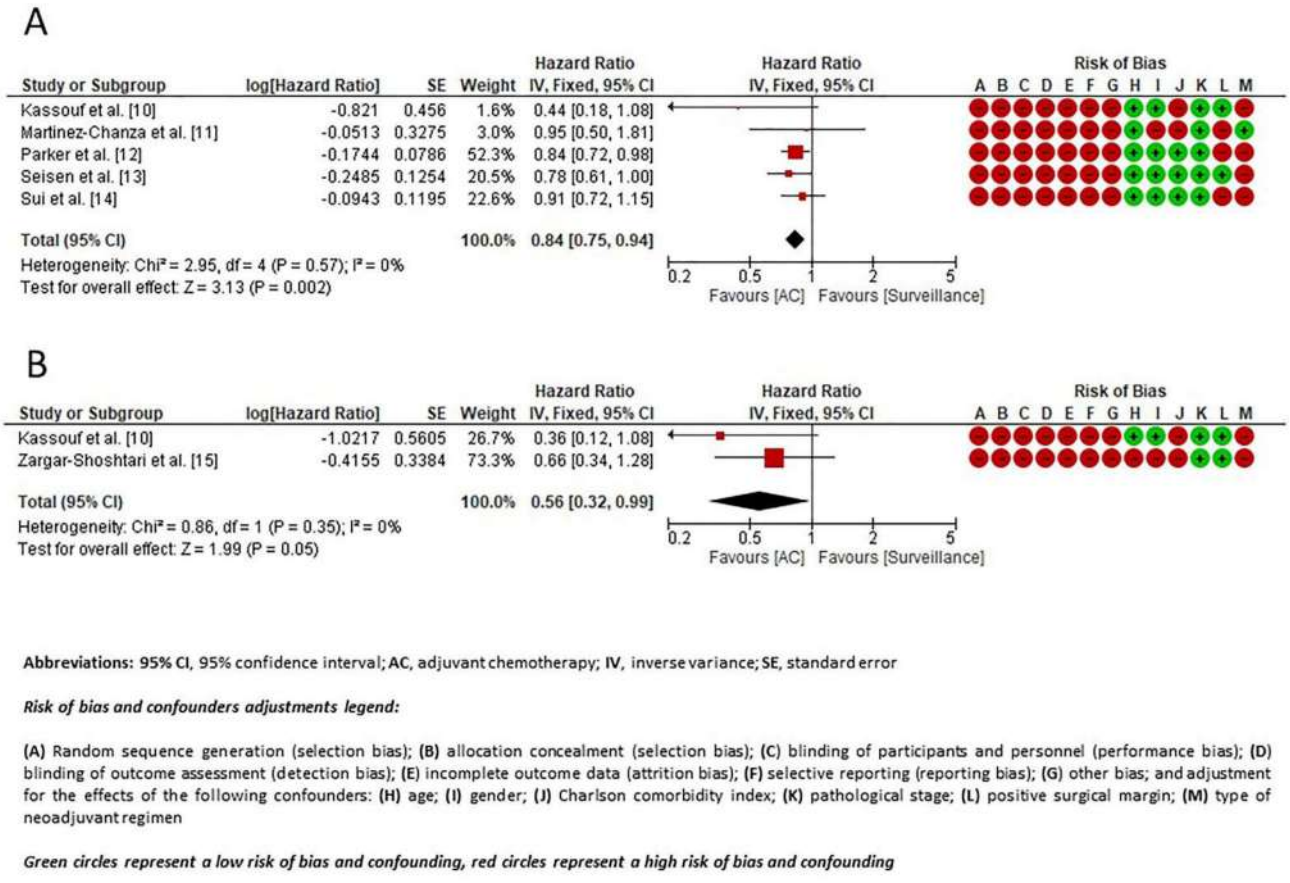
3.3. Risk-of-Bias Assessment

All studies carried a high RoB, which was primarily related to their retrospective design. In the majority of studies, multivariate analyses were adjusted for the effect of at least three major confounders, with age, gender, and CCI being the most common.

3.4. Meta-Analysis Results

For each outcome of interest (OS and DSS), we performed a main analysis that included data from all available publications. Subsequently, we conducted subgroup analysis of OS in the pN+ subpopulation. Subgroup analysis of DSS in the pN+ subpopulation could not be reliably performed due to insufficient data.

OS data were reported in five articles [10–14]. No significant heterogeneity was observed among the studies (I^2 = 0%; p = 0.61). Therefore, a fixed-effect model was used. A forest plot of HR and 95% CI for OS is presented in Figure 2A. The use of AC was associated with significantly better OS (HR 0.84, 95% CI 0.75–0.94; p = 0.002) in the pooled analysis. The funnel plot revealed no publication bias (Figure 3A).



Abbreviations: **95% CI**, 95% confidence interval; **AC**, adjuvant chemotherapy; **IV**, inverse variance; **SE**, standard error

Risk of bias and confounders adjustments legend:

(A) Random sequence generation (selection bias); **(B)** allocation concealment (selection bias); **(C)** blinding of participants and personnel (performance bias); **(D)** blinding of outcome assessment (detection bias); **(E)** incomplete outcome data (attrition bias); **(F)** selective reporting (reporting bias); **(G)** other bias; and adjustment for the effects of the following confounders: **(H)** age; **(I)** gender; **(J)** Charlson comorbidity index; **(K)** pathological stage; **(L)** positive surgical margin; **(M)** type of neoadjuvant regimen

Green circles represent a low risk of bias and confounding, red circles represent a high risk of bias and confounding

Figure 2. Forest plot of the hazard ratio for (**A**) overall survival, and (**B**) disease-specific survival.

Data for DSS were reported in two studies [10,15]. No significant heterogeneity was observed among the studies (I^2 = 0%; p = 0.35). Therefore, a fixed-effect model was used. A forest plot of HR and 95% CI for OS is presented in Figure 2B. Receiving AC was associated with significantly better DSS (HR 0.56, 95% CI 0.32–0.99; p = 0.05) in the pooled analysis. The funnel plot revealed no publication bias (Figure 3B).

In subgroup analysis, which only included patients with pN+, AC was not significantly associated with better OS compared to the surveillance group (HR 0.89, 95% CI 0.58–1.35; p = 0.58) (Figure 4). Significant heterogeneity was observed among the studies (I^2 = 59%; p = 0.08). Therefore, a random-effect model was used.

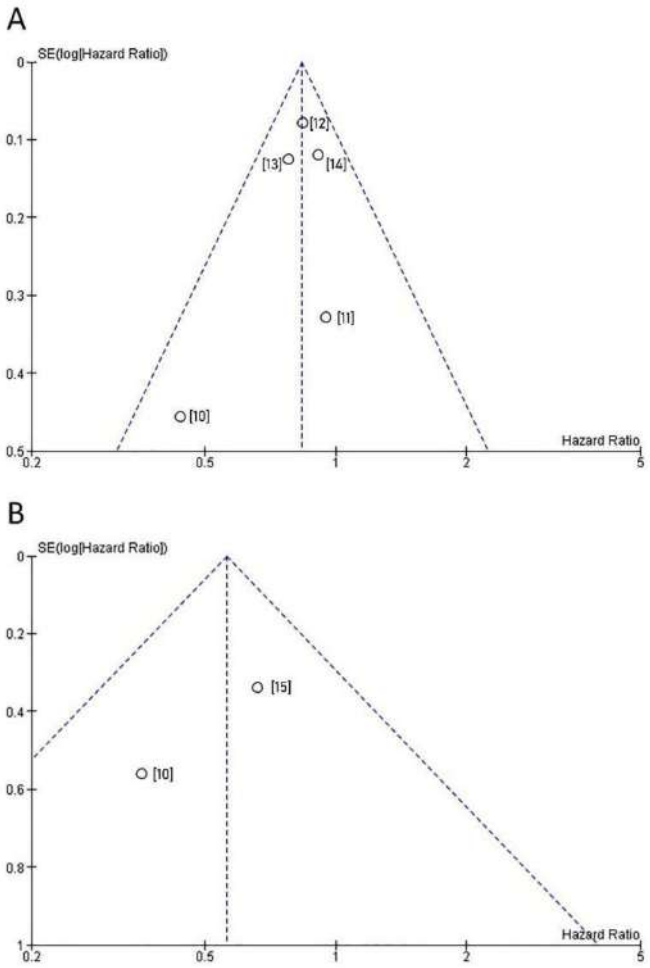
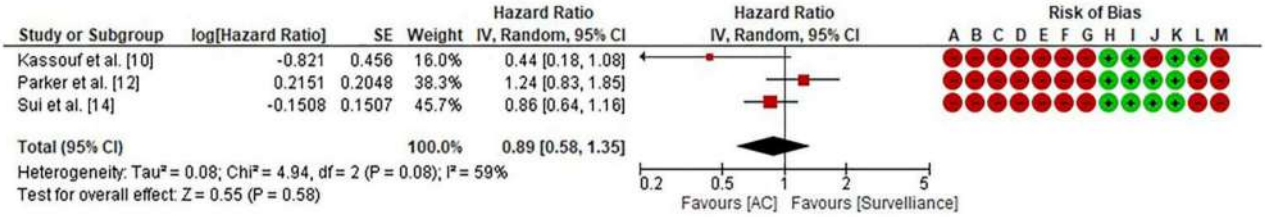


Figure 3. Funnel plot for evaluation of publication bias for (**A**) overall survival, and (**B**) disease-specific survival. Circles represent hazard ratios (x axis) and standard errors (y axis) of particular studies (references are placed in square brackets).



Abbreviations: **95% CI**, 95% confidence interval; **AC**, adjuvant chemotherapy; **IV**, inverse variance; **SE**, standard error

Risk of bias and confounders adjustments legend:

(A) Random sequence generation (selection bias); **(B)** allocation concealment (selection bias); **(C)** blinding of participants and personnel (performance bias); **(D)** blinding of outcome assessment (detection bias); **(E)** incomplete outcome data (attrition bias); **(F)** selective reporting (reporting bias); **(G)** other bias; and adjustment for the effects of the following confounders: **(H)** age; **(I)** gender; **(J)** Charlson comorbidity index; **(K)** pathological stage; **(L)** positive surgical margin; **(M)** type of neoadjuvant regimen

Green circles represent a low risk of bias and confounding, red circles represent a high risk of bias and confounding

Figure 4. Forest plot of the hazard ratio for overall survival in a subpopulation of pN+ patients.

4. Discussion

In this systematic review and meta-analysis, we provide a summary of the accumulated evidence in a tentatively understood area of MIBC management. We evaluated the impact of AC administration on the survival outcomes of patients with advanced disease (pT3–T4 and/or pN+) at RC after NAC. To the best of our knowledge, this is the first meta-analysis to address this issue. Our analyses demonstrated that AC was associated with significantly better OS (HR 0.84, 95% CI 0.75–0.94; $p = 0.002$) and DSS (HR 0.56, 95% CI 0.32–0.99; $p = 0.05$) compared to the surveillance approach.

The available literature regarding the role of AC in patients with MIBC who had received NAC and demonstrated advanced disease (pT3–T4 and/or pN+) comprises several retrospective studies and population-based registries, whereas no prospective RCT has been published to date. Initial results (only abstract available) from the Retrospective International Study of Cancers of the Urothelium (RISC) group showed that AC administration in patients with residual disease after NAC was associated with a significant reduction in relapse risk compared to surveillance (HR = 0.35 for AC, 95% CI 0.17–0.74). The subset analysis of patients with residual pathologic T4 and/or N+ after NAC also revealed a significant improvement in time to relapse in the AC group (HR = 0.43 for AC, 95% CI 0.21–0.89). Therefore, the authors concluded that AC might delay recurrence in patients with residual disease after NAC in an advanced MIBC setting. Notably, the majority of the included patients changed regimens between NAC and AC [16]. Previously, Millikan et al. suggested that, with the absence of a pathological response to the initial NAC, further therapy with the same regimen may not afford additional survival benefits. Their conclusions were based on the observation that patients either receiving two courses of neoadjuvant MVAC (a chemotherapy combination that includes methotrexate, vinblastine, doxorubicin and cisplatin) followed by RC and three additional cycles of chemotherapy, or undergoing an initial RC followed by five cycles of AC, exhibited comparable progression-free survival (PFS) and DSS [17]. These highlighted reports suggest that, in the case of the absence of a pathological response after NAC, changes in therapeutic regimens should be considered if further AC is subsequently planned. Among the six studies included in this systematic review and meta-analysis, only one provided detailed data regarding changes between pre- and post-RC chemotherapy. In a study by Zargar-Shoshtari et al., 86% of patients received different chemotherapy combinations before and after surgery, but AC was not associated with significant improvements in either RFS or DSS compared to the surveillance approach. A clinically noteworthy observation was made with respect to patients who had been treated with cisplatin-based AC. In these patients, the time to recurrence was the longest when compared with the two other AC regimens (i.e., adjuvant carboplatin and other regimens)—23 vs. 8.4 vs. 5.2, respectively. However, the number of cases in this sub-analysis was small, and differences were not statistically representative [15].

We performed an additional subgroup analysis for OS, only including data from studies reporting HRs for the population with pN+ disease. Contrary to the main analysis, we found that AC was not significantly associated with better OS compared to surveillance (HR 0.89, 95% CI 0.58–1.35; $p = 0.58$) in the pooled analysis. The observed heterogeneity between the studies might be primarily related to the different sizes of the populations. In one included study, Parker et al. provided additional subgroup analyses for the pT3 pN0 pM0 and pT4 pN0 pM0 stages, and demonstrated that AC administration after NAC and RC only significantly improved OS in patients with pT4 pN0 pM0 disease (HR 0.56 for AC, 95% CI 0.33–0.97) [12]. Thus, the increased survival benefit of additional AC could theoretically be observed in patients with the highest T-stage tumors without concomitant nodal metastases. However, identification of additional predictive biomarkers moving beyond the pathological stage to personalize treatment and clinical decisions seems to be a reasonable direction for further research.

As checkpoint inhibitors are increasingly utilized for the treatment of metastatic MIBC, the potential decision as to whether AC should be administered in patients with adverse pathology features after NAC may become debatable, taking into consideration that up

to 50% of patients after RC are ineligible for cisplatin-containing chemotherapy [1,18]. Currently, trials with checkpoint immunotherapy are specifically targeting patients at a high risk of recurrence, including patients with significant residual disease after prior NAC (pT2–T4 and/or pN+; NCT02632409, NCT02450331). Comparison of these two treatment modalities (AC vs. checkpoint inhibitors) in patients receiving NAC could identify the best form of treatment in terms of oncological efficacy.

The important question remains as to whether survival benefits surpass the possible significant negative effect of AC after NAC on patient-reported outcomes. According to a study by Zhang et al. with chemotherapy-naïve patients, self-reported anxiety and depression were increased, while quality of life (QoL) was not decreased in MIBC patients during AC [19]. Data regarding the toxicity and impact on patient-reported outcomes of AC after NAC and RC were not presented in any of the included studies. As there are no additional data available in the literature, this issue should be addressed in future trials.

This study has potential limitations that need to be considered for the interpretation of the results. First, the strength of the conclusions that can be drawn from this meta-analysis is limited by the fact that all included studies were retrospective, with their own limitations, such as selection bias. However, extraction of data from multivariable analyses was possible in almost all eligible articles (mainly adjusted for the effects of major confounders); thus, we were able to minimize bias and establish an acceptable level of comparability. Second, the small sample size of some of the eligible publications may render the results less reliable. Future larger studies may overcome this limitation, providing more robust evidence. Third, we could not fully exclude the bias resulting from possible overlap of some patients in particular articles. Fourth, the absence of detailed data on the NAC and AC regimens and the number of cycles given in some included studies, as well as other important confounders (e.g., VH), potentially limit the conclusions for the current daily practice.

5. Conclusions

The administration of AC in patients with MIBC and pT3–T4 residual disease after NAC might have a positive impact on OS and DSS. However, this may not apply to N+ patients. Further prospective studies are required in order to make a definitive statement.

Author Contributions: Conceptualization, all authors.; methodology, W.K. and Ł.N.; software, Ł.N.; validation, all authors; formal analysis, all authors; investigation, all authors; resources, all authors; data curation, W.K., Ł.N. and A.P.; writing—original draft preparation, all authors; writing—review and editing, W.K., A.P., J.D. and R.Z.; visualization, W.K. and Ł.N.; supervision, W.K., A.P., J.D. and R.Z.; project administration, W.K.; funding acquisition, not applicable. All authors have read and agreed to the published version of the manuscript.

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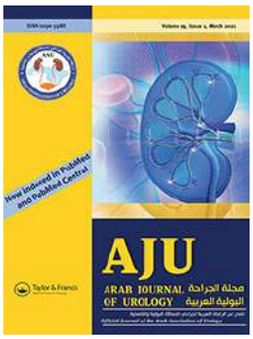
9. ARTYKUŁ CZWARTY:

Assessment of the oncological outcomes of three different bacillus Calmette–Guérin strains in patients with high-grade T1 non-muscle-invasive bladder cancer



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MANAGEMENT OF NMIBC: ORIGINAL ARTICLE

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Assessment of the oncological outcomes of three different bacillus Calmette–Guérin strains in patients with high-grade T1 non-muscle-invasive bladder cancer

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ABSTRACT

Objective: : To determine whether there are significant differences in oncological outcomes between three different bacillus Calmette–Guérin (BCG) strains used for adjuvant intravesical immunotherapy in patients with high-grade T1 (T1HG) non-muscle-invasive bladder cancer (NMIBC).

Patients and methods: : Data of 590 patients with a diagnosis of primary T1HG NMIBC were retrospectively reviewed. The study included 138 (23.4%) patients who were treated with the Moreau, 272 (46.1%) with the TICE, and 180 (30.5%) with the RIVM strains. All patients included in the analysis received at least five instillations of an induction course and at least two installations of a maintenance course. Due to existing differences in baseline patient characteristics, the association between oncological outcomes and strain groups was investigated by complementary analysis with the implementation of inverse probability weighting (IPW).

Results: : The 5-year recurrence-free survival (RFS) rate was 70.5%, 66.7% and 55.2% for the Moreau, TICE and RIVM groups, respectively ($P = 0.016$). The 5-year progression-free survival (PFS) rates were 84.4%, 85% and 77.8% in the Moreau, TICE and RIVM groups, respectively ($P = 0.215$). The IPW-adjusted Cox proportional hazard regression analysis did not show any differences in RFS between the Moreau and TICE groups ($P = 0.69$), whereas the RIVM strain was significantly associated with worse RFS compared to the Moreau (hazard ratio [HR] 1.69 for RIVM; $P = 0.034$) and TICE (HR 1.87 for RIVM; $P = 0.002$) strains. The IPW-adjusted analysis did not show any significant differences between study groups in terms of PFS.

