WROCŁAW UNIVERSITY
OF ENVIRONMENTAL
AND LIFE SCIENCES

FACULTY OF VETERINARY MEDICINE
DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY

Beatriz Hernández Suárez MSc

The role of tumor associated proteins in DNA damage and Unfolded Protein Responses and their use as target for the development of novel canine cancer therapy

Supervisor

Aleksandra Pawlak DVM PhD, associate professor

Second supervisor

David A. Gillespie PhD, FRSE

Wroclaw 2023

This research has been funded by the project „UPWr 2.0: International and interdisciplinary development programme for Wrocław University of Environmental and Life Sciences", co-financed by the European Social Fund under the Operational Program Knowledge Education Development, under contract No. POWR.03.05.00-00-Z062 / 18 of June 4, 2019.

Acknowledgements

Doing a PhD is a very demanding and stressful experience, at in some cases it could seems like you are doing it all alone. But the truth is that it is a cooperative journey where all people you know, before and during this period, they play a key role to help you to finally assemble the dissertation. Being said this, I would like to thank all these people who help me along the way to become *Dr. Hernández-Suárez*.

My first big thank is for my supervisor Prof. Aleksandra Pawlak, who has been the pilar from the beginning to the end. I want you to thank you for believing in me and invite me to come to your laboratory as your PhD student. Thank you for encouraging me to apply for funding, to apply for scholarships and for pushing me to write articles, I thought that I was not able to do it, but here is the evidence that proved that I could make it. Thank you for your optimism and support even in the harder times even when I did not believe in myself.

My second big thank is for my second supervisor Dr. David Gillespie, who has believed in my abilities since he knew me during my Master. Thank you, David, for your guidance and help since the beginning, for your support during all these years. You helped me and trained me during my master's project, you gave my first job, and now you co-supervised this dissertation. All my scientific career is thanks to you, and I will always be grateful for that.

My PhD adventure has started at the department of Pharmacology and Toxicology, and I could not be prouder of have been part of it for these four years. A special thanks to Prof Obmińska-Mrukowicz, you have shown to be a perfect leader, the department will not be the same without you as the head of the department. Also, I want to thank my colleagues, Blazej, Marta and Angelika, for the good times and nice conversations with coffee. Thank you, Julia, for your curiosity and inspiring questions. Thank you, Karolina, for help me practicing my polish while doing western blots. Thank you Pawel for the coffees. Thank you Agnieska, Asia and Ewa, for your help during this last year. Thank you Kasia, Abubakar, Marta T, Karolina K, Łukasz, Desiye, and Jason for making the PhD room alive and friendly. Thank you to all members of the department, for your kindness during those four years. And thank you to all colleagues from other departments, especially to Asia from Immunology department, for all her help with the western blots. Bardzo dziękuję kolegom i przepraszam, że nie mówię po polsku.

I want to thank the UPWr for the opportunity to be part of the POWER programme. I want to thank the International Relations Office - Krzysztof Kafarski, Maria Błaszczuk and Sylwia Popiel, for their help during the process, especially during the first year of the programme. Also, I would like to thank Kamila Dumańska, for her help with all the EOD system and financial issues. To Daria from the Doctoral School, for your help with all documents. I want to say thank you to all the professor that have been part of the POWER programme, we have had very enriching and interesting classes.

I want to thank Prof Anna Matczuk for the inspiring and helpful tutoring classes.

Thank you Karolina Bykowska, our polish teacher, not only for the polish lesson but for help us to discover Poland. Dziękuję bardzo, lubię podróżować z tobą po Polsce.

More especially, I want to thank my classmates from POWER, especially to Joncer, Chantura, Meriem, Kasia and Ali, for being not only colleagues but my friends. Thank you to Thiru and Waheed, for your friendship and support.

During my PhD, I was lucky to have the opportunity to be abroad twice, I want to thank again the UPWr for giving me the chance to do it and enriching this way my scientific carrer. Also, I want to thank all the people who I have meet during such adventures.

Quiero dar las gracias al equipo de Dr. Andrés Aguilera en el CABIMER (Sevilla), por acogerme con las manos abiertas y hacerme sentir parte del equipo desde el primer momento, incluso siendo mi vista de sólo tres meses. Quiero agradecer en primer lugar al Dr. Andrés Aguilera por darme la oportunidad de incorporarme a su equipo. También, quiero dar las gracias a Sonia Barroso, por guiarme y enseñarme la técnica del DNA combing así cómo introducirme en el club *curly*. Quiero agradecer a todos los doctorandos y miembros del equipo por su ayuda y por el buen ambiente, en especial a Mariangeles, José Antonio, y Pablo por los desayunos tempraneros, a Cristina G por sus castigos de chocolate, a Cristina A y Mar por nuestras charlas sobre nuestros proyectos, a Teresa y a Patricia por dejarme contarles mis batallitas en Polonia, a Iván, Javi, Laura por llevarme a la playa, y a Sonia P y Eugenia por dejarme participar en los videos de despedidas. Gracias por esos tres meses.

Je veux vraiment remercier à l'équipe 5 dans CRCT, j'ai passé le meilleur temps dans mon étage avec vous. Merci beaucoup Stefania et Stéphane pour m'avoir permis d'aller avec vous pendant an année. Mercie Anne pour être mon guide dans le labo. Merci Estelle pour les commentaires intéressant dans les lab meetings. Et merci Katy pour les glaces ! Aux copains merci pour m'avoir accepté dans le « jungle ». Merci Pauline pour être très gentille. Merci Lulu pour être ma copine d'équipe poubelle. Merci Alice pour les soirées dans les guinguettes avec tes amis. Merci Sanae pour tes intéressantes questions et te sympathie. Merci Quentin pour appliquer les "BA tips". Merci Leïla pour être l'énergie de l'équipe, on peut dire la mitochondrie du labo. Merci Seb pour être le chat de l'équipe, toute bonne équipe a besoin d'une mascotte. Merci Axel pour les chansons et chorégraphies, le radio du labo je la manque beaucoup. Merci beaucoup à tous pour toute la science que j'ai appris avec vous, et pour tout le bonne humeur et l'ambiance de très belles équipe, vous êtes magnifiques.

And last but not least, I want to thank all people in my personal life that have also impact in my success with my PhD.