Conclusions: : The results of the present study suggest that the Moreau and TICE strains might be superior to the RIVM strain in terms of RFS in patients with T1HG NMIBC.

Abbreviations: CIS: carcinoma *in situ*; IPW: inverse probability weighting; IQR: interquartile range; HR: hazard ratio; HG: high grade; LVI: lymphovascular invasion; MP: muscularis propria; NMIBC: non-muscle-invasive bladder cancer; PFS: progression-free survival; RCT: randomised controlled trial; RFS: recurrence-free survival; T1HG, high-grade T1; (re-)TURB: (re-staging) transurethral resection of bladder; VH: variant histology

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Non-muscle-invasive bladder cancer; bacillus Calmette–Guérin; strain; recurrence; progression

Introduction

In the management of high-risk (HR) non-muscle-invasive bladder cancer (NMIBC), transurethral resection of bladder (TURB) followed by adjuvant intravesical BCG immunotherapy has been considered the most effective form of treatment [1]. Although BCG has been used as an immunotherapeutic agent for almost 40 years, it is still unknown why treatment results vary among patients [2]. One of the unanswered question remains whether attainable BCG strains are equal in terms of oncological outcomes. International guidelines do not include any clear recommendations regarding the preferable use of certain BCG strains

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and there are only several direct head-to-head comparisons between commonly used strains in the available literature [1,3,4].

Identification of the most efficient BCG strain and assessing its optimal administration regimen could improve oncological outcomes in patients with NMIBC. Further, due to current global shortages of BCG supplies, possible schedule modification, including the administration of selected strains, has been considered a valuable strategy for treatment optimisation [5].

The aim of the present study was to determine whether there were significant differences in oncological outcomes between three different BCG strains used for adjuvant intravesical immunotherapy in patients with high-grade T1 (T1HG) NMIBC.

Patients and methods

This retrospective observational cohort study was approved by an Institutional Review Board for institutional data sharing from all of the participating sites. Data of 590 patients with a diagnosis of primary T1HG NMIBCs, treated with maintenance BCG immunotherapy at seven tertiary high-volume care centres between 2001 and 2019 were retrospectively reviewed. Three BCG strains were used: Moreau, TICE, and RIVM. The selection of the strain type resulted primarily from differences in supply and distribution at each participating centre. Therefore, it was not a planned randomised allocation.

The main analysis included 138 (23.4%) patients who were treated with the Moreau strain, 272 (46.1%) with the TICE strain, and 180 (30.5%) with the RIVM strain. Patients included in the analysis received a minimum of five instillations of an induction course and at least two installations of a maintenance course. BCG instillations were given in accordance to international guidelines and local protocols at that time with ≥1 year of planned maintenance. All patients underwent re-staging TURB (re-TURB) performed after initial TURB and before BCG introduction.

The basic sociodemographic characteristics of patients included age at the time of surgery, gender, and smoking history. The available pathological data comprised the tumour stage, tumour grade, tumour size, tumour focality, the presence of concomitant carcinoma *in situ* (CIS), and the presence of muscularis propria (MP) involvement in primary TURB specimen. Data about immediate single-instillation chemotherapy, lymphovascular invasion (LVI), variant histology (VH), and prostatic involvement of the tumours were not uniformly reported, and therefore not included in the analysis.

Surgical specimens were evaluated by dedicated uropathologists in each participating centre without the application of any central assessment protocol. Tumours included in the analysis were staged according to the American Joint Committee on Cancer TNM staging classification and graded according to the WHO 2004 grading system.

There was no standardised follow-up schedule due to the retrospective nature of the study and multicentric data acquisition. Patients were followed-up according to international guidelines at that time.

Concomitant CIS was defined as the coexistence of CIS in conjunction with the exophytic tumour. A recurrence was defined as a recurrence of a tumour of any stage and grade confirmed by TURB and histological or cytological evaluation. Viable tumours at re-TURB and tumour recurrence in the upper urinary tract were not considered as recurrence. Progression was defined as tumour relapse at tumour stage ≥T2 in the bladder or stromal invasion of the prostatic urethra or as distant (e.g. lymph nodes) progression. The patients with T2 lesions at re-TURB were not included in the analysis as they were not suitable for BCG therapy.

The primary database comprised 1511 patients with high-risk NMIBC. The following exclusion criteria were incorporated: incomplete data on major variables; tumours other than T1HG NMIBC; recurrent tumours; incomplete primary TURB and evident residual disease; less than five BCG induction instillations; less than two BCG maintenance installations; a follow-up period of <6 months; other than a full dose concentration of BCG for a given strain; BCG strain other than Moreau, TICE or RIVM; and the modification of the BCG strain during treatment. Finally, 590 cases were included in the analysis.

The primary endpoints of this study were 5-year recurrence-free survival (RFS) and 5-year progression-free survival (PFS).

Statistical analysis

Descriptive statistics of the categorical variables were presented as counts and percentages. Medians and interquartile ranges (IQRs) were reported for continuous variables. Study groups were compared using chi-squared and Mann–Whitney *U*-tests. Kaplan–Meier curves were plotted for RFS and PFS, and differences between times of survival in each group were evaluated with a likelihood-ratio test. Additionally, Cox regression analyses were performed for both RFS and PFS. Patients without an event or death before an event were censored at the last date of follow-up. Times to events were calculated taking the date of BCG initiation as time zero.

Due to differences in baseline patient characteristics, the association between survival outcomes and particular strain groups was investigated by complementary analysis with the implementation of inversed probability weighting (IPW) adjusted for gender, smoking status, age, the presence of MP in primary TURB specimen,

tumour focality, tumour size, and incidence of concomitant CIS [6]. We used IPW-adjusted Cox proportional hazard regression analysis to calculate the IPW-adjusted hazard ratio (HR) and 95% CI for all included strain pairs.

All *P* values were two-sided, with $P < 0.05$ considered statistically significant. Statistical analyses were performed using STATISTICA 13.3 (TIBCO Software Inc., Palo Alto, CA, USA) and the R platform (R project, Vienna, Austria).

Results

Baseline patient characteristics are presented in table 1. Groups did not differ statistically in terms of gender and the presence of concomitant CIS; however, significant differences in smoking history, tumour size, tumour focality, and the presence of MP in the TURB specimen were observed.

The median (IQR) follow-up for the whole population was 40 (25–60) months. The patients were enrolled prospectively and the groups were not matched in terms of observation time. For this reason, our primary analyses were performed for 5-year RFS and PFS.

Recurrence occurred in 37 (26.8%) patients in the Moreau group, 95 (34.9%) in the TICE group, and 71 (39.4%) in the RIVM group ($P = 0.471$). Progression of the cancer was observed in 17 (12.3%) patients in the Moreau group, 39 (14.3%) in the TICE group, and in 30 (16.7%) in the RIVM group ($P = 0.976$). There were 12 (35.3%), 14 (8.7%) and eight (4.4%) cancer-specific deaths in the Moreau, TICE and RIVM groups, respectively ($P = 0.269$)

The 5-year RFS rate was 70.5%, 66.7% and 55.2% for the Moreau, TICE and RIVM groups, respectively. The observed differences in RFS between the study groups were statistically significant for both 5-year ($P = 0.016$) (figure 1A) and the whole observation period ($P = 0.013$) (figure 1B). The 5-year PFS rate was 84%, 85% and 77.8% in the Moreau, TICE and RIVM groups, respectively (figure 1C), being statistically non-significant ($P = 0.108$). No differences were also found for the whole observation period for PFS ($P = 0.215$) (figure 1D).

Considering the IPW-unadjusted multivariate analysis (table 2), the RIVM strain was associated with significantly worse RFS compared to either the Moreau (HR 1.61, 95% CI 1.05–2.47for RIVM; $P = 0.029$) or TICE strains (HR 1.66, 95% CI 1.15–2.38 for RIVM; $P = 0.006$). No other significant differences in both RFS and PFS were found between particular strain groups.

To reduce the bias of unweighted estimators and adjust for covariates imbalance between groups without losing the patients, we performed complementary analysis using IPW. After IPW adjustment, absolute standardised mean differences for all adjusted clinicopathological variables were <10%, which indicated that patients in all strain groups were subsequently comparable (figure 2).

The IPW-adjusted Cox proportional hazard regression analysis did not show any difference in RFS between the Moreau and TICE groups (HR 0.91, 95% CI 0.55–1.49 for TICE; $P = 0.69$), whereas the RIVM strain was significantly associated with worse RFS compared to the Moreau (HR 1.69, 95% CI 1.03–2.78 for RIVM; $P = 0.034$) and TICE strains (HR 1.87, 95% CI 1.25–2.81 for RIVM; $P = 0.002$) (figure 3A). In terms of PFS, the

Table 1. The patients' baseline characteristics (chi-squared and Mann–Whitney *U*-test *P* values of the differences between the two study groups).

Variable	All patients $N = 590$	Moreau $n = 138$ (23.4%)	TICE $n = 272$ (46.1%)	RIVM $n = 180$ (30.5%)	*P*
Age, years, median (IQR)	66.9 (58–75)	61.1 (55–66)	71.1 (65–78)	66.1 (56–74)	<0.001a
Gender, *n* (%)	493 (83.6)	114 (82.6)	232(85.3)	147 (81.7)	0.979
Male	97 (16.4)	24 (17.4)	40 (14.7)	33 (18.3)	
Female					
Smoking history, *n* (%)	171 (29)	52 (37.6)			<0.001a
Never	241 (40.8)	43 (31.2)	63 (23.1)	56 (31.1)	
Former	161 (27.3)	43 (31.2)	136 (50)	62 (34.4)	
Current	17 (2.9)	0 (0)	57 (21)	61 (33.9)	
Unknown			16 (5.9)	1 (0.6)	
Concomitant CIS, *n* (%)	119 (20.2)	28 (20.3)	46 (16.9)	45 (25)	0.654
Yes	466 (79)	110 (79.7)	222 (81.6)	134 (74.4)	
No	5 (0.8)	0 (0)	4 (1.5)	1 (0.6)	
Unknown					
Tumour size, *n* (%)	294 (49.8)	79 (57.2)	123 (45.2)	92 (51.1)	0.021a
<3 cm	251 (42.6)	59 (42.3)	117 (43)	75 (41.7)	
>3 cm	45 (7.6)	0 (0)	32 (11.8)	13 (7.2)	
Unknown					
Tumour focality, *n* (%)	275 (46.6)	88 (63.8)	100 (36.8)	87 (48.3)	<0.001a
Solitary	281 (47.6)	50 (36.2)	150 (55.1)	81 (45)	
Multiple	34 (5.8)	0 (0)	22 (8.1)	12 (6.7)	
Unknown					
MP in the primary TURB specimen, *n* (%)	432 (73.2)	104 (75.4)	208 (76.5)	120 (66.7)	0.005a
Yes	121 (20.5)	32 (23.2)	53 (19.5)	36 (20)	
No	37 (6.3)	2 (1.4)	11 (4)	24 (13.3)	
Unknown					
Total number of BCG instillations; median (IQR)	15 (9–18)	15 (12–21)	14 (9–18)	15 (11–18)	0.083

aStatistically significant *P* value.

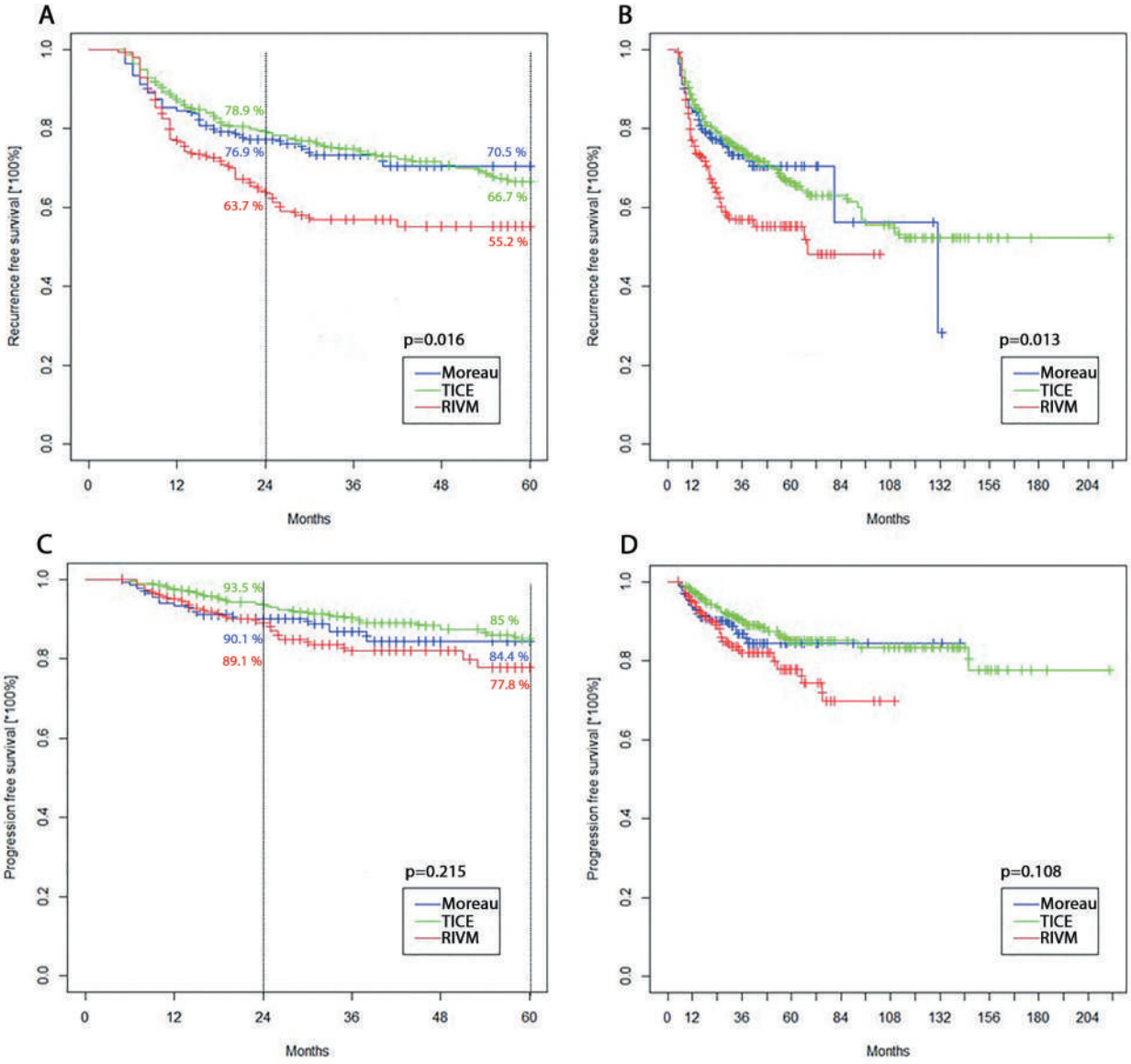

Figure 1. **A**. RFS for the 5-year follow-up ($P = 0.016$). **B**. RFS for the whole observation period ($P = 0.013$). **C**. PFS for the 5-year follow-up ($P = 0.215$). **D**. PFS for the whole observation period ($P = 0.108$).

IPW-adjusted Cox proportional hazard regression analysis did not show any statistical difference between the Moreau and TICE groups (HR 0.74, 95% CI 0.37–1.47 for TICE; $P = 0.399$), as well as the Moreau and RIVM groups (HR 1.34, 95% CI 0.67–2.57 for RIVM; $P = 0.413$). Also, no statistical difference in PFS was observed between the TICE and RIVM groups (HR 1.79, 95% CI 0.94–3.38 for RIVM; $P = 0.071$) (figure 3B).

Discussion

The original BCG strain was developed by Calmette and Guérin in 1921 from an attenuated strain of *Mycobacterium bovis*. During the following decades the sub-culturing process in various laboratories all over the world resulted in genetic evolution of the original strain [3]. Subsequently, results of several studies based on animal and *in vitro* models indicated that certain BCG sub-strains might differ in terms of oncological outcomes [7,8].

In the present study, a uniform group of patients with primary T1HG tumours was retrospectively analysed. We compared the oncological outcomes between three different BCG strains, including the Moreau, TICE and RIVM strains. Although study groups differed initially in terms of some clinicopathological variables (such as tumour size, tumour focality, presence of MP in the initial TURB specimen), which was primarily related to multicentric data collection and presence of some missing data, potential bias resulting from covariates imbalance was minimised after implementation of IPW. In the analysis of the study population (both IPW-unadjusted and -adjusted) significant differences in RFS were found between particular strain groups. Patients treated with the RIVM strain were more likely to experience disease recurrence

Table 2. Multivariable analysis assessing factors associated with RFS and PFS.

	Recurrence-free survival (RFS)			Progression-free survival (PFS)		
Variable	*HR*	*95% CI*	*p-value*	*HR*	*95% CI*	*p-value*
Age	0.99	0.98–1.01	0.668	0.99	0.97–1.02	0.579
Gender (**female** vs male)	1.20	0.79–1.81	0.377	1.13	0.58–2.21	0.709
Smoking (never/**any**)	1.13	0.81–1.57	0.475	1.03	0.61–1.73	0.916
MP in primary TURB (**yes**/no)	0.91	0.61–1.32	0.586	0.83	0.47–1.45	0.497
Concomitant CIS (**yes** vs no)	1.58	0.94–2.29	0.081	1.73	0.87–3.55	0.072
Size (≤3 cm vs **>3 cm**)	1.02	0.75–1.39	0.941	1.25	0.76–2.07	0.375
Focality (solitary vs **multiple**)	1.11	0.81–1.51	0.516	1.25	0.75–2.09	0.384
BCG strain: HR (95% CI); p-value	*vs. Moreau*	*vs. TICE*	*vs. RIVM*	*vs. Moreau*	*vs. TICE*	*vs. RIVM*
Moreau	X	1.03 (0.68–1.58); p = 0.881	0.62 (0.40–0.95); p = 0.029a	X	1.34 (0.72–2.56); p = 0.357	0.81 (0.42–1.56); p = 0.523
TICE	0.97 (0.63–1.47); p = 0.881	X	0.60 (0.42–0.87); p < 0.001a	0.74 (0.39–1.39); p = 0.357	X	0.60 (0.34–1.06); p = 0.081
RIVM	1.61 (1.05–2.47); p = 0.029a	1.66 (1.15–2.38); p < 0.001a	X	1.23 (0.64–2.35); p = 0.523	1.65 (0.94–2.91); p = 0.081	X

aStatistically significant *P* value. HR >1 worse outcome for the bolded option, HR <1 better outcome for the bolded option.

compared to the Moreau or TICE strains; however, no significant differences in PFS were observed between any of the strains.