First, to the people I have met during my time in Wroclaw. To Miquel, for discover me the "10 minutes a day". To Justyna, for the Polish-Spanish exchange. To Parnita, for our interesting conversations and multiple plans. Thank you to Marianna, the best Pilates instructor I have ever met, thank you for doing bilingual classes only for me, I will never forget: *noga, sufitu, kręgi,* very useful polish words. Also, I want to thank Paul and the hiking group for the fresh air and the good moments in the mountains, especially to Elvira and Gustavo, los argentinos más maravillosos. And to Ekostraż, to the volunteers and all the animals.

Quiero agradecer a mis profesores de la Universidad de La Laguna tanto durante la licenciatura cómo durante el máster, ya que sin ellos no habría llegado hasta aquí. En especial al Profesor Mariano Hernández Ferrer, por haberme apoyado durante estos años, y al grupo del Dr. Raimundo Freire por acogerme para realizar las prácticas del máster. También quiero dar las gracias a mis amigos y compañeros carrera: Miguel, Guille, Eva, Lucas, Walter, Romina, Manu, Alexander, Javi, Marlene, Ángel, Yordana, Sandra, Vero, Nayra, Mónica, y al resto de mi promoción. Gracias por acompañarme durante la carrera y por seguir haciéndolo.

A mi profesora de francés Laura, por sus divertidas clases y por unir al grupo de amigos de francés: Patri, Ita y Alberto. Merci beaucoup à les quatre!

A mis amigas del instituto, Sandra, Mónica, Miriam, Yami, y Cris, por las reuniones navideñas y el club de lectura.

A Alba P por tu sencillez y felicidad contagiosa.

A mis amigas de toda la vida: Elena, Gara, y Patri, por seguir a pesar de la distancia y el tiempo.

A mis amigos que son familia, Carlota, Miguel, Laura, Alba, David, Guille, Yeray y Carol, por el apoyo, los reencuentros, los días de juegos y las videollamadas, gracias.

A Pedro, por descubrirme tantas cosas nuevas, por acompañarme en viajes inesperados y por tu apoyo incondicional.

Y finalmente, gracias a mi familia, a mis padres, a mis hermanas Laura y Sara, a mi tío Andre, y a mi gata Katia, por estar siempre ahí, por apoyarme incluso en mis locuras, por cuidarme y por ser la mejor familia que uno podría desear, gracias.

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List of publications included in the doctoral dissertation

- **Publication 1 (P1):**

Hernández-Suárez B*, Gillespie DA, Pawlak A. DNA damage response proteins in canine cancer as potential research targets in comparative oncology. Vet Comp Oncol. 2022 Jun;20(2):347-361. doi: 10.1111/vco.12795. Epub 2022 Jan 10. PMID: 34923737; PMCID: PMC9304296.

IF: 2.385, MEiN: 200

- **Publication 2 (P2):**

Hernández-Suárez B*, Gillespie D.A, Dejnaka E, Kupczyk P, Obmińska-Mrukowicz B, Pawlak A. Studying the DNA Damage Response pathway in hematopoietic canine cancer cell lines – a necessary step for finding targets to generate new therapies to treat cancer in dogs. Front. Vet. Sci. 2023 Aug; vol. 10 doi: 10.3389/fvets.2023.1227683

IF: 3.471, MEiN: 70

- **Publication 3 (P3):**

Hernández-Suárez B*, Gillespie D.A, Obmińska-Mrukowicz B, Pawlak A. An initial characterization of the Unfolded Protein Response pathway in hematopoietic canine cancer cell lines -a necessary step for the introduction of new therapies in dogs with cancer. Journal of Veterinary Research 2023. doi:10.2478/jvetres-2023-0042

IF: 2.058, MEiN: 140

Abstract

Cancer is a genetic disease that affects both humans and dogs. To minimize the risk of cancer almost all body cells have different mechanisms and pathways to protect themselves from neoplastic transformation. Two particularly important mechanisms are referred to as the DNA damage response (DDR) and Unfolded Protein Response (UPR) pathways respectively. Unfortunately, little is known about those two pathways in dogs, despite their potential importance and expected similarity to humans. Therefore, the aim of the research presented in this thesis was to determine methods and validate reagents that can be used to study the DDR and UPR pathways in canine cells. To achieve this goal, RNA sequencing analysis was performed in CLBL-1 and GL-1 cell lines to prove the presence of transcripts for major players in both pathways in canine cells. Further, a panel of lymphoma and leukemia cell lines (CLBL-1, CLB70, CNK-89, and GL-1) was analyzed by western blotting using a selection of antibodies to study protein expression levels and qPCR to study the mRNA level of ATR and Claspin proteins. To determine replication dynamics DNA combing assay was used, and finally, flow cytometry analysis was performed to study apoptosis.

So far, in studies using canine cells, only proteins such as BRCA1, BRCA2, p53, TopBP1, or Rad51 from the DNA damage response pathway have been investigated, while there is no information on the ATR-Chk1 pathway in dogs. In our research on the DDR pathway, we found a high degree of homology in key DDR components between dogs and humans. The expression levels of the DDR proteins varied between the cancer cell lines we studies, being generally higher in CLBL-1 and CLB70 than in GL-1. After activation of the DDR pathway through the use of a DNA-damaging drug – etoposide - increased activatory phosphorylation of Chk1 was observed in the tested cancer cell lines together with a marked accumulation of Rad51 protein. A high increase of Rad51 was observed after only 2 hours of treatment with etoposide, it was the reason to analyze replication dynamics in the canine cancer cell lines by pulse-labelling with thymidine analogues followed by a DNA combing assay. The results of this analysis showed that DNA replication forks synthesize DNA more rapidly in GL-1 cells than CLBL-1, but that both cell lines have a tendency to asymmetric fork progression.

Similar to the DDR pathway, we found a high degree of homology between key UPR components in humans and dogs. The expression of UPR markers p-eIF2α and CHOP increased after treating the canine cancer cells with the endoplasmic reticulum stress inducers thapsigargin and MG132. Because UPR activation can lead to cell apoptosis, the level of apoptosis in thapsigargin and MG132-treated canine cancer cell cultures was examined using flow cytometry. Despite a clear induction of endoplasmic reticulum stress evidenced by an increase in the level of CHOP expression, no significant increase in the low basal rate of spontaneous apoptosis was found under the conditions tested.