The BCG strains analysed in our present study have rarely been reported in the literature or they have not been directly compared yet in patients with T1HG NMIBC. D'Andrea *et al*. [9] in their retrospective study did not show any difference in RFS and PFS between the TICE and Moreau strains. Although primary analysis was conducted for the heterogeneous population of patients with different NMIBC stages, additional exploratory analysis adjusted for pathological stage (including the T1 subgroup) also revealed no association with RFS and PFS between the TICE and Moreau strains. Interestingly, a trend in RFS in favour of Moreau was observed if adequate BCG treatment was administered, but patients receiving BCG-Moreau were more likely to experience disease recurrence without adequate maintenance treatment [9]. On the contrary, we observed a slight trend towards BCG-TICE (HR 0.91 for TICE).

The Dutch Cooperative Trial evaluated mitomycin vs BCG-TICE vs BCG-RIVM in 469 patients with pTA/pT1 NMIBC and CIS of the urinary bladder after TURB, and no significant difference in terms of recurrence between analysed strains was found [10]. Contrary to that study, our present results indicated the possible inferiority of the RIVM strain.

Although several randomised controlled trials (RCTs) reported differences between various BCG strains, they were generally flawed by a small sample size and/or not involving patients receiving maintenance treatment [11–13]. However, because of the fact that maintenance BCG provides superior RFS benefit when compared to an induction course only, results from these trials may not be applicable to current practices. One example is a study by Rentsch *et al*. [14]. In their RCT including 132 patients with high-risk NMIBC, who received either BCG-Connaught ($n = 71$) or BCG-TICE ($n = 60$), the authors found a significantly higher 5-year RFS rate for patients treated with BCG-Connaught. However, no statistically significant difference was observed for PFS and overall survival. Additionally, the authors evaluated immunogenicity of the two strains in a mouse model and found that BCG-Connaught induced more robust T-cell recruitment to the bladder compared with BCG-TICE ($P < 0.05$), which might explain the differential efficacy of the Connaught and TICE strains. Even though the study was well-designed, it has to be once more highlighted that patients received only a BCG induction course [14].

Finally, a meta-analysis of prospective RCTs and recent network meta-analysis including 10 different BCG strains did not confirm the superiority of any BCG strain over any other [15,16]. Similarly, recent data from a prospective study conducted by Unda-

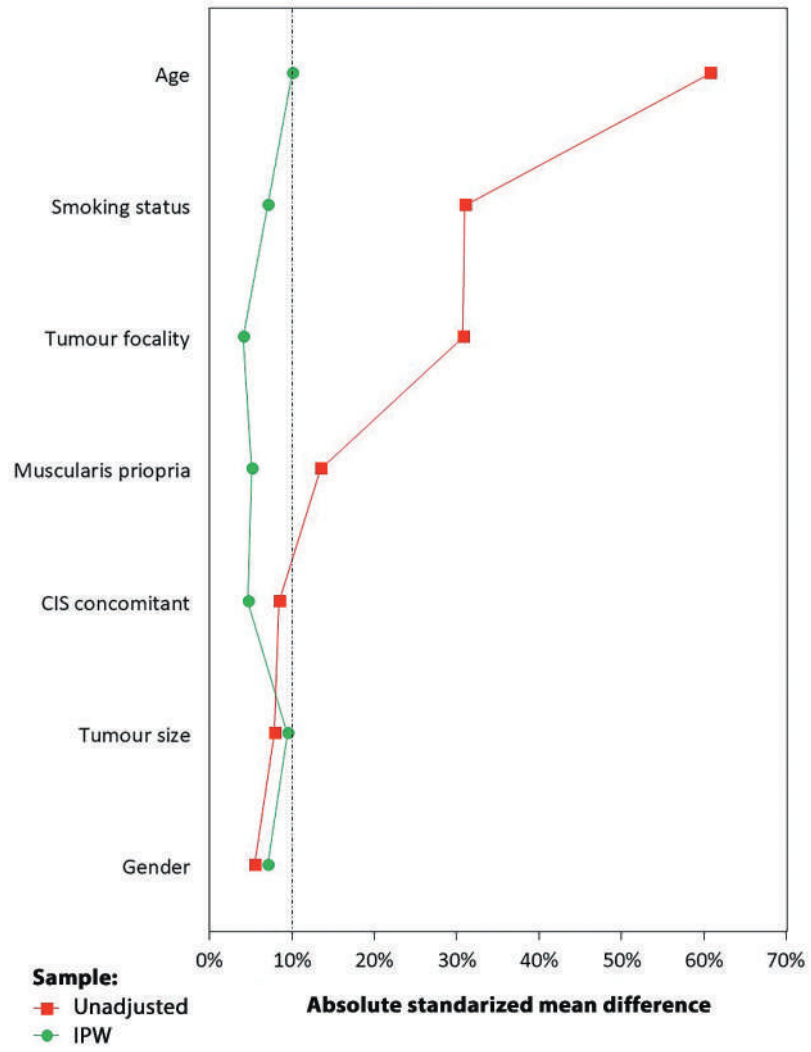

Figure 2. Covariates balance before adjustment and after IPW.

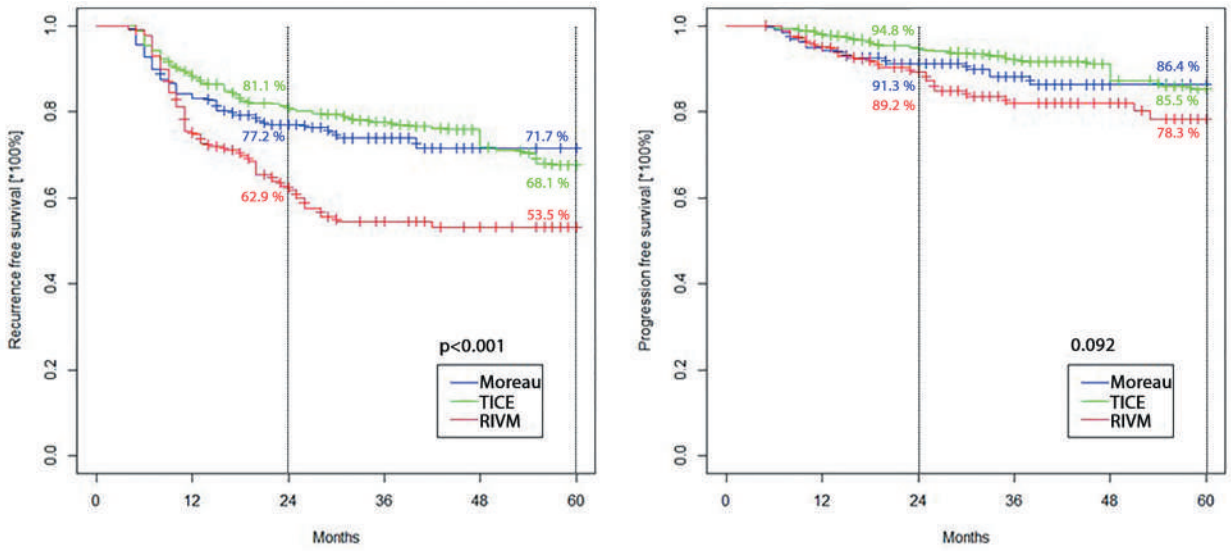

Figure 3. **A**. RFS for the 5-year follow-up after IPW ($P < 0.001$). **B**. PFS for the 5-year follow-up after IPW ($P = 0.092$).

Urzaiz *et al*. [17], as well as data from a *post hoc* analysis of a large Phase II prospective trial assessing BCG and interferon-α in both BCG-naive and BCG-failure patients [18] did not present any clear differences in oncological outcomes between various BCG strains. However, the quality of data (Level of Evidence 2a) did not allow for the drawing of definitive conclusions.

To our best knowledge, we directly compared oncological outcomes of different BCG strains in the largest cohort of patients with T1HG tumours to date. Strains

presented in the present study, especially Moreau, are seldom reported in other trials. Also, patients included in our present study received a maintenance course, which was rarely presented in previous reports. Finally, contrary to the majority of other papers, both RFS and PFS were analysed in the present study. Despite several strengths, the present study has certain limitations. First, some centres collected clinical and pathological data prospectively, but most data were collected retrospectively. Thus, to overcome limitations of a retrospective design, as well as existing differences in baseline patients' characteristics, we performed IPW-adjusted analyses. Second, the data included in the present study was mainly gathered from outpatient BCG departments. Therefore, meticulous details on patients who were not qualified or were dropped from BCG were not recorded and therefore not included in the analysis. Third, to preserve the homogeneity of the population, we included only patients that received at least five induction and two maintenance instillations, representing an adequate BCG exposure. However, this might mean that some relevant patients (e.g. patients with poor outcome at re-TURB after BCG induction) were not included. Fourth, there was no central specimens review and no substaging of T1 tumours. Fifth, all data used in the present paper originated from high-volume oncological centres, thus the results of this study may not be applicable to centres with lesser experience in bladder cancer treatment. Sixth, data regarding the experience of the surgeons, technique details were not available and therefore were not included in the analysis. Also, data about the WHO 1973 grade, immediate single-instillation chemotherapy, LVI, VH and prostatic involvement of the tumours were not uniformly reported and/or were unreliable, and therefore not included in the analysis. Finally, we did not perform cancer-specific survival analysis because the number of events (cancer-specific deaths) was low, and therefore, not statistically representative.

Conclusions

The results of the present study suggest that the Moreau and TICE BCG strains might be superior to the RIVM strain in terms of RFS in patients with T1HG NMIBC.

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Disclosure statement

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10. ARTYKUŁ PIĄTY:

Ubiquitin-Specific Proteases as Potential Therapeutic Targets in Bladder Cancer-In Vitro Evaluation of Degrasyn and PR-619 Activity Using Human and Canine Models

biomedicines

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Article

Ubiquitin-Specific Proteases as Potential Therapeutic Targets in Bladder Cancer—In Vitro Evaluation of Degrasyn and PR-619 Activity Using Human and Canine Models

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Abstract: Background: The inhibition of ubiquitin-specific proteases (USPs) is a novel and promising direction in the development of molecularly targeted therapies in oncology. The aim of the present study was to examine whether Degrasyn could be a potential therapeutic agent against bladder cancer (BC). Also, we aimed to determine whether Degrasyn is more effective in terms of anti-cancer activity compared to the non-selective DUB inhibitor PR-619. To facilitate the translational value of the obtained results, our experiments were performed using both human and canine in vitro models of BC. Methods: Human T24 (urothelial grade III BC) and SV-HUC-1 (non-tumorigenic urothelial cell line), as well as canine K9TCC-PU-NK and RDSVS-TCC1 (both derived from invasive grade III urothelial bladder tumors) cell lines, were used in the present study. Cell proliferation was determined using the MTT assay and Ki-67 proliferation assay, and the level of apoptosis induced by Degrasyn and PR-619 was evaluated by Annexin V-FITC staining and caspase 3/7 activation assay. Western blot was used to assess DNA damage and key proteins involved in apoptosis. Results: Degrasyn inhibited the proliferation of all BC cell lines in a concentration- and time-dependent manner. Lower concentrations of Degrasyn were more potent against human and canine BC cell lines compared to PR-619. Degrasyn induced caspase-dependent apoptosis and triggered DNA damage. PR-619 did not show a significant pro-apoptotic effect. Conclusions: Our results demonstrate that Degrasyn significantly impairs the growth of in vitro models of human and canine BC. Selective USP inhibition with Degrasyn seems to be more effective in reducing BC cell proliferation and inducing apoptosis and DNA damage than non-selective USP inhibition with PR-619.

Keywords: bladder cancer; urothelial carcinoma; ubiquitin-specific proteases; Degrasyn; PR-619

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1. Introduction

Bladder cancer (BC) is the most common malignancy of the urinary system, with approximately 500,000 individuals diagnosed annually worldwide [1]. BC is a heterogeneous disease associated with various clinical outcomes. It may be divided into two main staging subgroups: non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC) [2]. NMIBC includes lesions limited to the mucosal or submucosal layers of the bladder wall, whereas MIBC comprises tumors infiltrating and/or exceeding the lamina muscularis propria of the bladder wall [2].

The majority of NMIBCs can be managed with total curative intent, while the treatment of MIBCs is very challenging and multimodal. Despite the standard treatment (involving radical cystectomy with pelvic lymph node dissection), almost 50% of patients with MIBC will eventually develop metastatic disease, which is associated with poor survival rates [3].

The systemic management of advanced or metastatic MIBC primarily consists of platinum-based chemotherapy; however, chemotherapy provides an approximately 50% response rate with only a 20% 5-year overall survival rate [3]. One of the most important discoveries in recent years, facilitating the treatment of advanced or metastatic MIBC, has been the introduction and approval of drugs targeting immune checkpoints: programmed death receptor 1 (PD-1) and programmed death receptor 1 ligand (PD-L1) inhibitors. Nevertheless, despite their efficacy, which has been proven in an increasing number of clinical trials, only a small proportion of patients clearly benefit from such treatment (which is mainly dependent on PD-1/PD-L1 expression levels) [4]. Due to the above-mentioned limitations of current systemic MIBC therapies, great emphasis is constantly placed on the investigation of novel therapeutic targets and drugs.

Since its discovery almost 40 years ago, the ubiquitin-proteasome system (UPS) has been the subject of increased research interest, as protein ubiquitination and related post-translational modifications regulate diverse aspects of cellular biology. Generally, the process of ubiquitination requires three different enzymes: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase) [5]. It can be reversed by deubiquitinating enzymes (DUBs), a group of over 100 enzymes divided into five families: ubiquitin-specific proteases (USPs); ovarian tumor proteases (OTUs); ubiquitin C-terminal hydrolases (UCHs); Machado–Joseph disease protein domain proteases (MJDs); and JAMM motif proteases [5]. Particular DUBs catalyze the removal of ubiquitin particles from different ubiquitinated proteins, which leads to the regulation of the stability and activity of the target particles [5,6]. This process is crucial for controlling various cellular pathways, such as DNA repair, gene expression, protein localization, kinase activation, cell cycle progression, or cell apoptosis [6]. The growing body of evidence suggests that dysregulation of the DUBs system can contribute to the development of a variety of cancers, including BC [6].

Being the largest family among DUBs with over 50 members, USPs have attracted major attention as potential molecular targets in cancer therapy. To date, pharmacological studies have identified several novel DUB inhibitors for potential clinical use, mainly non-selective multiple DUBs/USPs inhibitors [7]. In the BC setting, a few preclinical studies demonstrated that the non-selective pan-DUB/USP inhibitor PR-619 appears to be a potential therapeutic agent, especially in combination with chemotherapeutics [8,9]. Recently, more selective DUBs/USPs inhibitors, such as Degrasyn (also known as WP1130), have been investigated in some types of cancer with promising results. Degrasyn is a small molecule compound identified as a selective USP (USP5, USP9X, and USP14) inhibitor. The effectiveness of Degrasyn against BC has not been investigated to date.

BC is not only a significant health issue in humans, but it also occurs in companion animals, including dogs (representing 1–2% of all canine tumors) [10]. With estimates that 4–6 million pet dogs develop cancer in the United States annually, this equates to more than 60,000 cases of BC in dogs each year. In contrast to humans, the majority of dogs (90% of cases) are diagnosed with advanced or metastatic MIBC, which is frequently characterized by an aggressive clinical course and chemotherapy resistance [10]. Therefore, the generally poor survival outcomes in dogs with BC implicate an urgent need for the development of novel treatment compounds simultaneously with human medicine. Successful findings from research using both human and canine models can be potentially translated across the species, accelerating the development of novel therapeutic agents effective against BC in either human or veterinary medicine.

As the inhibition of DUBs/USPs functions is a novel and promising direction in the development of molecularly targeted therapies in oncology, the main aim of the present study was to examine whether Degrasyn could be a potential therapeutic agent effective against bladder cancer (BC). Also, we aimed to determine whether Degrasyn is more effective in terms of anti-cancer activity compared to the non-selective DUB inhibitor PR-619. To facilitate the translational value of the obtained results, our experiments were performed using both human and canine in vitro models based on multidisciplinary collaboration.

2. Materials and Methods

2.1. Cell Lines and Cell Culture

Human T24 (urothelial grade III BC) and SV-HUC-1 (immortalized and non-tumorigenic urothelial cells) cell lines were used in the present study. They were acquired from the American Type Culture Collection (ATCC, Rockville, MD, USA). T24 and SV-HUC-1 cell lines were maintained in McCoy's 5a Modified Medium and F-12K Medium (ATCC, Rockville, MD, USA), respectively. As a representative in vitro model of canine BC, K9TCC-PU-NK and RDSVS-TCC1 cell lines (both derived from invasive grade III urothelial bladder tumors) were also involved in this study. K9TCC-PU-NK and RDSVS-TCC1 cell lines were generous gifts from Deepika Dhawan (Purdue University College of Veterinary Medicine, USA) and Maciej Parys (Royal (Dick) School of Veterinary Studies and The Roslin Institute, University of Edinburgh, Scotland), respectively. Both canine BC cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium (Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland).

All culture media used in our study were supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma-Aldrich, Steinheim, Germany), and 10% heat-inactivated fetal bovine serum (FBS, Gibco, Grand Island, NY, USA). All cell cultures were performed at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Chemicals and Reagents

Degrasyn (WP1130) and PR-619 were purchased from Selleck Chemicals (Houston, TX, USA). FBS, L-glutamine, penicillin and streptomycin solution, 0.25% trypsin EDTA, trypan blue, poly-L-lysine, ribonuclease A, propidium iodide (PI), RIPA buffer, SigmaFAST Protease Inhibitor Cocktail, and TBST buffer were acquired from Sigma-Aldrich (Steinheim, Germany). Annexin V-FITC was obtained from Immunostep (Salamanca, Spain). The CellEvent® Caspase3/7 Green Flow Cytometry Assay by ThermoFisher (Waltham, MA, USA) was used. Anti-USP5 (sc-390943), anti-USP9X/Y (sc-365353), anti-USP14 (sc-515812), anti-Bcl-2 (sc-7382), and anti-β actin (sc-47778) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), whereas the anti-Bcl-xl (#2764) antibody was purchased from Cell Signaling (Cell Signaling Technology, Danvers, MA, USA). The anti-γH2A.X (ab26350) antibody was from Abcam (Cambridge, UK), and anti-mouse/HRP and anti-rabbit/HRP antibodies were bought from Dako (Glostrup, Denmark).

2.3. Cell Proliferation Assay

Cell proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, Steinheim, Germany). Cells were plated at a density of 1×10^5 cells per mL in 96-well plates (Thermo Fisher Scientific, Roskilde, Denmark) and allowed to attach overnight. Subsequently, Degrasyn and PR-619 were added in increasing concentrations (0.6, 1.25, 2.5, 5, 10, and 20 µM). After incubation for 24, 48, and 72 h with either the compounds or in the medium alone, 20 µL of MTT solution (5 mg/mL) was added to each well. After an additional 4 h, 80 µL of the lysis buffer (225 mL DMF, 67.5 g SDS, and 275 mL distilled water) was added to each well. After dissolving the content, the absorbance was measured at a reference wavelength of 570 nm using a spectrophotometric microplate reader Spark (Tecan). The values were obtained from 3 independent experiments (different plates, different days). Based on the results, the concentration that inhibited 50% of the cell proliferation (IC_{50}) was calculated for all incubation intervals.

2.4. Ki-67 Flow Cytometry Proliferation Assay

For the cell proliferation assay, cells were plated in 100 mm cell culture plates. When the cultures reached 60% confluence, the media were discarded from the wells and replaced with culture media only or media with added compounds (Degrasyn, PR-619) with increasing concentrations for 24 h. The growth medium was then removed from the wells, the cells were rinsed twice with PBS, and 0.25% trypsin-EDTA was added to detach the cells. After

5 min of incubation at 37 °C and 5% CO_2, the fresh culture medium was added to inactivate the trypsin, and cells were collected in flow cytometry tubes. Cells were then labeled using the Ki-67 Proliferation Kit BD Pharmingen(BD Biosciences) according to the manufacturer's instructions and analyzed using a flow cytometer (Cytoflex, Beckman Coulter). Unstained control cells were used for gating to determine the percentage of the proliferation of the Ki-67-positive cells in the samples. Percentages of proliferating (Ki-67 positive) cells were used to calculate the means ± SD for each cell line. The presented results were obtained from three independent experiments.

2.5. Western Blot Analysis

Western blot was performed to assess the expression of USP5, USP9X, and USP14 in analyzed cell lines. After 24 h of incubation with increasing concentrations of Degrasyn and PR-619, the expression of proteins involved in apoptosis (Bcl-2 and Bcl-xl) and a marker of DNA damage (γH2A.X) was also evaluated. Briefly, cells were rinsed with cold phosphate-buffered saline (PBS), suspended in lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1% NP-40, protease inhibitors set), and incubated for 20 min on ice. Subsequently, after centrifuging at 10,000 rpm at 4 °C for 12 min, sodium dodecyl sulfate (SDS) sample buffer was added to the suspensions to clear the supernatants. After boiling for 5 min at 95 °C, the samples were subjected to SDS-polyacrylamide gel electrophoresis on 8–15% gel. For USP5, USP9X, USP14, γH2A.X, Bcl-2, and Bcl-xl expression analysis, transfers onto nitrocellulose membranes were performed using a Semidry Transfer Cell or wet transfer method (Bio-Rad, Hercules, CA, USA). After transfer, the membranes were blocked with 1% casein in TBS (or 3% BSA for γH2Ax) at room temperature for 1 h. After blocking, the membranes were incubated overnight at 4 °C with primary antibodies: anti-USP5 (dilution 1:500), anti-USP9X/Y (dilution 1:500), anti-USP14 (dilution 1:500), anti-γH2A.X (dilution 1:1000), anti-Bcl-2 (dilution 1:1000), anti-Bcl-xl (dilution 1:1000), and murine monoclonal anti-β-actin antibody (dilution 1:2000). The membranes were then incubated with the secondary antibody for 1 h at room temperature. Membrane visualizations were performed using ChemiDoc Touch Instruments (Bio-Rad, Hercules, CA, USA).

2.6. Quantification of Apoptosis

To assess the level of apoptosis induced by Degrasyn and PR-619, cells were seeded at a density of 1×10^5/mL in 96-well plates (TPP, Trasadingen, Switzerland) and incubated for 24 h with increasing concentrations of compounds. Cells were then collected, suspended in a binding buffer, and stained with Annexin V-FITC for 10 min at room temperature. Subsequently, PI was added, and flow cytometric analysis was immediately performed using a flow cytometers (FACS Calibur, Becton Dickinson, Biosciences, San Jose, CA, USA; CytoFlex, Beckman Coulter, CA, USA). CellQuest 3.lf. (Becton Dickinson, San Jose, CA, USA) and CytExpert 2.4 (Beckman Coulter, CA, USA) software were used for data analysis.

Subsequently, to assess caspases 3/7 activation, the cells were incubated with medium alone or with increasing concentrations of compounds (Degrasyn, PR-619) in conditions similar to the previous test. Following cell collection, the cells were twice PBS-washed and stained in accordance with the manufacturer's instructions. For active caspase 3/7 detection, CellEventCaspase-3/7 Green Detection Reagent Solution was added to the samples. The cells were then suspended 1 more time in the wash buffer after being washed twice. For flow cytometric analysis, a flow cytometers (FACS Calibur, Becton Dickinson, Biosciences, San Jose, CA, USA; CytoFlex, Beckman Coulter, CA, USA) were used, and CellQuest 3.lf (Becton Dickinson, San Jose, CA, USA) and CytExpert 2.4 (Beckman Coulter, CA, USA) software were used for data analysis. The presented results were obtained from three independent experiments.

2.7. Statistical Analysis

Statistical analyses were performed using the GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA). All data were presented as means with standard deviations

(SD). Data from the 2 groups were analyzed by a 2-tailed Student's t-test, and data from multiple groups were analyzed using a 1-way ANOVA followed by Tukey's posthoc test. Results were considered significant at $p < 0.05$.

3. Results

3.1. Expression of USP5, USP9X, and USP14 in Human and Canine Bladder Cancer Cells

At the first step of the research, we analyzed USP5, USP9X, and USP14 expression levels in selected cell lines, as they are targets of the inhibitory effect of Degrasyn. As illustrated in Figure 1, both human cell lines (T24 and SV-HUC-1) were characterized by higher expression levels of the USP5 protein compared to canine BC cell lines (K9TCC-PU-NK and RDSVS-TCC1). Compared to control non-tumorigenic urothelial cells (SV-HUC-1 cell line), higher expression of USP5 protein was observed in the T24 cell line (representing a human BC cell line). Similar expression levels of USP9X and USP14 proteins were observed in either human or canine cell lines.

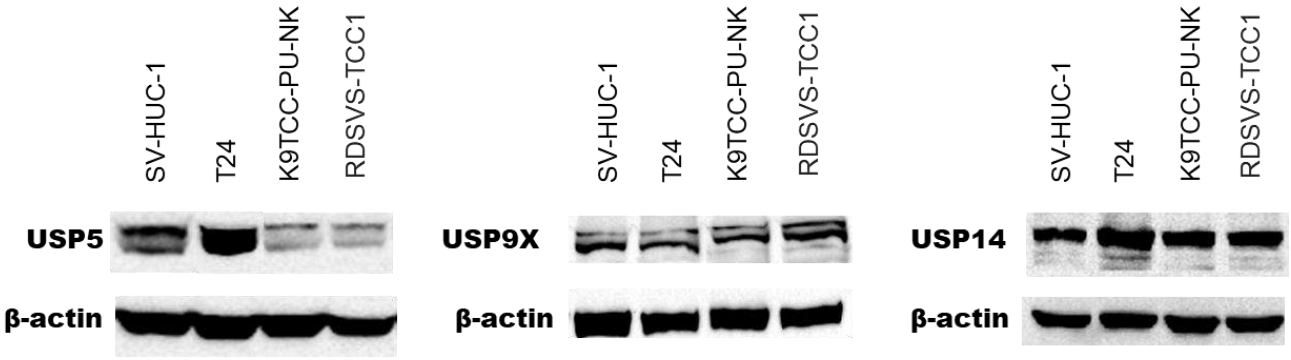

Figure 1. Representative graphical results of Western blot analyses showing expression of USP5, USP9X and USP14 in tested cell lines. USP = ubiquitin-specific protease.

3.2. Determination and Comparison of the Anti-Proliferative Effects of Degrasyn and PR-619 on Bladder Cancer Cells

Subsequently, in order to evaluate the hypothesis of the antiproliferative activity of Degrasyn against BC cells, we performed an MTT assay. The MTT assay demonstrated that Degrasyn inhibited the proliferation of all cell lines used in the present study in a concentration- and time-dependent manner (Table 1). The number of actively dividing cells clearly decreased between 24 and 48 h of incubation, but the difference between 48 h and 72 h was marginal. Relative to 24 h of incubation time, we observed significantly less cytotoxicity of Degrasyn on the human control SV-HUC-1 cell line compared to the T24 cell line (IC_{50} values 2.33 μM and 3.07 μM, respectively, $p < 0.05$). However, the IC_{50} values did not significantly differ for longer incubation times (Table 1). For all incubation times, the human BC line (T24) was more sensitive to the anti-proliferative effect of Degrasyn compared to the canine BC cell lines (K9TCC-PU-NK and RDSVS-TCC1).

Table 1. Comparison of Degrasyn concentrations that inhibited 50% of the cell proliferation (IC_{50} values (μM)) for all tested cell lines after 24, 48, and 72 h of exposure, evaluated with MTT assay.

Time/Cell Line	SV-HUC-1	T24	K9TCC-PU-NK	RDSVS-TCC1
24 h	$3.07^{a} \pm 0.17$	$2.33^{b} \pm 0.19$	$4.65^{c} \pm 0.27$	$4.36^{c} \pm 0.22$
48 h	$1.59^{a} \pm 0.09$	$1.43^{a} \pm 0.06$	$3.50^{b} \pm 0.16$	$3.76^{b} \pm 0.56$
72 h	$1.51^{a} \pm 0.08$	$1.37^{a} \pm 0.12$	$3.52^{b} \pm 0.04$	$3.21^{c} \pm 0.10$

Note: The concentration range used was 0.6, 1.25, 2.5, 5, 10, and 20 μM. For each cell line, values without common letters (a, b, c) in the superscript differ statistically ($p < 0.05$). Results are presented as means ± standard deviations (SD) of three independent experiments (three wells each).

Furthermore, we compared the antiproliferative activity of Degrasyn and PR-619. The MTT assay confirmed the antiproliferative activity of PR-619 against all analyzed cell lines. The effect was both concentration- and time-dependent (Table 2). The IC_{50} of Degrasyn was significantly lower than the IC_{50} of PR-619 in corresponding time intervals (Figure 2). Lower concentrations of Degrasyn (1.25–10 µM) were more potent toward human and canine cell lines compared to PR-619 (Figure 3, Supplementary Figure S1). Nevertheless, the highest concentrations of both Degrasyn and PR-619 used in this study killed almost 100% of cells in all analyzed cell lines.

Table 2. Comparison of PR-619 concentrations that inhibited 50% of the cell proliferation (IC_{50} values (µM)) for all tested cell lines after 24, 48, and 72 h of exposure, evaluated with MTT assay.

Time/Cell Line	SV-HUC-1	T24	K9TCC-PU-NK	RDSVS-TCC1
24 h	7.28 ^{a} ± 0.12	6.78 ^{a} ± 0.78	13.19 ^{b} ± 0.58	13.41 ^{b} ± 0.56
48 h	4.23 ^{a} ± 0.11	3.70 ^{a} ± 0.37	7.19 ^{b} ± 0.26	8.56 ^{b} ± 1.58
72 h	3.85 ^{a} ± 0.06	3.23 ^{a} ± 0.31	6.15 ^{b} ± 0.60	7.31 ^{b} ± 0.61

Note: The concentration range used was 1.25, 2.5, 5, 10, and 20 µM. For each cell line, values without common letters (a, b) in the superscript differ statistically ($p < 0.05$). Results are presented as means ± standard deviations (SD) of three independent experiments (three wells each).

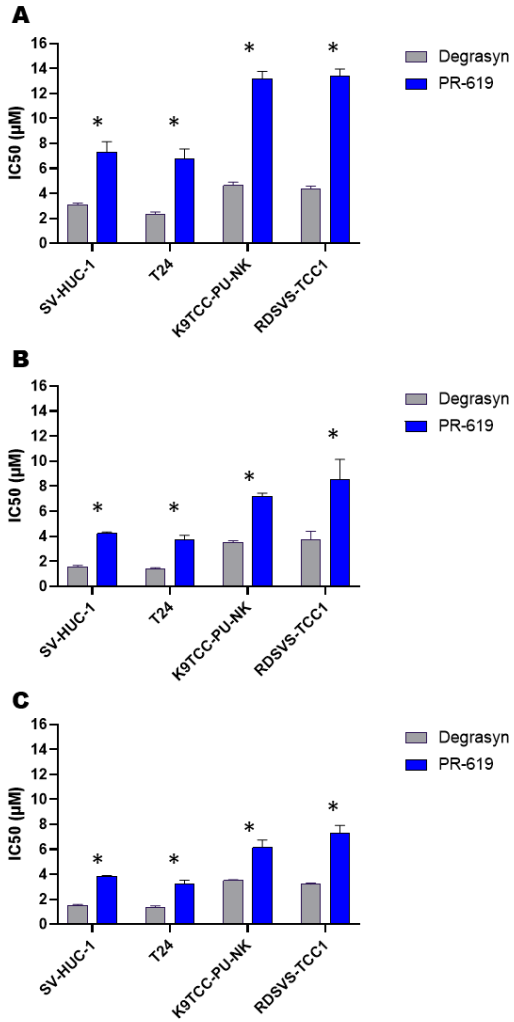

Figure 2. Comparison of Degrasyn and PR-619 half maximal inhibitory concentrations (IC_{50}) values after (**A**) 24 h incubation; (**B**) 48 h incubation; (**C**) 72 h incubation. The values are presented as means ± standard deviations (SD) of three independent experiments. * Difference between Degrasyn and PR-619 considered statistically significant ($p < 0.05$).

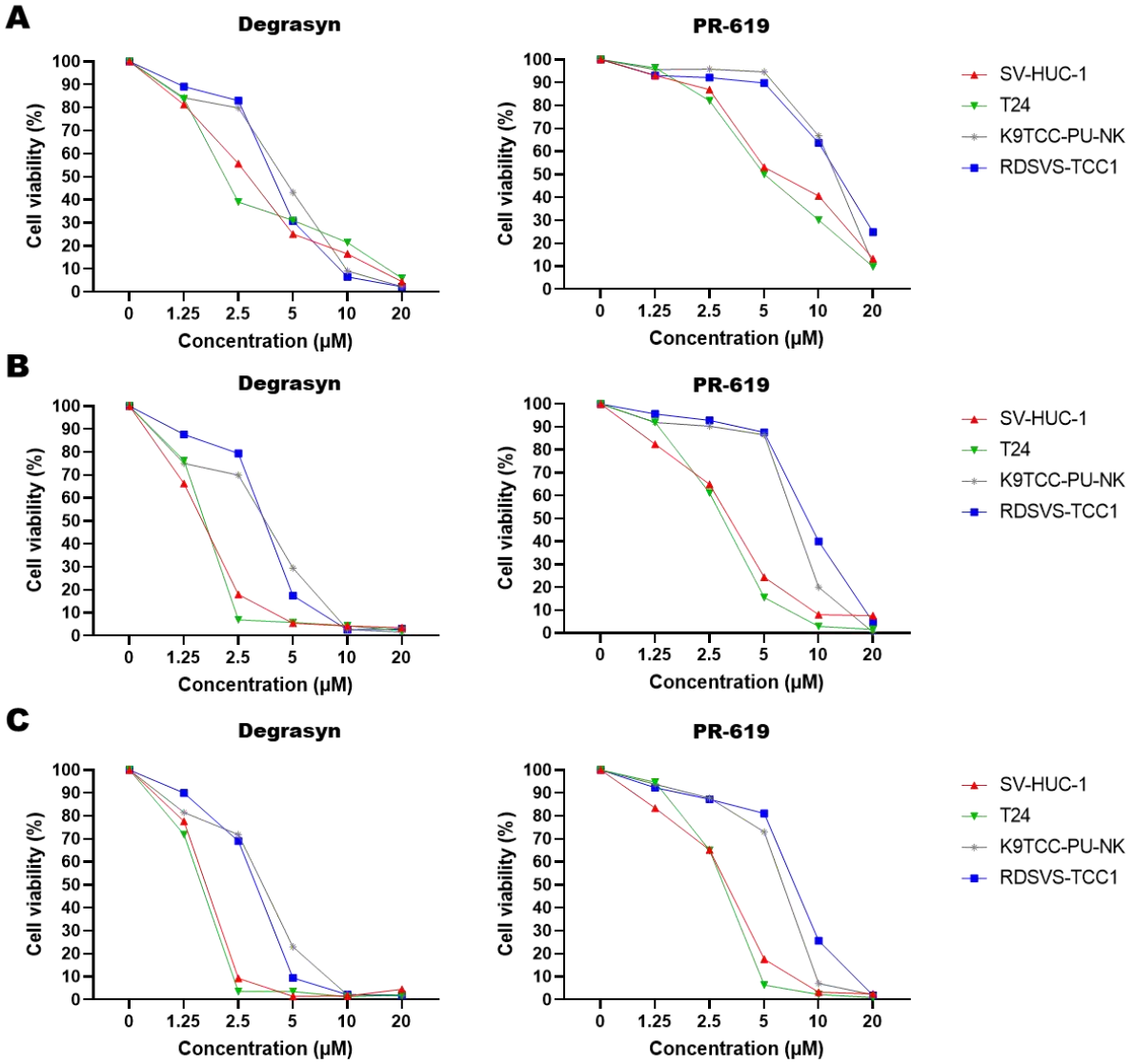

Figure 3. Analysis of Degrasyn and PR-619 influence on the cell proliferation of SV-HUC-1, T24, K9TCC-PU-NK, and RDSVS-TCC1 cell lines (MTT assay) after 24 (**A**), 48 (**B**), and 72 (**C**) hours with increasing concentrations of the drugs (data for 1.25, 2.5, 5, 10, and 20 μM). Values are presented as means ± standard deviations (SD) of three independent experiments.

3.3. Ki-67 Flow Cytometry Proliferation Assay

The percentage of Ki-67 positive cells (as compared to the control) was significantly decreased after incubation with 2.5–5 μM Degrasyn and 5–10 μM PR-619 in the T24 cell line ($p < 0.05$) (Figure 4, Supplementary Figure S2). For other cell lines, the percentage of Ki-67 positive cells (as compared to control) was significantly decreased ($p < 0.05$) after incubation only with the highest concentrations of compounds (5 μM Degrasyn and 10 μM PR-619) (Figure 4, Supplementary Figure S2). Generally, the proportion of Ki-67 positive cells was significantly lower ($p < 0.05$) in SV-HUC-1 and canine BC cell lines than in the T24 cell line for all used concentrations of Degrasyn and PR-619 ($p < 0.05$).