The presented results for the first time initially characterize the DDR and UPR pathways in canine cells as well as provide information about the tools and techniques that can be used to study these pathways in dogs. We observed that the regulation of these pathways in dogs is similar to that which has been described in humans and other species. This, together with our validation in dogs of reagents originally designed for use in human or mouse cells, will facilitate research on the role of DNA damage and endoplasmic reticulum stress in carcinogenesis in dogs. The availability of appropriately verified research tools will also enable the development of new targeted therapies in veterinary oncology.

Streszczenie

Nowotwór jest chorobą genetyczną, która dotyka zarówno ludzi, jak i psy. Aby zminimalizować ryzyko rozwinięcia się nowotworu, prawie wszystkie komórki organizmu wyposażone są w wyspecjalizowane mechanizmy molekularne mające zapewnić ochronę przed rozwojem nowotworu. Jednymi z takich mechanizmów są szlak reakcji w odpowiedzi na uszkodzenia DNA (ang. *DNA damage response, DDR*) i szlak odpowiedzi na niesfałdowane białka (ang. *Unfolded protein response, UPR*). Niestety, niewiele wiadomo o tych dwóch szlakach u psów, poza ich potencjalną rolą i oczekiwanym podobieństwem do mechanizmów obserwowanych u ludzi. Dlatego, celem prezentowanej pracy było opracowanie metod badawczych i walidacja odczynników, które można zastosować w badaniach szlaków DDR i UPR w komórkach psa. Aby osiągnąć ten cel, najpierw wykonano sekwencjonowanie RNA z użyciem psich linii komórkowych CLBL-1 i GL-1, aby udowodnić obecność transkryptów dla głównych białek biorących udział w obu badanych szlakach sygnałowych. Następnie, panel linii komórkowych chłoniaka i białaczki psa (CLBL-1, CLB70, CNK-89 i GL-1) analizowano metodą Western blotting przy użyciu wybranych przeciwciał w celu zbadania poziomów ekspresji białek zaangażowanych w badane szlaki oraz zastosowano technikę qPCR w celu określenia poziomu mRNA dla białek ATR i klaspiny. Do oceny dynamiki replikacji wykorzystano test DNA combing assay, a na koniec przeprowadzono analizę metodą cytometrii przepływowej w celu zbadania poziomu apoptozy.

Do tej pory w badaniach z wykorzystaniem psich komórek, ze szlaku odpowiedzi na uszkodzenie DNA, badano jedynie takie białka jak: BRCA1, BRCA2, p53, TopBP1 czy Rad51, podczas gdy brak jest danych literaturowych na temat szlaku ATR-Chk1 u psów. W prezentowanych badaniach w odniesieniu do szlaku DDR stwierdzono wysoki odsetek homologii pomiędzy poszczególnymi elementami tego szlaku u człowieka i psa. Poziomy ekspresji białek szlaku DDR różniły się między badanymi liniami komórkowymi i były wyższe w liniach komórkowych CLBL-1 i CLB70 niż w linii GL-1. Po aktywacji szlaku DDR poprzez zastosowanie leku uszkadzającego DNA – etopozydu przez 2 godziny, w testowanych psich liniach komórkowych zaobserwowano wzrost poziomu fosforylacji kinazy Chk1 i ekspresji białka Rad51. Tak duży wzrost w tak krótkim czasie był powodem do podjęcia analizy dynamiki replikacji metodą DNA combing assay. Badanie wykazało, że linia GL-1 replikuje szybciej niż CLBL-1, ale obie linie komórkowe mają tendencje do asymetrii.

Podobnie jak w przypadku szlaku DDR, stwierdzono wysoki procent homologii pomiędzy komponentami szlaku UPR między ludźmi i psami. Poziom ekspresji jednych z ważniejszych białek szlaku UPR: p-eIF2α i CHOP wzrósł zgodnie z oczekiwaniami po potraktowaniu komórek induktorami stresu retikulum endoplazmatycznego - tapsygarginą i MG132. Ponieważ aktywacja UPR może prowadzić do apoptozy, poziom apoptozy komórek traktowanych tapsygarginą i MG132 zbadano za pomocą cytometrii przepływowej. Pomimo wyraźnej indukcji stresu retikulum endoplazmatycznego obserwowanego jako wzrost poziomu ekspresji białka CHOP w badanych komórkach, w badaniu nie stwierdzono istotnego stopnia indukcji procesu apoptozy.

Przedstawione wyniki po raz pierwszy wstępnie charakteryzują szlaki DDR i UPR w komórkach psów oraz dostarczają informacji o narzędziach, które można wykorzystać w badaniu tych szlaków u psów. Podobieństwo opisanych szlaków u psów i ludzi oraz możliwość zastosowania odczynników dedykowanych do pracy z komórkami ludzkimi rzuca nowe światło na możliwość wykorzystania psa jako modelu do badań mechanizmów związanych z uszkodzeniem DNA i stresem retikulum endoplazmatycznego. Dostępność odpowiednio zweryfikowanych narzędzi badawczych umożliwi w przyszłości opracowanie nowych terapii celowanych w onkologii weterynaryjnej.

Abbreviations List

53BP1 - TP53-binding protein 1

ACTB – β-actin gene

AKT - AKT serine/threonine kinase, also known as protein kinase B

ATF4 - Activating transcription factor 4

ATF6 - Activating transcription factor 6

ATR - ataxia telangiectasia mutated and Rad3-related

BACH1 - BRCA1-associated C-terminal helicase

BAK - Bcl-2 homologous antagonist/killer

BAX - Apoptosis regulator BAX, also known as bcl-2-like protein 4

BCL-2 - B-cell lymphoma-2

BIM - Bcl-2 Interacting Mediator of cell death

BRCA1– Breast cancer gene 1

BRCA2 - breast cancer gene 2

BRCT – BRCA C-terminal domains

CDKs – Cyclin-dependent kinases

CldU - 5-chloro-2'-deoxyuridine

Chk1 - checkpoint kinase 1

CHOP - C/EBP homologous protein, also known as growth arrest- and DNA damage-inducible gene 153 (GADD153)

CMT – Canine mammary tumor

DDR – DNA damage response

eIF2α - eukaryotic translation initiation factor 2A

ER – Endoplasmic reticulum

ERO1α - Endoplasmic Reticulum Oxidoreductase 1 Alpha

GADD34 - growth arrest and DNA damage-inducible protein GADD34

GRP78 - glucose-regulated protein 78, also known as BiP

HKGs – Housekeeping genes

HR - homologous recombination

IdU - 5-iodo-2'-deoxyuridine

IRE1α - inositol-requiring enzyme 1 alpha

P21 - Cyclin-dependent kinase inhibitor 1

P27 - Cyclin-dependent kinase inhibitor 1B

P53 - Cellular tumor antigen p53

PALB2 - partner and localizer of breast cancer 2 (BRCA2)