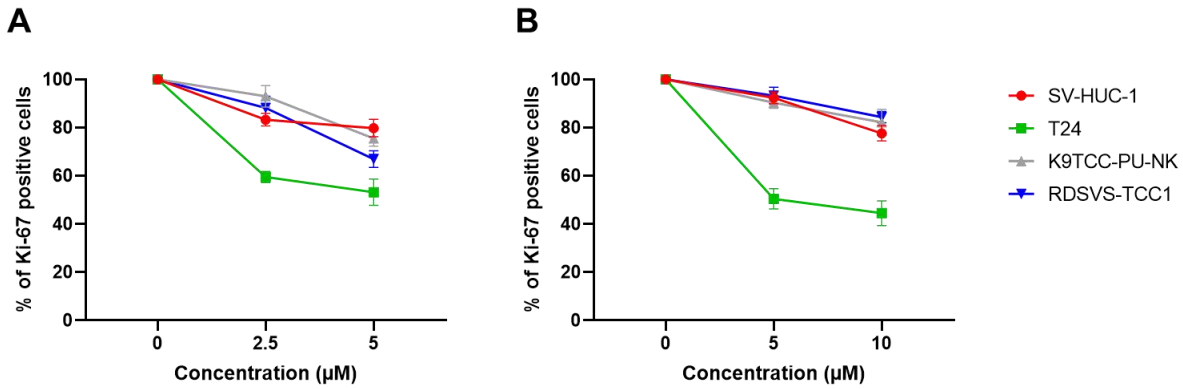

Figure 4. Ki-67 flow cytometry proliferation assay. Percentages of proliferating (Ki-67 positive) cells in reference to control (medium only) after the incubation with increasing concentrations of (**A**) Degrasyn and (**B**) PR-619. Values are presented as means ± standard deviations (SD) of three independent experiments.

3.4. Determination and Comparison of Pro-Apoptotic Effect of Degrasyn and PR-619 on Bladder Cancer Cells

In the next step, we determined whether the inhibition of cell proliferation by Degrasyn demonstrated in the MTT assay was potentially related to the induction of apoptosis. For this purpose, Annexin V-FITC and PI staining were performed. After 24 h of incubation with Degrasyn at increasing concentrations (up to approximately the IC_{50} value for particular cell lines), we detected a significant increase in the number of apoptotic cells (stained only with Annexin V-FITC for early apoptotic cells and double stained with Annexin-FITC and PI for late apoptotic cells) in all cell lines except the K9TCC-PU-NK cell line (Figure 5). The effect of Degrasyn on apoptosis level was concentration-dependent, and the percentage of apoptotic cells was the highest in T24 and RDSVS-TCC1 cell lines (for the highest used concentrations representing an approximate IC_{50} value) (Figure 6). In contrast, PR-619 did not show a significant pro-apoptotic effect in the analyzed cell lines. For the highest used concentrations representing approximate IC_{50} values of both compounds, we detected a lower increase in the number of apoptotic cells (stained only with Annexin V-FITC for early apoptotic cells and double stained with Annexin-FITC and PI for late apoptotic cells) after 24 h of incubation with PR-619 compared to Degrasyn in all analyzed cell lines (Figure 5). The percentages of early and late apoptotic cells after 24 h of incubation with 2.5 μM Degrasyn and 5 μM PR-619 were: 24.1 ± 2.8% and 14.8 ± 5.8% in the SV-HUC-1 cell line, respectively; 32.2 ± 9.2% and 9.8 ± 1.5% in the T24 cell line, respectively; 11.8 ± 3.4% and 8.4 ± 0.8% in the K9TCC-PU-NK cell line, respectively; 42.4 ± 2.3% and 17.2 ± 6.5% in the RDSVS-TCC1 cell line, respectively.

To further assess the role of caspases in the apoptosis induced by Degrasyn and PR-619, we evaluated the activation of caspase 3/7. The results are presented in Figures 7 and 8. In the SV-HUC-1 cell line, the activation of caspase 3/7 was significantly higher compared to the control (medium only) only after incubation with the highest used concentration of Degrasyn (2.5 μM). For the T24 cell line, the activation of caspase 3/7 was significantly higher compared to the control (medium only) after incubation with 1.25 μM and 2.5 μM Degrasyn). In both canine BC cell lines (K9TCC-PU-NK and RDSVS-TCC1), the activation of caspase 3/7 was significantly higher after incubation with 2.5 μM and 5 μM Degrasyn as compared to control (medium only). Generally, activation of caspase 3/7 was less pronounced in canine BC cell lines than in the T24 cell line after incubation with Degrasyn. Specifically, the number of cells with active caspase 3/7 after the incubation with the highest concentrations of Degrasyn reached 22.4 ± 5.4% in the SV-HUC-1 cell line, 36.6 ± 0.7% in the T24 cell line, 11.9 ± 1.8% in the K9TCC-PU-NK cell line, and 22.5 ± 4.3% in the RDSVS-TCC1 cell line. After the incubation with the highest concentration of PR-619, the percentage of cells with active caspase 3/7 was 19.6 ± 1.8% in the SV-HUC-1 cell line,

16.1 ± 4.9% in the T24 cell line, 13.3 ± 3.3% in the K9TCC-PU-NK cell line, and 16.2 ± 7.4% in RDSVS-TCC1 cell line.

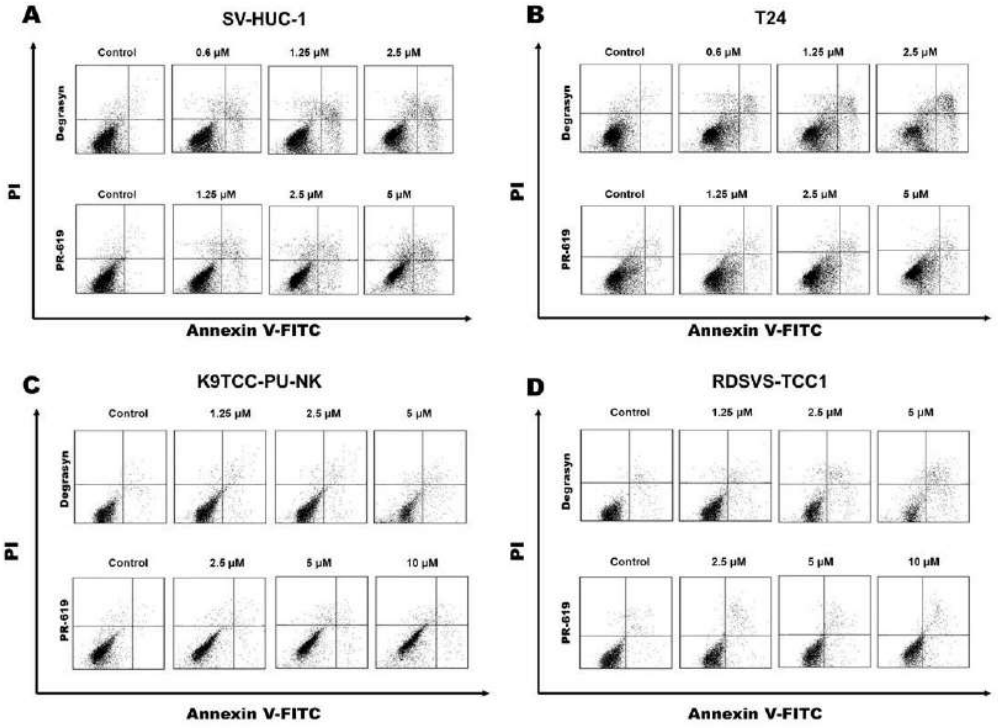

Figure 5. Evaluation of apoptosis induced by Degrasyn and PR-619. Representative dot-plots showing Annexin V-FITC/PI staining of (**A**) SV-HUC-1, (**B**) T24, (**C**) K9TCC-PU-NK, and (**D**) RDSVS-TCC1 cells after 24 h exposure to increasing concentrations of Degrasyn and PR-619.

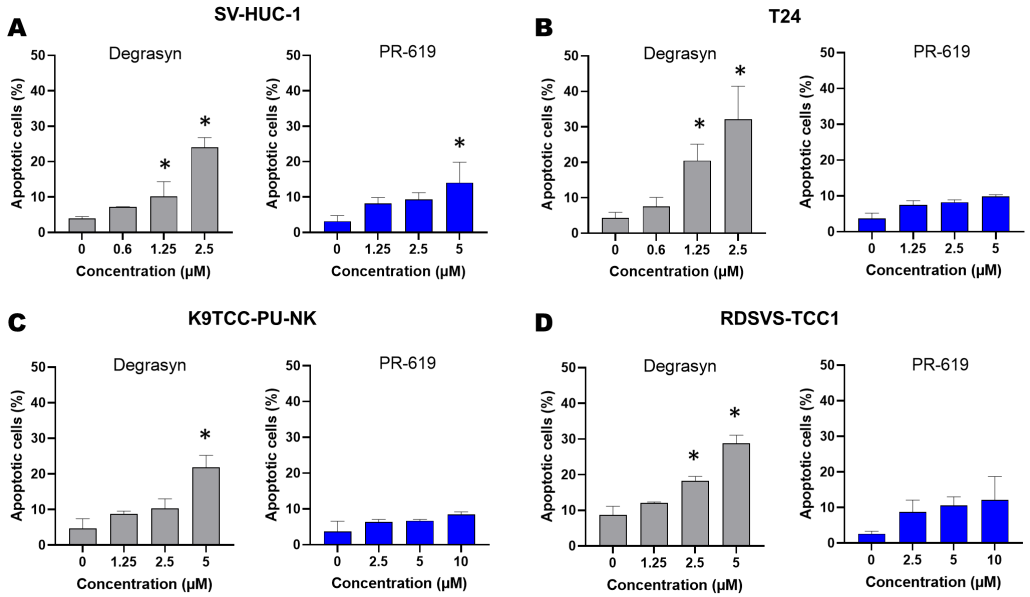

Figure 6. Evaluation of apoptosis induced by Degrasyn and PR-619. Percentage of apoptotic cells after 24 h exposure to increasing concentrations of Degrasyn and PR-619 in (**A**) SV-HUC-1, (**B**) T24, (**C**) K9TCC-PU-NK, and (**D**) RDSVS-TCC cell lines (Annexin V-FITC/PI staining). Values are expressed as the means ± standard deviations (SD) of three independent experiments. * Considered significant in comparison to control ($p < 0.05$).

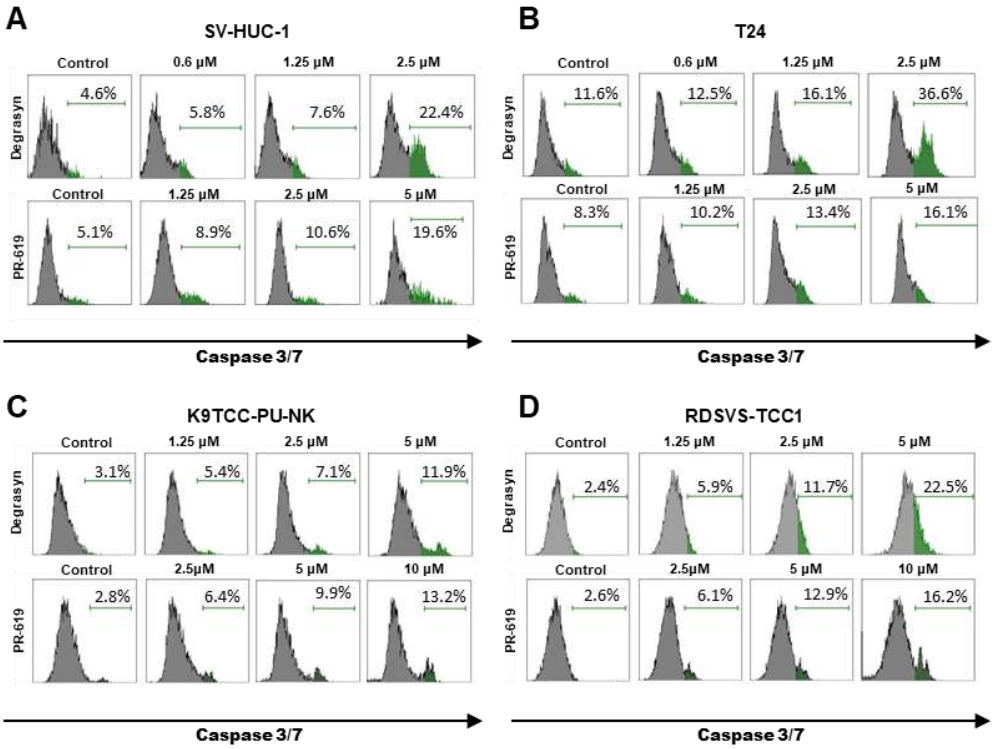

Figure 7. Evaluation of caspase 3/7 activation induced by Degrasyn and PR-619. Representative histograms showing the percentage of cells stained with CellEvent®Caspase-3/7 Green Detection Reagen after 24 h exposure to increasing concentrations of Degrasyn and PR-619 in (**A**) SV-HUC-1, (**B**) T24, (**C**) K9TCC-PU-NK, and (**D**) RDSVS-TCC cell lines.

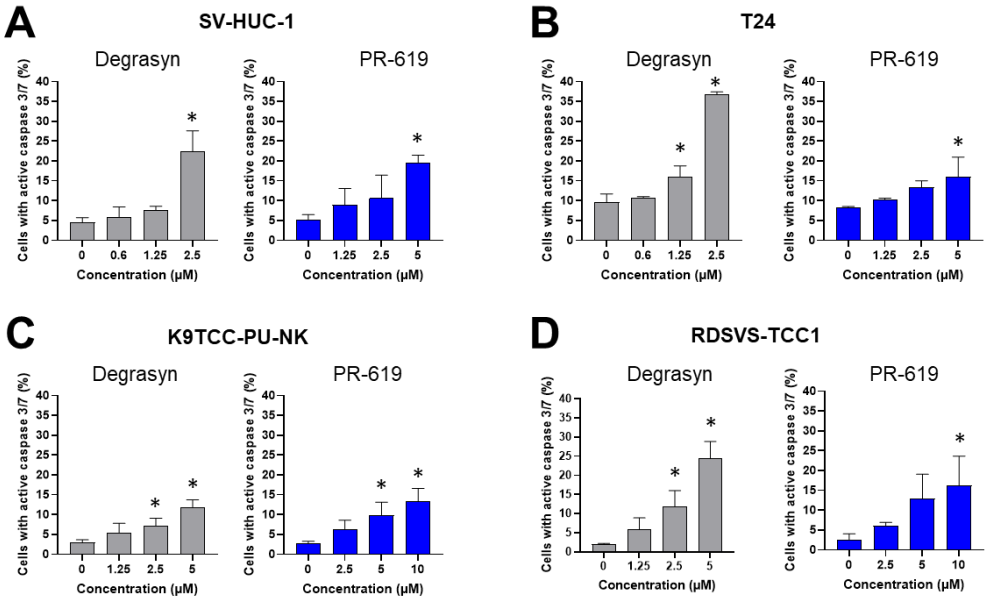

Figure 8. Evaluation of caspase 3/7 activation induced by Degrasyn and PR-619. Percentage of cells with active caspase 3/7 after 24 h exposure to increasing concentrations of Degrasyn and PR-619 in: (**A**) SV-HUC-1, (**B**) T24, (**C**) K9TCC-PU-NK, and (**D**) RDSVS-TCC cell lines. Values are expressed as the means ± standard deviations (SD) of three independent experiments. * Considered significant in comparison to control ($p < 0.05$).

3.5. Evaluation of USP5 Expression in T24 Cells after 24 h of Incubation with Degrasyn and PR-619

To determine the impact of Degrasyn and PR-619 on USP5 protein expression level, a Western blot was performed after a 24 h incubation with both compounds in the human T24 cell line (characterized by the highest expression of USP5 protein among analyzed cell lines). As shown in Figure 9, Degrasyn clearly decreased the USP5 protein expression; however, no visible effect on USP5 expression was observed after 24 h of incubation with PR-619.

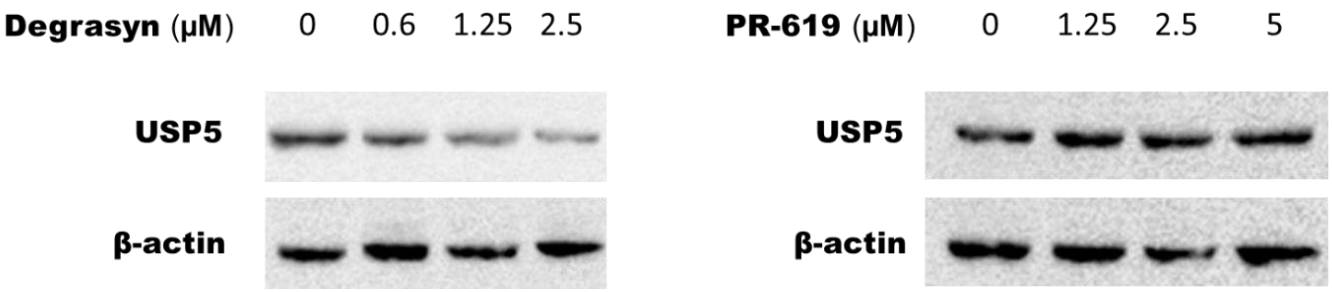

Figure 9. Western blot analysis for USP5 expression in T24 cells after 24 h of incubation with increasing concentrations of Degrasyn (0.6, 1.25, and 2.5 μM) and PR-619 (1.25, 2.5, and 5 μM). USP = ubiquitin-specific protease.

3.6. Evaluation of DNA Damage in Bladder Cancer Cells Induced by Degrasyn and PR-619

Because γH2AX is a recognized marker of DNA damage, we measured its levels after 24 h of incubation with increasing concentrations of Degrasyn and PR-619. Figure 10 shows that treatment of all used BC cell lines with Degrasyn increased the amount of γH2AX at higher concentrations of the drug. In the T24 cell line, a high expression of γH2AX was observed after incubation with 2.5 μM and 5 μM Degrasyn. In canine BC cell lines, a high expression of γH2AX was observed only after incubation with 5 μM Degrasyn. Incubation with PR-619 resulted in only a slight visible increase in the amount of γH2AX after incubation with the highest concentrations of the drug.

3.7. Evaluation of Proteins Involved in Apoptosis Induced by Degrasyn and PR-619

The involvement of key proteins in cell apoptosis after Degrasyn and PR-619 treatment was analyzed using Western blot (Figure 11). No expression of the anti-apoptotic protein Bcl-2 was observed in the SV-HUC-1 cell line, which could be explained by the fact that it is a non-cancerous cell line. The basal expression level of Bcl-2 and Bcl-xl was comparable between human and canine BC cell lines (T24, K9TCC-PU-NK, and RDSVS-TCC-1). Expression of Bcl-2 protein was slightly decreased after incubation with the highest concentrations of drugs (5 μM Degrasyn and 10 μM PR-619 in all BC cell lines. In all cell lines, Bcl-xl expression was slightly reduced after incubation with a 5 μM concentration of Degrasyn. No clearly visible changes in the expression levels of Bcl-xl were noticed in the analyzed cell lines after incubation with increasing concentrations of PR-619.

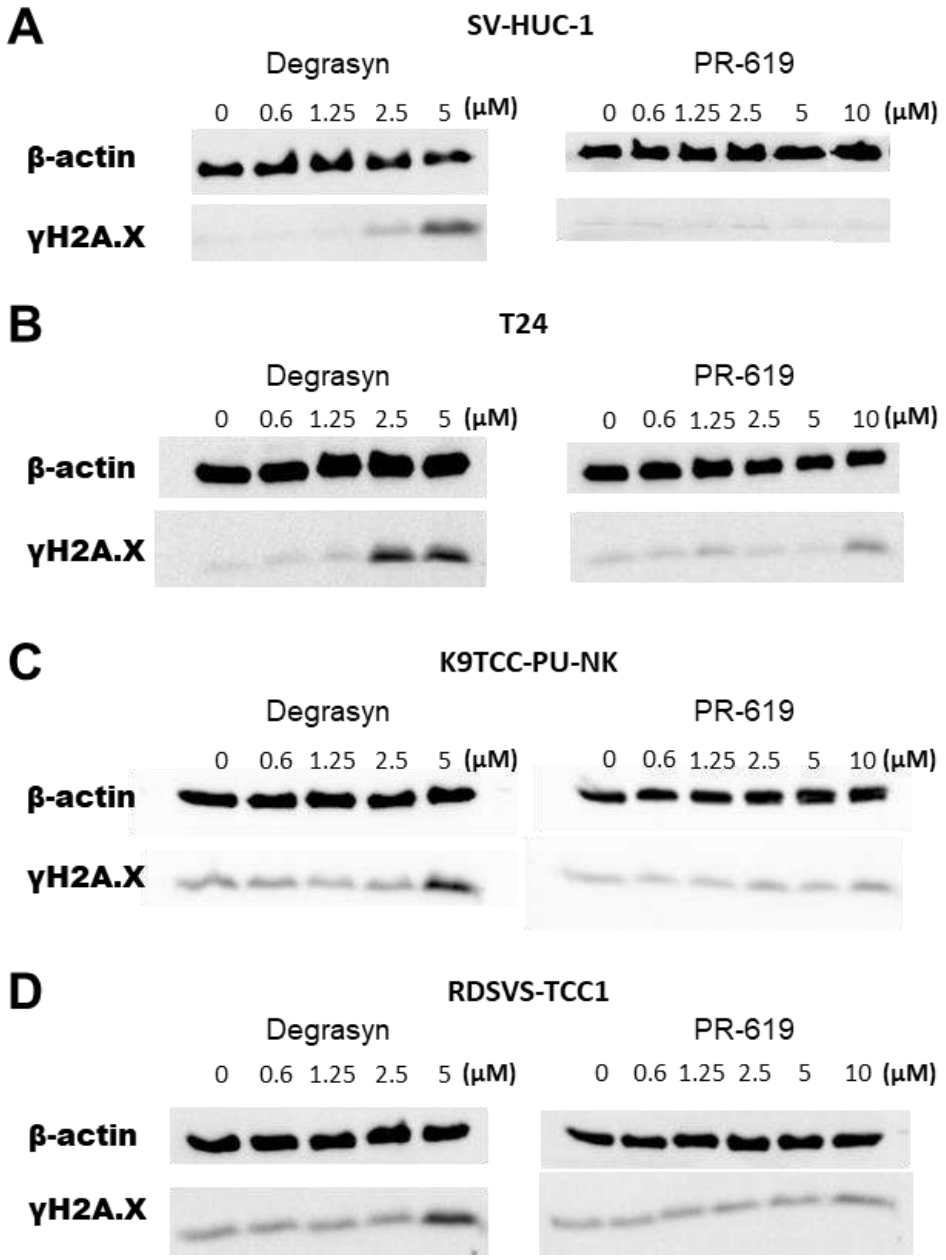

Figure 10. Evaluation of DNA damage generated by Degrasyn and PR-619. Representative blot of γH2A.X expression in (**A**) SV-HUC-1, (**B**) T24, (**C**) K9TCC-PU-NK, and (**D**) RDSVS-TCC cells after 24 h of treatment with increasing concentrations of Degrayn and PR-619. Actin serves as a loading control.

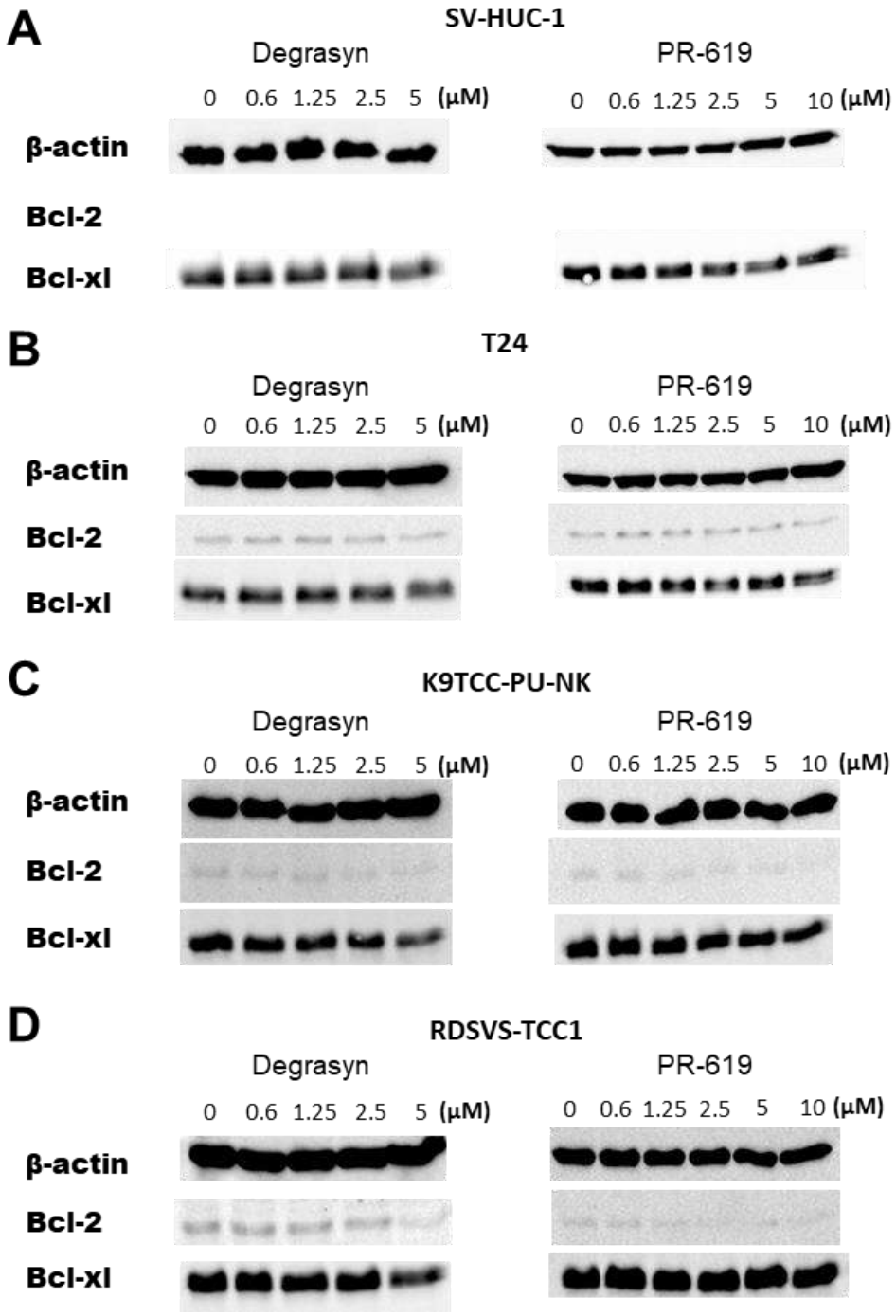

Figure 11. Evaluation of proteins involved in apoptosis induced by Degrasyn and PR-619. Representative blot of Bcl-2 and Bcl-xl expression in (**A**) SV-HUC-1, (**B**) T24, (**C**) K9TCC-PU-NK, and (**D**) RDSVS-TCC cells after 24 h of treatment with increasing concentrations of Degrayn and PR-619. Actin serves as a loading control.

4. Discussion

Given that DUBs and USPs target proteins contain a large number of cell homeostasis regulators as well as products of known oncogenes or tumor suppressor genes, DUBs and USPs inhibition has emerged as an appealing and promising research direction for the development of novel cancer therapies [11]. As Degrasyn (a selective inhibitor of USP5, USP9X, and USP14) has shown an inhibitory effect on some cancer types, our aim was to determine whether Degrasyn could be a potential therapeutic agent in BC. We

demonstrated that Degrasyn is an effective agent against human and canine BC cells. Degrasyn exhibited anti-proliferative, pro-apoptotic, and DNA-damage effects on BC cells that were more pronounced compared to the non-selective DUB inhibitor PR-619.

Initially, we confirmed the expression of USP5, USP9X, and USP14 proteins in selected BC cell lines, which provided the rationale for the utilization of Degrasyn in a BC setting. We observed the overexpression of USP5 protein in a human BC cell line (T24), as compared to normal urothelial cells. Accumulating data suggest that overexpression of USP5 contributes to tumorigenesis in various cancer types through deubiquitinating and stabilizing oncoproteins, such as p53 in melanoma, c-Maf in multiple myeloma, Slug in hepatocellular carcinoma, or histone deacetylase 2 (HDAC2) in ovarian cancer [12]. We evaluated the antiproliferative effect of Degrasyn using the MTT assay. The present study demonstrated that, from all investigated cell lines, the T24 cell line (human BC cells) was most sensitive to the anti-proliferative effect of Degrasyn. This could be potentially related to the observed overexpression of USP5 protein in human BC cells. Some previous studies demonstrated that the anti-cancer activity of Degrasyn could be mainly related to the USP5 inhibitory effect. Li et al. found that Degrasyn suppressed proliferation, reduced colony formation, and inhibited metastasis in pancreatic cancer cells through the USP5-WT1E-cadherin axis [13]. In another paper by Zhang et al., the authors showed that Degrasyn displayed marked activity in downregulating CCND1 protein and suppressing non-small cell lung carcinoma proliferation, which was consistent with the inhibitory effect elicited by the knockdown of USP5 expression [14].

Having analyzed the impact of Degrasyn on cell proliferation, we investigated if Degrasyn may cause any particular type of cell death. We demonstrated that Degrasyn might cause apoptosis in BC cells. Our results were consistent with previous reports on multiple myomas [15]. Additionally, comparing the results of the MTT assay with the analysis of apoptosis level at the same concentration and time of incubation, it can be concluded that at the lower concentrations of the compound, it had a mainly anti-proliferative effect, and with its increase, the reduction of cell viability was increasingly related to apoptosis. Anti-apoptotic effect of PR-619 was less pronounced. Prior research has shown that inhibition of USP can induce cell death via caspase-dependent apoptosis [16]. The activation of effector caspases 3/7, which are proteases responsible for protein cleavage, DNA condensation, and other apoptotic markers, is one of the critical steps during the process [17]. We were able to identify the activation of caspase 3/7 in analyzed cell lines treated with Degrasyn and proved that caspase activation was essential in the apoptosis induced by the agent. In addition, we observed decreased expression of Bcl-2 protein after incubation with the highest concentrations of Degrasyn (5 μM Degrasyn) in all BC cell lines. Also, Bcl-xl expression was slightly reduced after incubation with a 5 μM concentration of Degrasyn. As it has been proven that substrates for Degrasyn (e.g., USP9X) might regulate the Bcl-2 family axis [18], their inhibition may contribute to the anti-cancer effect of Degrasyn.

According to several studies, many USPs are involved in DNA damage repair pathways (DDR) [19,20]. Various ubiquitination pathways have distinct molecular structural characteristics and biological activities, including ubiquitin chains that are critical to the DDR process. Nakajima et al. demonstrated that USP5 is involved in the elimination of the ubiquitin signal from damaged sites and is required for efficient DNA double-strand break (DSB) repair [20]. In another study, Zheng et al. demonstrated that inhibition of USP5 in non-small cell lung cancer can cause DNA damage while modulating the transcriptional activity of p53 [19]. In our study, DNA damage (significant upregulation of the appearance of γH2A.X expression) was found to be an effect of Degrasyn action in analyzed BC cell lines. As the majority of invasive BCs have alterations in the p53 pathway, a p53-dependent mechanism of Degrasyn action might be related to DNA damage in BC cells; however, the detailed mechanism has to be further evaluated.

While there is great interest in DUBs/USPs as a way to provide a high-precision mechanism for the degradation of their substrate proteins, the number of selective compounds is

limited to date [7,21]. One of the most difficult challenges in developing inhibitors selective for a specific DUB or USP is the similarity between DUB family members (especially USPs), which results in compounds with poor selectivity profiles and limits their utility in elucidating DUB or USP function [7,11,21]. A majority of the reported DUBs/USPs inhibitors exhibited weak inhibitory activity (double-digit micromolar range) [7,11,21,22]. Recently, the non-selective multiple DUBs/USPs inhibitor PR-619 was investigated as a potential therapeutic agent in BC. Hsu et al. revealed that PR-619 enhanced cisplatin-induced cytotoxicity and alleviated cisplatin resistance in the cisplatin-resistant T24/R (cisplatin-resistant BC cells) cell line and concurrently suppressed c-Myc expression [9]. Similarly, Kuo et al. demonstrated that PR-619 could inhibit the BC cells' growth alone as well as effectively enhance the cisplatin-induced antitumor effect via concurrent suppression of the Bcl-2 level [8]. In the present study, we observed a significantly higher anti-proliferative effect of Degrasyn than PR-619 at corresponding concentrations. Further, we demonstrated a lower apoptosis induction in BC cells after 24 h of incubation with PR-619 compared to Degrasyn. Additionally, the effect on DNA damage induction was also less pronounced. Thus, our results are in line with existing evidence regarding the potential superiority of selective DUB/USP inhibitors in terms of anti-cancer activity as compared to non-selective inhibitors [7,11,21,22].

In our study, we also analyzed the anti-cancer activity of Degrasyn and PR-619 on two canine BC cell lines (representing in vitro models of invasive BC in dogs). Generally, canine MIBC shares many similarities with human MIBC, including protein and gene homology, pathophysiological mechanisms of cancer initiation and progression, drug targets, drug resistance, and potential prognostic and diagnostic biomarkers [23]. The multidisciplinary collaboration and utilization of canine BC cell lines in our study were justified by providing translational data that could be implemented not only in human oncology but also in veterinary oncology. Although the observed anti-proliferative effect was higher in human BC cells, for the first time, we demonstrated that both Degrasyn and PR-619 could be potential therapeutic agents in canine BC. In our study, differences between human and canine cell lines could be explained by different USP5 expressions and caspase 3/7 activation; however, several other unknown factors may contribute to them and need to be elucidated in further studies.

5. Conclusions

Our results demonstrate that Degrasyn significantly impairs the growth of in vitro models of human and canine BC. Selective USP inhibition with Degrasyn seems to be more effective in reducing BC cell proliferation and inducing apoptosis and DNA damage than non-selective USP inhibition with PR-619.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biomedicines11030759/s1, Figure S1: Comparison of Degrasyn and PR-619 influence on the cell proliferation of SV-HUC-1 (A), T24 (B), K9TCC-PU-NK (C), and RDSVS-TCC1 (D) cell lines (MTT assay) after 24 and 48 h with increasing concentrations of the drugs (data for 1.25, 2.5, 5, 10, and 20 μM). Values are presented as means ± standard deviations (SDs) of three independent experiments. * Considered significant in comparison to control ($p < 0.05$). Figure S2: Ki-67 flow cytometry proliferation assay. Representative histograms showing the percentage of Ki-67 positive cells (in reference to control) after 24 h exposure to increasing concentrations of Degrasyn and PR-619 in (A) SV-HUC-1, (B) T24, (C) K9TCC-PU-NK, and (D) RDSVS-TCC cell lines.

Author Contributions: Conceptualization, Ł.N. and A.P.; Data curation, Ł.N.; Formal analysis, Ł.N. and A.P.; Funding acquisition, Ł.N. and W.K.; Investigation, Ł.N., E.D., W.K. and A.P.; Methodology, Ł.N., E.D. and A.P.; Project administration, Ł.N.; Resources, Ł.N. and A.P.; Software, Ł.N.; Supervision, T.S. and A.P.; Validation, A.P.; Writing—original draft, Ł.N.; Writing—review & editing, W.K., B.M., T.S. and A.P. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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12. CURRICULUM VITAE

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- **08.01.2021 – OBECNIE**

  Młodszy asystent, Klinika Urologii Małoinwazyjnej i Robotycznej, Uniwersyteckie Centrum Urologii, Uniwersytecki Szpital Kliniczny im. Jana Mikulicza-Radeckiego

- **30.09.2019 – 30.10.2020**

  Lekarz stażysta, Dolnośląskie Centrum Onkologii, Pulmonologii i Hematologii we Wrocławiu

WYKSZTAŁCENIE

- **30.09.2019 – OBECNIE**

  Szkoła Doktorska, Uniwersyteckie Centrum Urologii, Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

- **30.09.2019 – OBECNIE**

  Program „ProHum, Interdyscyplinarna Szkoła Doktorska – planowanie badań eksperymentalnych, tworzenie i optymalizacja zwierzęcych modeli doświadczalnych z umiejętnościami transferowania ich do badań klinicznych w medycynie człowieka" – program realizowany wspólnie przez Uniwersytet Przyrodniczy we Wrocławiu i Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

- **30.09.2013 – 24.06.2019**

  Kierunek Lekarski, Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

ZNAJOMOŚĆ JĘZYKÓW OBCYCH

Język ojczysty: POLSKI

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- **Angielski:** C2
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❖ 42 artykuły opublikowane w Polskich i międzynarodowych czasopismach (11 jako pierwszy autor)

❖ Całkowity impact factor (IF): IF = 118,853 (punktacja MEiN = 3 455)

❖ Impact factor (IF) jako pierwszy autor: IF = 20,683 punktów.