PARP - Poly (ADP-ribose) polymerase

PCNA - Proliferating cell nuclear antigen

PERK - protein kinase RNA-like ER kinase

PPIA – peptidyl-prolyl cis-trans isomerase a gene

PTEN - phosphatase and tensin homolog deleted on chromosome 10

Rad51 – Rad51 helicase

RAD9 - Rad9 Checkpoint clamp component of 9-1-1 clamp: Rad9-Hus1-Rad1

RPA - Replication protein A

RPLP0 – 60S acidic ribosomal protein P0 gene

SIRT1 - NAD-dependent protein deacetylase sirtuin-1

TGs – Target genes

UPR – Unfolded protein response

1. Introduction

Cancer is a genetic disease that affects both humans and dogs. It is a disease primarily caused by the accumulation of mutations in the genome, which deregulate cell growth control and cell cycle checkpoints, leading ultimately to malignant transformation and carcinogenesis [1]. In fact, nowadays cancer is one of the leading cause of death in dogs [2].

Cells in the human body have evolved multiple mechanisms to protect themselves from carcinogenesis. Amongst these, two of the most important are the DNA damage response (DDR) and the Unfolded protein response (UPR) pathways respectively [3], [4].

The DDR pathway is a system that responds to multiple forms of DNA damage and replication stress that arises when DNA replication progression is impeded [5]. When such a situation occurs, the apical protein kinase Ataxia Telangiectasia-mutated and Rad3-related (ATR) is activated and with the help of Claspin it phosphorylates and activates the effector kinase checkpoint kinase 1 (Chk1). One of Chk1's functions is to induce cell cycle arrest in G2 phase [6]. During this block to mitotic entry, the triad BRCA1-PALB2-BRCA2 recruits the recombinase Rad51 to the damage site on the DNA, allowing the machinery of homologous recombination (HR) repair to fix the problem [7]. A simplified scheme of the DDR pathway is shown in **Figure 1A**.

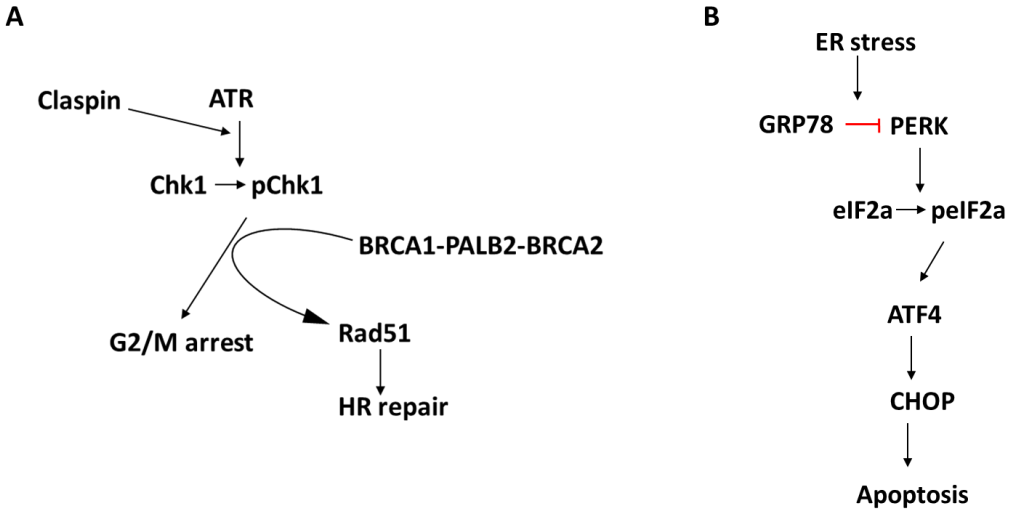

Fig 1. Generic schemes of DDR and UPR pathways in the cell. A) DNA damage response pathway is triggered under conditions of DNA damage or replication stress through activation of the apical kinase ATR. The adaptor protein Claspin helps ATR to activate Chk1 through phosphorylation. Chk1 kinase induces cell cycle arrest and the triad BRCA1-PALB2-BRCA2 recruits Rad51 to the DNA damage site allowing the homologous recombination to repair the errors. **B)** The Unfolded Protein Response pathway is activated under conditions of endoplasmic reticulum stress. Binding of the GRP78 chaperone binding to misfolded proteins causes its dissociation from the endoplasmic reticulum kinase PERK, allowing PERK to dimerize, autophosphorylate, and achieve its active form. PERK phosphorylates and inhibits eIF2α, a protein that controls protein translation. After phosphorylation by PERK, eIF2α is deactivated, and

global protein translation is inhibited. Exceptionally, ATF4 is translated preferentially under these conditions and induces the transcription and translation of CHOP. The primary purpose of these events is to limit the accumulation of misfolded proteins in the endoplasmic reticulum and restore cellular homeostasis. When ER stress conditions are excessively prolonged, CHOP has the potential to induce cell death through apoptosis.

The UPR is a system that restores the homeostasis of the cell. Cancer cells can suffer from exposure to many different types of stress such as deprivation of nutrients, hypoxia, and extracellular acidification [8] which can cause the accumulation of misfolded and unfolded proteins in the endoplasmic reticulum (ER). This situation causes the so-called ER stress that induces activation of the UPR pathway. The chaperone GRP78 (glucose-regulated protein 78) (also known as BiP) associates with the luminal domain of protein kinase PERK (protein kinase RNA-like ER kinase) when levels of misfolded proteins are low and acts as an inhibitor of PERK activation. Upon ER stress, GRP78 dissociates from PERK and redistributes to the resulting high levels of misfolded proteins. Consequently, a luminal domain of PERK is free to be bound by misfolded proteins, PERK is able to dimerize, undergo autophosphorylation, and attain its active state [9].