❖ Wskaźnik Hirscha (h-index) według: ResearchGate = 8; Web of Science Core Collection = 7

❖ Liczba cytowań według: ResearchGate = 156; Web of Science Core Collection = 134

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- **Nagrody, wyróżnienia**

❖ Kierunek lekarski ukończony z wyróżnieniem rektora

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❖ Członek Polskiego Towarzystwa Urologicznego (PTU)

❖ Członek Europejskiego Towarzystwa Urologicznego (EAU)

13. DOROBEK NAUKOWY (z wyłączeniem prac stanowiących cykl publikacji do Rozprawy Doktorskiej)

13.1. Lista publikacji

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   IF = 6,575; Punkty MEiN = 140

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13.2. Doniesienia zjazdowe

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14. OŚWIADCZENIA WSPÓŁAUTORÓW

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Ja, Wojciech Krajewski, wyrażam zgodę na włączenie poniższych prac naukowych, których jestem współautorem, do serii publikacji, na podstawie których będzie oparta praca doktorska Łukasza Nowaka.

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Ja, Aleksandra Pawlak, wyrażam zgodę na włączenie poniższych prac naukowych, których jestem współautorem, do serii publikacji, na podstawie których będzie oparta praca doktorska Łukasza Nowaka.

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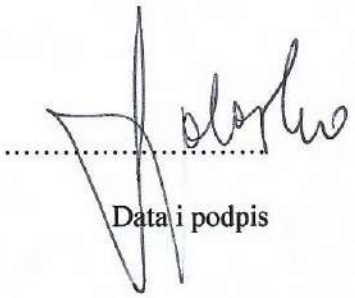
Wrocław, 27.03.2023

DEKLARACJA WSPÓŁAUTORA

Ja, Tomasz Szydełko, wyrażam zgodę na włączenie poniższych prac naukowych, których jestem współautorem, do serii publikacji, na podstawie których będzie oparta praca doktorska Łukasza Nowaka.

Nowak Ł, Krajewski W, Dejnaka E, Małkiewicz B, Szydełko T, Pawlak A. Ubiquitin-Specific Proteases as Potential Therapeutic Targets in Bladder Cancer—In Vitro Evaluation of Degrasyn and PR-619 Activity Using Human and Canine Models. Biomedicines. 2023; 11(3):759.

Nowak Ł, Krajewski W, Małkiewicz B, Szydełko T, Pawlak A. Characteristics and Applications of Canine In Vitro Models of Bladder Cancer in Veterinary Medicine: An Up-to-Date Mini Review. Animals (Basel). 2022;12(4):516.



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Wrocław, 27.03.2023

DEKLARACJA WSPÓŁAUTORA

Ja, Bartosz Małkiewicz, wyrażam zgodę na włączenie poniższych prac naukowych, których jestem współautorem, do serii publikacji, na podstawie których będzie oparta praca doktorska Łukasza Nowaka.

Nowak Ł, Krajewski W, Dejnaka E, Małkiewicz B, Szydełko T, Pawlak A. Ubiquitin-Specific Proteases as Potential Therapeutic Targets in Bladder Cancer—In Vitro Evaluation of Degrasyn and PR-619 Activity Using Human and Canine Models. Biomedicines. 2023; 11(3):759.

Nowak Ł, Krajewski W, Małkiewicz B, Szydełko T, Pawlak A. Characteristics and Applications of Canine In Vitro Models of Bladder Cancer in Veterinary Medicine: An Up-to-Date Mini Review. Animals (Basel). 2022;12(4):516.

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Ja, Romuald Zdrojowy, wyrażam zgodę na włączenie poniższych prac naukowych, których jestem współautorem, do serii publikacji, na podstawie których będzie oparta praca doktorska Łukasza Nowaka.

Krajewski W, Nowak Ł, Moschini M, Poletajew S, Chorbińska J, Necchi A, Montorsi F, Briganti A, Sanchez-Salas R, Shariat SF, Palou J, Babjuk M, Teoh JY, Soria F, Pradere B, Ornaghi PI, Pawlak A, Dembowski J, Zdrojowy R. Impact of Adjuvant Chemotherapy on Survival of Patients with Advanced Residual Disease at Radical Cystectomy following Neoadjuvant Chemotherapy: Systematic Review and Meta-Analysis. J Clin Med. 2021 Feb 8;10(4):651.

Nowak Ł, Krajewski W, Poterek A, Śliwa A, Zdrojowy R. The prognostic value of programmed cell death protein ligand 1 in patients with non-muscle-invasive bladder cancer treated with bacille Calmette-Guérin immunotherapy: Current status. Arab J Urol. 2020;19(1):67-70

Nowak Ł, Krajewski W, Moschini M, Chorbińska J, Poletajew S, Tukiendorf A, Muilwijk T, Joniau S, Tafuri A, Antonelli A, Orlando R, Di Trapani E, Alvarez-Maestro M, Simone G, Zamboni S, Simeone C, Marconi MC, Mastroianni R, Piszczek R, Xylinas E, Zdrojowy R; European Association of Urology - Young Academic Urologists (EAU-YAU): Urothelial Carcinoma Working Group. Assessment of the oncological outcomes of three different bacillus Calmette-Guérin strains in patients with high-grade T1 non-muscle-invasive bladder cancer. Arab J Urol. 2021 Jan 13;19(1):78-85.

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prof. dr hab. Romuald Zdrojowy
27.03.2023

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DEKLARACJA WSPÓŁAUTORA

Ja, Joanna Chorbińska, wyrażam zgodę na włączenie poniższych prac naukowych, których jestem współautorem, do serii publikacji, na podstawie których będzie oparta praca doktorska Łukasza Nowaka.

Krajewski W, Nowak Ł, Moschini M, Poletajew S, Chorbińska J, Necchi A, Montorsi F, Briganti A, Sanchez-Salas R, Shariat SF, Palou J, Babjuk M, Teoh JY, Soria F, Pradere B, Ornaghi PI, Pawlak A, Dembowski J, Zdrojowy R. Impact of Adjuvant Chemotherapy on Survival of Patients with Advanced Residual Disease at Radical Cystectomy following Neoadjuvant Chemotherapy: Systematic Review and Meta-Analysis. J Clin Med. 2021 Feb 8;10(4):651.

Nowak Ł, Krajewski W, Moschini M, Chorbińska J, Poletajew S, Tukiendorf A, Muilwijk T, Joniau S, Tafuri A, Antonelli A, Orlando R, Di Trapani E, Alvarez-Maestro M, Simone G, Zamboni S, Simeone C, Marconi MC, Mastroianni R, Piszczek R, Xylinas E, Zdrojowy R; European Association of Urology - Young Academic Urologists (EAU-YAU): Urothelial Carcinoma Working Group. Assessment of the oncological outcomes of three different bacillus Calmette-Guérin strains in patients with high-grade T1 non-muscle-invasive bladder cancer. Arab J Urol. 2021 Jan 13;19(1):78-85.

30.03.2023 Joanna Chorbińska

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Wrocław, 27.03.2023

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Ja, Ewa Dejnaka, wyrażam zgodę na włączenie poniższych prac naukowych, których jestem współautorem, do serii publikacji, na podstawie których będzie oparta praca doktorska Łukasza Nowaka.

Nowak Ł, Krajewski W, Dejnaka E, Małkiewicz B, Szydełko T, Pawlak A. Ubiquitin-Specific Proteases as Potential Therapeutic Targets in Bladder Cancer—In Vitro Evaluation of Degrasyn and PR-619 Activity Using Human and Canine Models. Biomedicines. 2023; 11(3):759.

27.03.23 Ewa Dejnaka

Data i podpis

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DEKLARACJA WSPÓŁAUTORA

Ja, Janusz Dembowski, wyrażam zgodę na włączenie poniższych prac naukowych, których jestem współautorem, do serii publikacji, na podstawie których będzie oparta praca doktorska Łukasza Nowaka.

Krajewski W, Nowak Ł, Moschini M, Poletajew S, Chorbińska J, Necchi A, Montorsi F, Briganti A, Sanchez-Salas R, Shariat SF, Palou J, Babjuk M, Teoh JY, Soria F, Pradere B, Ornaghi PI, Pawlak A, Dembowski J, Zdrojowy R. Impact of Adjuvant Chemotherapy on Survival of Patients with Advanced Residual Disease at Radical Cystectomy following Neoadjuvant Chemotherapy: Systematic Review and Meta-Analysis. J Clin Med. 2021 Feb 8;10(4):651.

Dr hab.n.med. Janusz Dembowski
prof. UMW
Chirurg Specjalista Urolog
[illegible]

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IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU

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Wrocław, 27.03.2023

DEKLARACJA WSPÓŁAUTORA

Ja, Adrian Poterek, wyrażam zgodę na włączenie poniższych prac naukowych, których jestem współautorem, do serii publikacji, na podstawie których będzie oparta praca doktorska Łukasza Nowaka.

Nowak Ł, Krajewski W, Poterek A, Śliwa A, Zdrojowy R. The prognostic value of programmed cell death protein ligand 1 in patients with non-muscle-invasive bladder cancer treated with bacille Calmette-Guérin immunotherapy: Current status. Arab J Urol. 2020;19(1):67-70

30.03.2023 Robert Śliwa

Data i podpis

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Ja, Sławomir Poletajew, wyrażam zgodę na włączenie poniższych prac naukowych, których jestem współautorem, do serii publikacji, na podstawie których będzie oparta praca doktorska Łukasza Nowaka.

Krajewski W, Nowak Ł, Moschini M, Poletajew S, Chorbińska J, Necchi A, Montorsi F, Briganti A, Sanchez-Salas R, Shariat SF, Palou J, Babjuk M, Teoh JY, Soria F, Pradere B, Ornaghi PI, Pawlak A, Dembowski J, Zdrojowy R. Impact of Adjuvant Chemotherapy on Survival of Patients with Advanced Residual Disease at Radical Cystectomy following Neoadjuvant Chemotherapy: Systematic Review and Meta-Analysis. J Clin Med. 2021 Feb 8;10(4):651.

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3. 04. 2023

Poletajew

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IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU

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Wrocław, 27.03.2023

DEKLARACJA WSPÓŁAUTORA

Ja, Andrzej Tukiendorf, wyrażam zgodę na włączenie poniższych prac naukowych, których jestem współautorem, do serii publikacji, na podstawie których będzie oparta praca doktorska Łukasza Nowaka.

Nowak Ł, Krajewski W, Moschini M, Chorbińska J, Poletajew S, Tukiendorf A, Muilwijk T, Joniau S, Tafuri A, Antonelli A, Orlando R, Di Trapani E, Alvarez-Maestro M, Simone G, Zamboni S, Simeone C, Marconi MC, Mastroianni R, Piszczek R, Xylinas E, Zdrojowy R; European Association of Urology - Young Academic Urologists (EAU-YAU): Urothelial Carcinoma Working Group. Assessment of the oncological outcomes of three different bacillus Calmette-Guérin strains in patients with high-grade T1 non-muscle-invasive bladder cancer. Arab J Urol. 2021 Jan 13;19(1):78-85.

5.04.2023 Tukiendorf.....

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DEKLARACJA WSPÓŁAUTORA

Ja, Anna Śliwa, wyrażam zgodę na włączenie poniższych prac naukowych, których jestem współautorem, do serii publikacji, na podstawie których będzie oparta praca doktorska Łukasza Nowaka.

Nowak Ł, Krajewski W, Poterek A, Śliwa A, Zdrojowy R. The prognostic value of programmed cell death protein ligand 1 in patients with non-muscle-invasive bladder cancer treated with bacille Calmette-Guérin immunotherapy: Current status. Arab J Urol. 2020;19(1):67-70

10.04.2023

Anna Śliwa .....

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Wrocław, 27.03.2023

CO-AUTHOR DECLARATION

I, Marco Moschini, hereby agree that the below mentioned scientific papers, which I have co-authored, may be incorporated into a series of publications on which the doctoral thesis of Łukasz Nowak will be based.

Krajewski W, Nowak Ł, Moschini M, Poletajew S, Chorbińska J, Necchi A, Montorsi F, Briganti A, Sanchez-Salas R, Shariat SF, Palou J, Babjuk M, Teoh JY, Soria F, Pradere B, Ornaghi PI, Pawlak A, Dembowski J, Zdrojowy R. Impact of Adjuvant Chemotherapy on Survival of Patients with Advanced Residual Disease at Radical Cystectomy following Neoadjuvant Chemotherapy: Systematic Review and Meta-Analysis. J Clin Med. 2021 Feb 8;10(4):651.

Nowak Ł, Krajewski W, Moschini M, Chorbińska J, Poletajew S, Tukiendorf A, Muilwijk T, Joniau S, Tafuri A, Antonelli A, Orlando R, Di Trapani E, Alvarez-Maestro M, Simone G, Zamboni S, Simeone C, Marconi MC, Mastroianni R, Piszczek R, Xylinas E, Zdrojowy R; European Association of Urology - Young Academic Urologists (EAU-YAU): Urothelial Carcinoma Working Group. Assessment of the oncological outcomes of three different bacillus Calmette-Guérin strains in patients with high-grade T1 non-muscle-invasive bladder cancer. Arab J Urol. 2021 Jan 13;19(1):78-85.

2023-04-01
Moschini Marco
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Wrocław, 27.03.2023

CO-AUTHOR DECLARATION

I, Marek Babjuk, hereby agree that the below mentioned scientific papers, which I have co-authored, may be incorporated into a series of publications on which the doctoral thesis of Łukasz Nowak will be based.

Krajewski W, Nowak Ł, Moschini M, Poletajew S, Chorbińska J, Necchi A, Montorsi F, Briganti A, Sanchez-Salas R, Shariat SF, Palou J, Babjuk M, Teoh JY, Soria F, Pradere B, Ornaghi PI, Pawlak A, Dembowski J, Zdrojowy R. Impact of Adjuvant Chemotherapy on Survival of Patients with Advanced Residual Disease at Radical Cystectomy following Neoadjuvant Chemotherapy: Systematic Review and Meta-Analysis. J Clin Med. 2021 Feb 8;10(4):651.

6.04.2023
Babjuk Marek

Date and signature

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Wrocław, 27.03.2023

CO-AUTHOR DECLARATION

I, Paola Irene Ornaghi, hereby agree that the below mentioned scientific papers, which I have co-authored, may be incorporated into a series of publications on which the doctoral thesis of Łukasz Nowak will be based.

Krajewski W, Nowak Ł, Moschini M, Poletajew S, Chorbińska J, Necchi A, Montorsi F, Briganti A, Sanchez-Salas R, Shariat SF, Palou J, Babjuk M, Teoh JY, Soria F, Pradere B, Ornaghi PI, Pawlak A, Dembowski J, Zdrojowy R. Impact of Adjuvant Chemotherapy on Survival of Patients with Advanced Residual Disease at Radical Cystectomy following Neoadjuvant Chemotherapy: Systematic Review and Meta-Analysis. J Clin Med. 2021 Feb 8;10(4):651.

11th April 2023
Paola Irene Ornaghi

Date and signature

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Wrocław, 27.03.2023

CO-AUTHOR DECLARATION

I, Benjamin Pradere, hereby agree that the below mentioned scientific papers, which I have co-authored, may be incorporated into a series of publications on which the doctoral thesis of Łukasz Nowak will be based.

Krajewski W, Nowak Ł, Moschini M, Poletajew S, Chorbińska J, Necchi A, Montorsi F, Briganti A, Sanchez-Salas R, Shariat SF, Palou J, Babjuk M, Teoh JY, Soria F, Pradere B, Ornaghi PI, Pawlak A, Dembowski J, Zdrojowy R. Impact of Adjuvant Chemotherapy on Survival of Patients with Advanced Residual Disease at Radical Cystectomy following Neoadjuvant Chemotherapy: Systematic Review and Meta-Analysis. J Clin Med. 2021 Feb 8;10(4):651.

14-04-2023
Pradere.....

Date and signature

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Wrocław, 27.03.2023

CO-AUTHOR DECLARATION

I, Francesco Soria, hereby agree that the below mentioned scientific papers, which I have co-authored, may be incorporated into a series of publications on which the doctoral thesis of Łukasz Nowak will be based.

Krajewski W, Nowak Ł, Moschini M, Poletajew S, Chorbińska J, Necchi A, Montorsi F, Briganti A, Sanchez-Salas R, Shariat SF, Palou J, Babjuk M, Teoh JY, Soria F, Pradere B, Ornaghi PI, Pawlak A, Dembowski J, Zdrojowy R. Impact of Adjuvant Chemotherapy on Survival of Patients with Advanced Residual Disease at Radical Cystectomy following Neoadjuvant Chemotherapy: Systematic Review and Meta-Analysis. J Clin Med. 2021 Feb 8;10(4):651.

2023 - 04 - 10

Francesco Soria

.....

Date and signature

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Wrocław, 27.03.2023

CO-AUTHOR DECLARATION

I, Jeremy Yuen-Chun Teoh, hereby agree that the below mentioned scientific papers, which I have co-authored, may be incorporated into a series of publications on which the doctoral thesis of Łukasz Nowak will be based.

Krajewski W, Nowak Ł, Moschini M, Poletajew S, Chorbińska J, Necchi A, Montorsi F, Briganti A, Sanchez-Salas R, Shariat SF, Palou J, Babjuk M, Teoh JY, Soria F, Pradere B, Ornaghi PI, Pawlak A, Dembowski J, Zdrojowy R. Impact of Adjuvant Chemotherapy on Survival of Patients with Advanced Residual Disease at Radical Cystectomy following Neoadjuvant Chemotherapy: Systematic Review and Meta-Analysis. J Clin Med. 2021 Feb 8;10(4):651.

2023-04-9
Yuen Chun

Date and signature

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Wrocław, 27.03.2023

CO-AUTHOR DECLARATION

I, Juan Palou, hereby agree that the below mentioned scientific papers, which I have co-authored, may be incorporated into a series of publications on which the doctoral thesis of Łukasz Nowak will be based.

Krajewski W, Nowak Ł, Moschini M, Poletajew S, Chorbińska J, Necchi A, Montorsi F, Briganti A, Sanchez-Salas R, Shariat SF, Palou J, Babjuk M, Teoh JY, Soria F, Pradere B, Ornaghi PI, Pawlak A, Dembowski J, Zdrojowy R. Impact of Adjuvant Chemotherapy on Survival of Patients with Advanced Residual Disease at Radical Cystectomy following Neoadjuvant Chemotherapy: Systematic Review and Meta-Analysis. J Clin Med. 2021 Feb 8;10(4):651.

2023-04-05

J. Palou

Date and signature

UNIWERSYTET MEDYCZNY
IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU

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Wrocław, 27.03.2023

CO-AUTHOR DECLARATION

I, Shahrokh François Shariat, hereby agree that the below mentioned scientific papers, which I have co-authored, may be incorporated into a series of publications on which the doctoral thesis of Łukasz Nowak will be based.