Active PERK then phosphorylates eukaryotic translation initiation factor 2A (eIF2α), at a specific residue, serine 51 (S51). eIF2α controls protein translation but when S51 is phosphorylated its function is inhibited and global translation is downregulated [10]. Although the translation of most poly-adenylated mRNAs is diminished after PERK-mediated eIF2α phosphorylation, there are exceptions. One of these is the transcription factor ATF4, whose translation is markedly enhanced under conditions of ER stress. The resulting increased levels of ATF4 stimulate the transcription of a range of downstream target genes, including the gene encoding the transcription factor CHOP (C/EBP homologous protein, also known as growth arrest- and DNA damage-inducible gene 153 (GADD153)) [10]. Under normal conditions, CHOP is ubiquitinated and expressed at very low level. Its missions consist of regulating the expression of anti-apoptotic and pro-apoptotic genes, it induces inhibition of BCL-2 family members and upregulation of BIM, which regulates BAX-BAK [11]. Like ATF4, CHOP is efficiently translated during ER stress, and during prolonged ER stress situations high levels of CHOP can promote cell death via apoptosis or by suppression of cell cycle regulator protein p21 [10], [12] (**Figure 1B**). Other physiological functions of CHOP are to control reactive oxygen species formation by activation of ERO1α (Endoplasmic Reticulum Oxidoreductase 1 Alpha) and restoring protein translation by activation of the protein GADD34 (growth arrest and DNA damage-inducible protein GADD34) which dephosphorylates eIF2α [13]. The increase of reactive oxygen species generates more misfolded proteins, and together with the reactivation of protein translation, CHOP is impeding the proper homeostasis restoration. CHOP has been described as promoting autophagy by interacting with the SIRT-1-AKT signaling pathways [14].

Those two pathways seem to be key to protecting the cell from neoplastic transformation, but they are a double edge sword in cancer. Their normal role is to protect the cell by correcting damage in the DNA, restoring proteostasis, or by inducing apoptosis if these outcomes cannot be achieved. In cancers, those systems are often deregulated,

and cancer cells can use those mechanisms to avoid cell death causing the development of tumors. Genome instability contributes to the ability to escape from immune system control and promote inflammation, which facilitates the spread of cancer [15], [16]. In those cases, tumor cells can hijack the DDR and UPR systems and exploit them to survive. The malfunctioning of the DDR pathway can be due to mutations and loss of DDR routes, making the cancer cell to rely only on one of the DDR pathways, usually the ATR-Chk1 route. The total dependency on only one route makes cancer cells somehow more vulnerable to inhibitors, making ATR-Chk1 an attractive therapeutic target based on synergic lethality and in combination with other therapies like radiotherapy [5]. Hypoxia, glucose deprivation, and the increase of protein translation due to genomic instability are conditions that increase in the ER stress and UPR activation [17]. In cancer cells, the highly active UPR helps tumors to grow and progress, facilitating invasive metastatic behavior [18]. Rare mutations have been found in UPR in cancer, mostly related to loss of function. The inhibition of UPR pathways components PERK and IRE1α have shown effective antitumor properties, but besides its promising therapeutic use, more research is needed as many side effects related to diabetes have been observed [19].

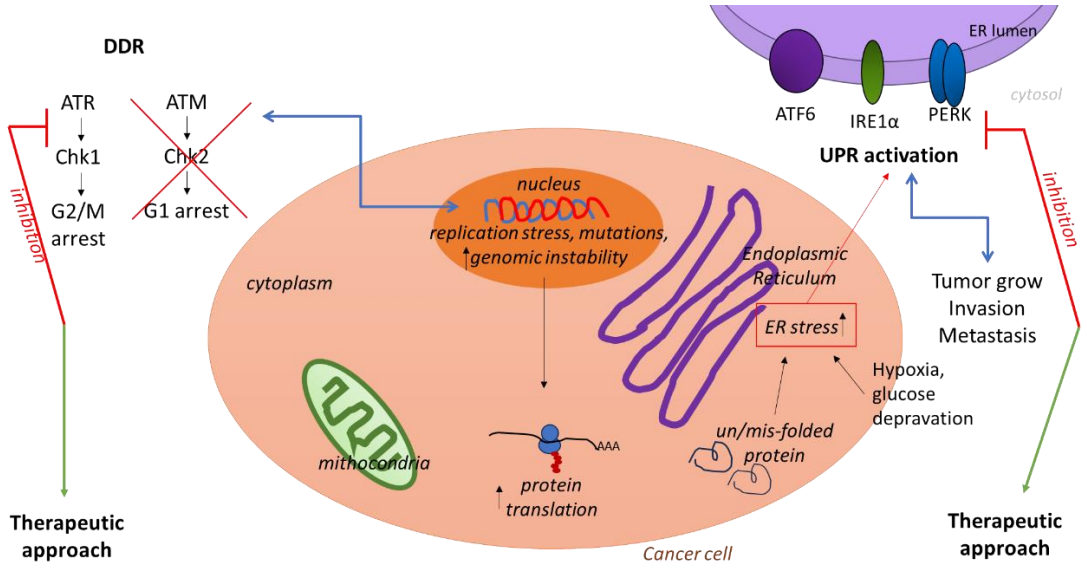

Fig. 2. Cancer cells often have an abundance of DNA damage, mutations, and replication stress. Due to mutations, most cancer cells lost some routes of the DDR pathway, generating high dependence on the ATR-Chk1 route. This dependency is an opportunity to target as a therapeutical approach and kill the cells by synergic lethality. The cancer cell environment causes hypoxia, glucose deprivation, and high levels of unfolded and misfolded proteins that could generate ER stress. High genomic instability could also influence in the increment of protein translation, which activates UPR. The high ER stress and high translation levels are inducing an overactivation of UPR which have been found to be related to tumor progression and metastasis. Inhibition of UPR components as PERK or IRE1α has effective antitumor properties.

Interestingly, ATR and Chk1 are the only DDR pathway elements on which most cancer cells fully rely due to the loss of other important DDR players like p53, which is found altered in almost 50% of human cancer and has been reported in a variety of canine cancers too [20], [21]. Therefore, inhibition of ATR and Chk1 kinases could present an attractive target for new-generation therapies [6], [22].

Increased expression of UPR components have been described in different type of cancers in humans [23], [24], and alterations in its expression and activity are linked to cancer progression and therapy resistance [25]. Consequently, targeting and inhibiting UPR proteins present an attractive therapeutic opportunity in many cancers. The connection of UPR with apoptosis reinforces the use of this pathway as a potential target. In this case, enhancement of ATF4-CHOP under hypoxia conditions in the cancer cells may help to overcome the excessive synthesis of antiapoptotic proteins such as Bcl-2 and improve the current anticancer therapies [10].

Human and canine cancer are similar in many aspects. The development of the disease is spontaneous and has a high incidence with age. Moreover, cancers in both species are heterogenic, have similar metastasis behavior, and have a similar response to antineoplastic therapies. It is also confirmed that the DDR system seems to be altered both in humans and dogs. DDR system in dogs is very conservative compared to other mammals. 94% of the canine genome is conserved between dogs, rats, mice, and humans. It is worth mentioning that the canine proteins are better related to humans than mice, especially when referred to DDR proteins [26] and UPR components [27] (**Table 1**).

Table 1. Percentage of protein homology between human and dog species.

DDR	**ATR**	94,75 %
	Claspin	84,47 %
	Chk1	96,2 %
	Rad51	99,12 %
UPR	eIF2α	96,07 %
	CHOP	92,2 %

Basic Local Alignment Search Tool (BLAST) of protein sequences.* Antibodies immunogen sequences were analyzed in BLAST® from National Center for Biotechnology Information (NCBI) (www2) [28]. Data published in a different form in **P2 and **P3**.

To study DDR, we have decided to focus on the ATR-Chk1 pathway because those proteins are among the most important in the response to replication stress. Firstly, replication stress is the major cause of genomic instability, which is the principal cause of cancer. Secondly - lots of cancer cells have p53 altered, which leaves cells with ATR-Chk1 as the only pathway to activate DDR, and thirdly, - mutation on DDR replication pathway-related proteins as Chk1 or ATR are rarely found in cancers and for being the only DDR pathway accessible for most of the cancer cells due to the loss of p53, their inhibition present an attractive target for new generation therapies [6], [22]. Surprisingly, the ATR-Chk1 pathway is not very well known in dogs. Many Chk1 and ATR inhibitors are already in clinical trials in humans [29], but there is only one study regarding the use of those inhibitors in dogs [30]. We signalized the importance of checking those inhibitors in other canine cancer types and their implications related to other proteins of the DDR system, to find targets for novel anticancer therapies.

In the study of the UPR pathway, we have decided to focus on two of the UPR markers: the phosphorylation of eIF2α and the protein CHOP because they are the main regulators of ER stress and both have been defined to be altered in human cancers [23], [25], [31]. Even though the UPR system has been studied in dogs, most of the described research about this system is not in cancer but in other diseases such as degenerative myelopathy, congestive heart failure, and even dengue infections [27], [32], [33]. Just a few studies in lymphoma and leukemia cell lines have analyzed the responses of UPR to different new proposed anticancer therapies for dogs. For example, valosin-containing protein inhibitor (CB-5083) have been proposed to treat canine lymphoma, and the research showed that activation of UPR measured by phosphorylation level of eIF2α and an increase in ATF4 expression occurs but failed in restore the homeostasis of the cells [34]. Due to the promising results obtained in humans, and the similarities between the UPR components in dogs and humans, we signalize the importance of doing research on this pathway and the use of inhibitors or potentiators to treat canine cancer.

Both pathways are important in cancer, especially both present a potential therapeutic use. There is a lack of information about the molecular mechanisms of those pathways in dogs, as well as the methodology to do such research. In this thesis, our work focuses on the search for reagents and tools that could work efficiently on canine cancer cells in order to facilitate further research and bring the first clues in the identification of potential targets in DDR and UPR that should be further analyzed to generate new anticancer therapies in dogs.

2. Aim and hypotheses

The aim of the presented research was to find new molecular targets whose study could facilitate the development of a new anticancer therapy in canine cancers. For this purpose, it was planned to analyze changes in selected cellular pathways: the DNA Damage Response (DDR) pathway, and the Unfolded Protein Response (UPR) pathway. Before developing new *in vitro* therapeutic regimens, it is necessary to elucidate the observed molecular mechanisms, which will additionally contribute to new knowledge about the biology of selected types of canine cancer cells.

Hypothesis 1 – DDR protein components and downstream targets of the master regulatory ATR-Chk1 pathway show variations in expression and activity in canine cancer cells.

Hypothesis 2 - Protein components and downstream targets of the UPR pathway responsible for maintaining protein homeostasis show variations in expression and activity in canine cancer cells.

Both hypotheses approach the final goal of finding DDR and UPR pathway alterations and possible targets that can be exploited for experimental cancer therapies in dogs and ultimately humans.

3. Methodology

3.1. Cells and cell culture

The following canine lymphoma/leukemia cell lines were used to perform the research presented in this thesis: CLBL-1 (B-cell lymphoma), CLB70 (B-cell chronic lymphocytic leukemia), GL-1 (B-cell leukemia) and CNK-89 (NK-cell lymphoma). The CLBL-1 cell line was a gift from Barbara Rütgen from the Institute of Immunology, Department of Pathobiology from the University of Vienna [35], the GL-1 cell line was received from Yasuhito Fujino and Hajime Tsujimoto from the Department of Veterinary Internal Medicine at the University of Tokyo [36], and the CLB70 cell line [37] and CNK-89 [38] were established with the participation of researchers from our laboratory.