Krajewski W, Nowak Ł, Moschini M, Poletajew S, Chorbińska J, Necchi A, Montorsi F, Briganti A, Sanchez-Salas R, Shariat SF, Palou J, Babjuk M, Teoh JY, Soria F, Pradere B, Ornaghi PI, Pawlak A, Dembowski J, Zdrojowy R. Impact of Adjuvant Chemotherapy on Survival of Patients with Advanced Residual Disease at Radical Cystectomy following Neoadjuvant Chemotherapy: Systematic Review and Meta-Analysis. J Clin Med. 2021 Feb 8;10(4):651.

2023 – 04 – 07

Shariat SF

Date and signature

UNIWERSYTET MEDYCZNY
IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU

Wydział Lekarski
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ul. Borowska 213, 50-556 Wrocław
Tel. +48 71 733-10-60

Wrocław, 27.03.2023

CO-AUTHOR DECLARATION

I, Rafael Sanchez-Salas, hereby agree that the below mentioned scientific papers, which I have co-authored, may be incorporated into a series of publications on which the doctoral thesis of Łukasz Nowak will be based.

Krajewski W, Nowak Ł, Moschini M, Poletajew S, Chorbińska J, Necchi A, Montorsi F, Briganti A, Sanchez-Salas R, Shariat SF, Palou J, Babjuk M, Teoh JY, Soria F, Pradere B, Ornaghi PI, Pawlak A, Dembowski J, Zdrojowy R. Impact of Adjuvant Chemotherapy on Survival of Patients with Advanced Residual Disease at Radical Cystectomy following Neoadjuvant Chemotherapy: Systematic Review and Meta-Analysis. J Clin Med. 2021 Feb 8;10(4):651.

2023-04-03
Sanchez-Salas

Date and signature

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Wrocław, 27.03.2023

CO-AUTHOR DECLARATION

I, Alberto Briganti, hereby agree that the below mentioned scientific papers, which I have co-authored, may be incorporated into a series of publications on which the doctoral thesis of Łukasz Nowak will be based.

Krajewski W, Nowak Ł, Moschini M, Poletajew S, Chorbińska J, Necchi A, Montorsi F, Briganti A, Sanchez-Salas R, Shariat SF, Palou J, Babjuk M, Teoh JY, Soria F, Pradere B, Ornaghi PI, Pawlak A, Dembowski J, Zdrojowy R. Impact of Adjuvant Chemotherapy on Survival of Patients with Advanced Residual Disease at Radical Cystectomy following Neoadjuvant Chemotherapy: Systematic Review and Meta-Analysis. J Clin Med. 2021 Feb 8;10(4):651.

2023.04.03
Briganti
.....

Date and signature

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Wrocław, 27.03.2023

CO-AUTHOR DECLARATION

I, Francesco Montorsi, hereby agree that the below mentioned scientific papers, which I have co-authored, may be incorporated into a series of publications on which the doctoral thesis of Łukasz Nowak will be based.

Krajewski W, Nowak Ł, Moschini M, Poletajew S, Chorbińska J, Necchi A, Montorsi F, Briganti A, Sanchez-Salas R, Shariat SF, Palou J, Babjuk M, Teoh JY, Soria F, Pradere B, Ornaghi PI, Pawlak A, Dembowski J, Zdrojowy R. Impact of Adjuvant Chemotherapy on Survival of Patients with Advanced Residual Disease at Radical Cystectomy following Neoadjuvant Chemotherapy: Systematic Review and Meta-Analysis. J Clin Med. 2021 Feb 8;10(4):651.

2023-04-01
Francesco Montorsi
.....

Date and signature

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Wrocław, 27.03.2023

CO-AUTHOR DECLARATION

I, Andrea Necchi, hereby agree that the below mentioned scientific papers, which I have co-authored, may be incorporated into a series of publications on which the doctoral thesis of Łukasz Nowak will be based.

Krajewski W, Nowak Ł, Moschini M, Poletajew S, Chorbińska J, Necchi A, Montorsi F, Briganti A, Sanchez-Salas R, Shariat SF, Palou J, Babjuk M, Teoh JY, Soria F, Pradere B, Ornaghi PI, Pawlak A, Dembowski J, Zdrojowy R. Impact of Adjuvant Chemotherapy on Survival of Patients with Advanced Residual Disease at Radical Cystectomy following Neoadjuvant Chemotherapy: Systematic Review and Meta-Analysis. J Clin Med. 2021 Feb 8;10(4):651.

2023-04-03
Necchi Andrea
.....

Date and signature

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Wrocław, 27.03.2023

CO-AUTHOR DECLARATION

I, Alessandro Antonelli, hereby agree that the below mentioned scientific papers, which I have co-authored, may be incorporated into a series of publications on which the doctoral thesis of Łukasz Nowak will be based.

Nowak Ł, Krajewski W, Moschini M, Chorbińska J, Poletajew S, Tukiendorf A, Muilwijk T, Joniau S, Tafuri A, Antonelli A, Orlando R, Di Trapani E, Alvarez-Maestro M, Simone G, Zamboni S, Simeone C, Marconi MC, Mastroianni R, Piszczek R, Xylinas E, Zdrojowy R; European Association of Urology - Young Academic Urologists (EAU-YAU): Urothelial Carcinoma Working Group. Assessment of the oncological outcomes of three different bacillus Calmette-Guérin strains in patients with high-grade T1 non-muscle-invasive bladder cancer. Arab J Urol. 2021 Jan 13;19(1):78-85.

14 - 04 - 2023

A. Antonelli

Date and signature

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Wrocław, 27.03.2023

CO-AUTHOR DECLARATION

I, Mario Álvarez-Maestro, hereby agree that the below mentioned scientific papers, which I have co-authored, may be incorporated into a series of publications on which the doctoral thesis of Łukasz Nowak will be based.

Nowak Ł, Krajewski W, Moschini M, Chorbińska J, Poletajew S, Tukiendorf A, Muilwijk T, Joniau S, Tafuri A, Antonelli A, Orlando R, Di Trapani E, Alvarez-Maestro M, Simone G, Zamboni S, Simeone C, Marconi MC, Mastroianni R, Piszczek R, Xylinas E, Zdrojowy R; European Association of Urology - Young Academic Urologists (EAU-YAU): Urothelial Carcinoma Working Group. Assessment of the oncological outcomes of three different bacillus Calmette-Guérin strains in patients with high-grade T1 non-muscle-invasive bladder cancer. Arab J Urol. 2021 Jan 13;19(1):78-85.

6.04.2023
Mario Álvarez-Maestro

Date and signature

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Wrocław, 27.03.2023

CO-AUTHOR DECLARATION

I, Ettore Di Trapani, hereby agree that the below mentioned scientific papers, which I have co-authored, may be incorporated into a series of publications on which the doctoral thesis of Łukasz Nowak will be based.

Nowak Ł, Krajewski W, Moschini M, Chorbińska J, Poletajew S, Tukiendorf A, Muilwijk T, Joniau S, Tafuri A, Antonelli A, Orlando R, Di Trapani E, Alvarez-Maestro M, Simone G, Zamboni S, Simeone C, Marconi MC, Mastroianni R, Piszczek R, Xylinas E, Zdrojowy R; European Association of Urology - Young Academic Urologists (EAU-YAU): Urothelial Carcinoma Working Group. Assessment of the oncological outcomes of three different bacillus Calmette-Guérin strains in patients with high-grade T1 non-muscle-invasive bladder cancer. Arab J Urol. 2021 Jan 13;19(1):78-85.

6.04. 2023
Di Trapani
.....

Date and signature

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Wrocław, 27.03.2023

CO-AUTHOR DECLARATION

I, Guiseppe Simone, hereby agree that the below mentioned scientific papers, which I have co-authored, may be incorporated into a series of publications on which the doctoral thesis of Łukasz Nowak will be based.

Nowak Ł, Krajewski W, Moschini M, Chorbińska J, Poletajew S, Tukiendorf A, Muilwijk T, Joniau S, Tafuri A, Antonelli A, Orlando R, Di Trapani E, Alvarez-Maestro M, Simone G, Zamboni S, Simeone C, Marconi MC, Mastroianni R, Piszczek R, Xylinas E, Zdrojowy R; European Association of Urology - Young Academic Urologists (EAU-YAU): Urothelial Carcinoma Working Group. Assessment of the oncological outcomes of three different bacillus Calmette-Guérin strains in patients with high-grade T1 non-muscle-invasive bladder cancer. Arab J Urol. 2021 Jan 13;19(1):78-85.

5.04.2023
Guiseppe Simone
.....

Date and signature

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Wrocław, 27.03.2023

CO-AUTHOR DECLARATION

I, Rossella Orlando, hereby agree that the below mentioned scientific papers, which I have co-authored, may be incorporated into a series of publications on which the doctoral thesis of Łukasz Nowak will be based.

Nowak Ł, Krajewski W, Moschini M, Chorbińska J, Poletajew S, Tukiendorf A, Muilwijk T, Joniau S, Tafuri A, Antonelli A, Orlando R, Di Trapani E, Alvarez-Maestro M, Simone G, Zamboni S, Simeone C, Marconi MC, Mastroianni R, Piszczek R, Xylinas E, Zdrojowy R; European Association of Urology - Young Academic Urologists (EAU-YAU): Urothelial Carcinoma Working Group. Assessment of the oncological outcomes of three different bacillus Calmette-Guérin strains in patients with high-grade T1 non-muscle-invasive bladder cancer. Arab J Urol. 2021 Jan 13;19(1):78-85.

14-04-2023
R. Orlando

Date and signature

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Wrocław, 27.03.2023

CO-AUTHOR DECLARATION

I, Claudio Simeone, hereby agree that the below mentioned scientific papers, which I have co-authored, may be incorporated into a series of publications on which the doctoral thesis of Łukasz Nowak will be based.

Nowak Ł, Krajewski W, Moschini M, Chorbińska J, Poletajew S, Tukiendorf A, Muilwijk T, Joniau S, Tafuri A, Antonelli A, Orlando R, Di Trapani E, Alvarez-Maestro M, Simone G, Zamboni S, Simeone C, Marconi MC, Mastroianni R, Piszczek R, Xylinas E, Zdrojowy R; European Association of Urology - Young Academic Urologists (EAU-YAU): Urothelial Carcinoma Working Group. Assessment of the oncological outcomes of three different bacillus Calmette-Guérin strains in patients with high-grade T1 non-muscle-invasive bladder cancer. Arab J Urol. 2021 Jan 13;19(1):78-85.

4.04.2023
Claudio Simeone
.....

Date and signature

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Wrocław, 27.03.2023

DEKLARACJA WSPÓŁAUTORA

Ja, Radosław Piszczek, wyrażam zgodę na włączenie poniższych prac naukowych, których jestem współautorem, do serii publikacji, na podstawie których będzie oparta praca doktorska Łukasza Nowaka.

Nowak Ł, Krajewski W, Moschini M, Chorbińska J, Poletajew S, Tukiendorf A, Muilwijk T, Joniau S, Tafuri A, Antonelli A, Orlando R, Di Trapani E, Alvarez-Maestro M, Simone G, Zamboni S, Simeone C, Marconi MC, Mastroianni R, Piszczek R, Xylinas E, Zdrojowy R; European Association of Urology - Young Academic Urologists (EAU-YAU): Urothelial Carcinoma Working Group. Assessment of the oncological outcomes of three different bacillus Calmette-Guérin strains in patients with high-grade T1 non-muscle-invasive bladder cancer. Arab J Urol. 2021 Jan 13;19(1):78-85.

3.04.2023
Piszczek

Data i podpis

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Wrocław, 27.03.2023

CO-AUTHOR DECLARATION

I, Stefania Zamboni, hereby agree that the below mentioned scientific papers, which I have co-authored, may be incorporated into a series of publications on which the doctoral thesis of Łukasz Nowak will be based.

Nowak Ł, Krajewski W, Moschini M, Chorbińska J, Poletajew S, Tukiendorf A, Muilwijk T, Joniau S, Tafuri A, Antonelli A, Orlando R, Di Trapani E, Alvarez-Maestro M, Simone G, Zamboni S, Simeone C, Marconi MC, Mastroianni R, Piszczek R, Xylinas E, Zdrojowy R; European Association of Urology - Young Academic Urologists (EAU-YAU): Urothelial Carcinoma Working Group. Assessment of the oncological outcomes of three different bacillus Calmette-Guérin strains in patients with high-grade T1 non-muscle-invasive bladder cancer. Arab J Urol. 2021 Jan 13;19(1):78-85.

31.03.2023

Zamboni
.....

Date and signature

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Wrocław, 27.03.2023

CO-AUTHOR DECLARATION

I, Maria Cristina Marconi, hereby agree that the below mentioned scientific papers, which I have co-authored, may be incorporated into a series of publications on which the doctoral thesis of Łukasz Nowak will be based.

Nowak Ł, Krajewski W, Moschini M, Chorbińska J, Poletajew S, Tukiendorf A, Muilwijk T, Joniau S, Tafuri A, Antonelli A, Orlando R, Di Trapani E, Alvarez-Maestro M, Simone G, Zamboni S, Simeone C, Marconi MC, Mastroianni R, Piszczek R, Xylinas E, Zdrojowy R; European Association of Urology - Young Academic Urologists (EAU-YAU): Urothelial Carcinoma Working Group. Assessment of the oncological outcomes of three different bacillus Calmette-Guérin strains in patients with high-grade T1 non-muscle-invasive bladder cancer. Arab J Urol. 2021 Jan 13;19(1):78-85.

1.04.2023 Maria Cristina Marconi

Date and signature

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Wrocław, 27.03.2023

CO-AUTHOR DECLARATION

I, Riccardo Mastroianni, hereby agree that the below mentioned scientific papers, which I have co-authored, may be incorporated into a series of publications on which the doctoral thesis of Łukasz Nowak will be based.

Nowak Ł, Krajewski W, Moschini M, Chorbińska J, Poletajew S, Tukiendorf A, Muilwijk T, Joniau S, Tafuri A, Antonelli A, Orlando R, Di Trapani E, Alvarez-Maestro M, Simone G, Zamboni S, Simeone C, Marconi MC, Mastroianni R, Piszczek R, Xylinas E, Zdrojowy R; European Association of Urology - Young Academic Urologists (EAU-YAU): Urothelial Carcinoma Working Group. Assessment of the oncological outcomes of three different bacillus Calmette-Guérin strains in patients with high-grade T1 non-muscle-invasive bladder cancer. Arab J Urol. 2021 Jan 13;19(1):78-85.

4.04.2023

Riccardo Mastroianni.....

Date and signature

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Wrocław, 27.03.2023

CO-AUTHOR DECLARATION

I, Evanguelos Xylinas, hereby agree that the below mentioned scientific papers, which I have co-authored, may be incorporated into a series of publications on which the doctoral thesis of Łukasz Nowak will be based.

Nowak Ł, Krajewski W, Moschini M, Chorbińska J, Poletajew S, Tukiendorf A, Muilwijk T, Joniau S, Tafuri A, Antonelli A, Orlando R, Di Trapani E, Alvarez-Maestro M, Simone G, Zamboni S, Simeone C, Marconi MC, Mastroianni R, Piszczek R, Xylinas E, Zdrojowy R; European Association of Urology - Young Academic Urologists (EAU-YAU): Urothelial Carcinoma Working Group. Assessment of the oncological outcomes of three different bacillus Calmette-Guérin strains in patients with high-grade T1 non-muscle-invasive bladder cancer. Arab J Urol. 2021 Jan 13;19(1):78-85.

31.03.2023 Xylinas
.....
Date and signature

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Tel. +48 71 733-10-60

Wrocław, 27.03.2023

CO-AUTHOR DECLARATION

I, Alessandro Tafuri, hereby agree that the below mentioned scientific papers, which I have co-authored, may be incorporated into a series of publications on which the doctoral thesis of Łukasz Nowak will be based.

Nowak Ł, Krajewski W, Moschini M, Chorbińska J, Poletajew S, Tukiendorf A, Muilwijk T, Joniau S, Tafuri A, Antonelli A, Orlando R, Di Trapani E, Alvarez-Maestro M, Simone G, Zamboni S, Simeone C, Marconi MC, Mastroianni R, Piszczek R, Xylinas E, Zdrojowy R; European Association of Urology - Young Academic Urologists (EAU-YAU): Urothelial Carcinoma Working Group. Assessment of the oncological outcomes of three different bacillus Calmette-Guérin strains in patients with high-grade T1 non-muscle-invasive bladder cancer. Arab J Urol. 2021 Jan 13;19(1):78-85.

7-04-2023

Date and signature

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Tel. +48 71 733-10-60

Wrocław, 27.03.2023

CO-AUTHOR DECLARATION

I, Steven Joniau, hereby agree that the below mentioned scientific papers, which I have co-authored, may be incorporated into a series of publications on which the doctoral thesis of Łukasz Nowak will be based.

Nowak Ł, Krajewski W, Moschini M, Chorbińska J, Poletajew S, Tukiendorf A, Muilwijk T, Joniau S, Tafuri A, Antonelli A, Orlando R, Di Trapani E, Alvarez-Maestro M, Simone G, Zamboni S, Simeone C, Marconi MC, Mastroianni R, Piszczek R, Xylinas E, Zdrojowy R; European Association of Urology - Young Academic Urologists (EAU-YAU): Urothelial Carcinoma Working Group. Assessment of the oncological outcomes of three different bacillus Calmette-Guérin strains in patients with high-grade T1 non-muscle-invasive bladder cancer. Arab J Urol. 2021 Jan 13;19(1):78-85.

8-04-2023
S. Joniau
.....
Date and signature

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Wrocław, 27.03.2023

CO-AUTHOR DECLARATION

I, Tim Muilwijk, hereby agree that the below mentioned scientific papers, which I have co-authored, may be incorporated into a series of publications on which the doctoral thesis of Łukasz Nowak will be based.

Nowak Ł, Krajewski W, Moschini M, Chorbińska J, Poletajew S, Tukiendorf A, Muilwijk T, Joniau S, Tafuri A, Antonelli A, Orlando R, Di Trapani E, Alvarez-Maestro M, Simone G, Zamboni S, Simeone C, Marconi MC, Mastroianni R, Piszczek R, Xylinas E, Zdrojowy R; European Association of Urology - Young Academic Urologists (EAU-YAU): Urothelial Carcinoma Working Group. Assessment of the oncological outcomes of three different bacillus Calmette-Guérin strains in patients with high-grade T1 non-muscle-invasive bladder cancer. Arab J Urol. 2021 Jan 13;19(1):78-85.

10-04-2023
Muilwijk
Date and signature