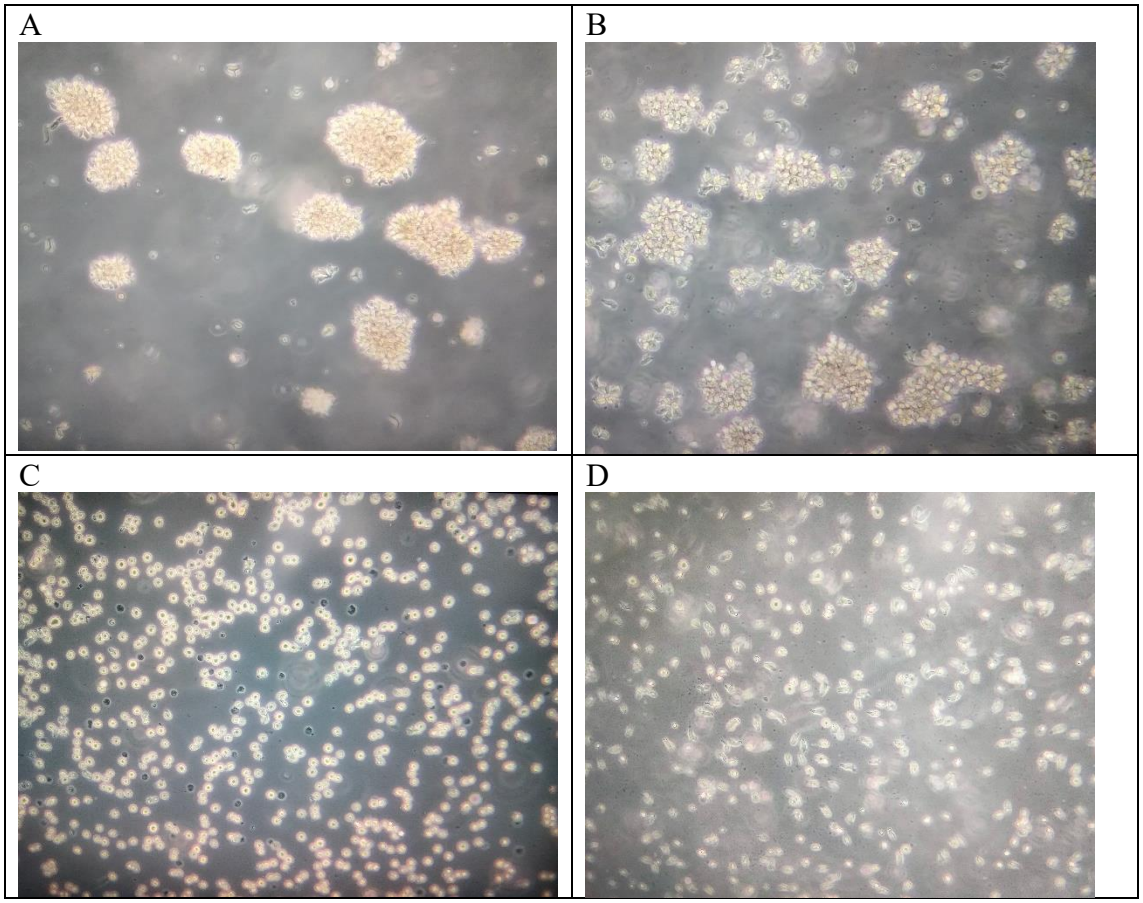

Figure 2. Cell lines in culture. A) CLBL-1 cell line, **B**) CLB70 cell line, **C**) GL-1 cell line and **D**) CNK-89 cell line.

The culture medium RPMI 1640 (Institute of Immunology and Experimental Therapy, Polish Academy of Science, Wrocław, Poland) was used to culture the CLBL-1 and GL-1 cell lines, and Advanced RPMI (Gibco, Grand Island, NY, USA) for the CLB70 and CNK-89 cell lines. The culture media were supplemented with 2 mM L-glutamine (Sigma Aldrich, Steinheim, Germany), 100 U/mL of penicillin, 100 µg/mL of streptomycin (Sigma Aldrich, Steinheim, Germany), and 10-20% heat-inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA). The cells were cultured in an atmosphere of 5% CO_2 and 95% humidified air, at 37 ºC in 25 cm^2 cell culture flasks (Corning, New York).

3.2. RNA sequencing

RNA was extracted from the selected cell lines CLBL-1 and GL-1 during unperturbed growth and sequenced by Novogene (UK). The expected number of Fragments Per Kilobase of transcript sequence per Millions of base pairs sequenced (FPKM) was used to calculate relative gene expression [39]. The DDR and UPR-related GO lists used for the intersection analysis were those presented in **Table 1**, the gene set information was obtained from the GSEA database [40], [41].

Table1. DDR and UPR-related GO lists.

Gene set	Species
AMUNDSON_DNA_DAMAGE_RESPONSE_TP53	Human
GOBP_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR	Human
GOBP_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR	Mouse
GOBP_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR_RESULTING_IN_CELL_CYCLE_ARREST	Human
GOBP_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR_RESULTING_IN_CELL_CYCLE_ARREST	Mouse
GOBP_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_RESULTING_IN_TRANSCRIPTION	Human
GOBP_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_RESULTING_IN_TRANSCRIPTION	Mouse
GOBP_NEGATIVE_REGULATION_OF_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR	Human
GOBP_NEGATIVE_REGULATION_OF_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR	Mouse
GOBP_POSITIVE_REGULATION_OF_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR	Human
GOBP_POSITIVE_REGULATION_OF_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR	Mouse
GOBP_POSITIVE_REGULATION_OF_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR_RESULTING_IN_TRANSCRIPTION_OF_P21_CLASS_MEDIATOR	Human
GOBP_REGULATION_OF_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR	Human
GOBP_REGULATION_OF_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR	Mouse
GOBP_REGULATION_OF_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR_RESULTING_IN_TRANSCRIPTION_OF_P21_CLASS_MEDIATOR	Human

GOBP_REGULATION_OF_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR_RESULTING_IN_TRANSCRIPTION_OF_P21_CLASS_MEDIATOR	Mouse
REACTOME_P53_DEPENDENT_G1_DNA_DAMAGE_RESPONSE	Human
REACTOME_SUMOYLATION_OF_DNA_DAMAGE_RESPONSE_AND_REPAIR_PROTEINS	Human
WP_DNA_DAMAGE_RESPONSE	Human
WP_DNA_DAMAGE_RESPONSE_ONLY_ATM_DEPENDENT	Human
WP_MIRNA_REGULATION_OF_DNA_DAMAGE_RESPONSE	Human
WP_MIRNAS_INVOLVED_IN_DNA_DAMAGE_RESPONSE	Human
GOBP_REGULATION_OF_PERK_MEDIATED_UNFOLDED_PROTEIN_RESPONSE	Human
GOBP_REGULATION_OF_PERK_MEDIATED_UNFOLDED_PROTEIN_RESPONSE	Mouse
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	Human
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	Mouse
REACTOME_UNFOLDED_PROTEIN_RESPONSE_UPR	Human
REACTOME_UNFOLDED_PROTEIN_RESPONSE_UPR	Mouse
WP_PHOTODYNAMIC_THERAPYINDUCED_UNFOLDED_PROTEIN_RESPONSE	Human
WP_UNFOLDED_PROTEIN_RESPONSE	Human

3.3. Treatments

DNA damage was induced by treatment with etoposide (Sigma Aldrich, USA), a topoisomerase II inhibitor, at a concentration of 20 μM for 2 hours.

The cells were treated with different drugs to induce ER stress and to test the activation of the pathway of interest. To induce ER stress, the cells were treated with thapsigargin (Sigma Aldrich, USA), a Ca^{2+} ATPase inhibitor, at a concentration of 2 μM for 2 hours, and MG132, proteasome inhibitor, at 10 μM for 16 hours. For flow cytometry analysis after ER stress induction, thapsigargin was used at a concentration of 1 μM for 5 hours and MG132 at 20 μM for 5 hours.

3.4. Western blot

3 x 10^5 cells/mL were cultured in 10 mL of media in a 25 cm^2 culture flask. After 48h of incubation, the samples were lysed in urea/ SDS buffer (composed of 900 μL of 7 M urea, 25 μL of 5 M NaCl, 25 μL 2 M Tris-HCl (pH=8), 50 μL 20% SDS), and run in 8-12% bis-tris acrylamide gels prepared using a BioRad Mini-PROTEAN Tetra Vertical Electrophoresis Cell system. The samples were transferred to a nitrocellulose membrane using BioRad Mini Trans-Blot® Cell for wet transfer and BioRad Trans-Blot® Turbo™ Transfer System device for semi-dry transfer.

The selection of the antibodies used in the study was based either on available literature data on reactivity with canine cells (**Table 2**) or a comparison of protein sequence

homology, and preliminary test results involving a comparison of the observed bands with the predicted molecular mass (kDa) of the protein of interest. Goat Anti-Mouse Immunoglobulins/HRP (#P0447 at 1:20000 concentration in TBS-T solution) and Goat Anti-Rabbit Immunoglobulins/HRP (#P0448 at 1:10000 concentration in TBS-T solution) were used as secondary antibodies. Both secondary antibodies were from Dako, now part of Agilent (USA, Santa Clara).

Table 2. Antibody list showing percentage protein identity between human and dog DDR and UPR components.

Protein	Clone	Ref. catalog number	Dilution used in the study	% of protein homology*	Literature
Chk1	G-4	sc-8408	1:1000 in 3% BSA in TBS-T	96.2	[42]–[45]
Phospho-Chk1 (SER345)	133D3	#2348	1:1000 in 3% BSA in TBS-T	96.2	[42], [44]–[47]
β-Actin	C4	sc-47778	1:1000 in 3% milk in TBS-T	97.22	[48]–[53]
ATR	C-1	sc-515173	1:800 in 3% BSA in TBS-T	94.75	[45], [46], [54]
Rad51	G-9	sc-377467	1:600 in 3% BSA in TBS-T	99.12	[47], [55], [56]
Claspin	B-6	sc-376773	1:800 in 3% BSA in TBS-T	84.47	[46]
Anti-gamma H2AX	9F3	ab26350	1:1000 in 3% BSA in TBS-T	99.17	[34], [49], [50], [54], [57]
Phospho-eIF2α (Ser51)	119A11	#3597	1:1000 in 3% BSA in TBS-T	96.07	[56], [58], [59] [27]
eIF2α	D-3	sc-133132	1:1000 in 3% BSA in TBS-T	96.07	[51], [52]

DDIT3/CHOP/ GADD153	B-3	sc-7351	1:500 in 3% BSA in TBS-T	92.3	[51], [60]
CHOP	L637F	#2895	1:1000 in 3% BSA in TBS-T	92.3	[52], [53], [59]

**Basic Local Alignment Search Tool (BLAST) of protein sequences.* Antibodies immunogen sequences were analyzed in BLAST® from National Center for Biotechnology Information (NCBI) (www2) [28].

3.5. qPCR

3.5.1. Bioinformatic sequence analysis and primer design

The *Canis lupus familiaris* nucleotide accession number sequences for mRNA of the target genes (TGs): Atr, Claspin, and six housekeeping genes (HKGs): Actb, Ppia, and Rplp0 were taken from the Nucleotide Center for Biotechnology Information (NCBI) database (NCBI, USA). The sequences were transferred into the Universal Probe Library. The designed primers and their amplified sequences were additionally verified for their specificity in the Nucleotide Basic Local Alignment Search Tool - Nucleotide-BLAST (NCBI, USA). Gene names, primer sequences for TGs and HKGs, amplicon size, as well their respective gene accession numbers are presented in **Table 3**.

Table 3. Gene names, forward (F) and reverse (R) primer sequences, amplicon nucleotide (nt) sizes with their respective gene accession numbers.

Gene name	**Forward (F) and Reverse (R) primer sequences**	**Amplicon size (nt)**	**Gene accession number**
Target Genes (TGs):			
ATR	F: ACCAGACAGCCTACAATGCT R: CCACTTTGCCCTCTCCACAT	77	XM_038432561.1
CLSPN	F: CGCACAAAGCCAGGTGAAAA R: CGTTCCTCATGCCTACGGAG	80	XM_539598.6
Housekeeping genes (HKGs):			
ACTB	F: CGCAAGGACCTCTATGCCAA R: CTTCTGCATCCTGTCAGCGA	78	NM_001195845.3
PPIA	F: TTTGGCAAGGTCAAGGAGGG R: TGGTCTTGCCATTCCTGGAC	73	XM_038689274.1

RPLP0	F: ACATGCTGAACATCTCCCCC R: CAGGGTTGTAGATGCTGCCA	80	XM_038436104.1

3.5.2. RNA isolation and reverse transcription

A flasks containing a total of $1x10^7$ cells in 10 mL from the CLBL-1, CLB70, and GL-1 lymphoma cell lines were centrifuged at 300 g, 4º C, and resuspended in 500 µL of TRIzol reagent (Invitrogen, USA). The cells were immediately transferred to a low-temperature freezer and stored in the Eppendorf tubes at -80º C for further analysis. Total RNA isolation was performed using Total RNA Zol-Out™ D (A&A Biotechnology, Poland) according to the protocol provided in the isolation kit. Briefly, the cells were thawed on ice for 30 minutes. After that, 167 µL of ultra-pure molecular biology water (A&A Biotechnology, Poland) was added, and the sample was mixed by inversion. Next, the cells were spun for 10 minutes at 10 000 rpm. The supernatant was mixed with 1 volume of 96-100% ethanol (Stanlab, Poland) and gently agitated until a homogenous solution was obtained. The supernatants from each tube were transferred into new tubes with RNA membrane binding column and were centrifuged through the column for 1 minute at 10 000 rpm and 4º C. The columns were rinsed with 700 µL washing A2W buffer for 2 minutes at 10 000 rpm. The RNA-isolated samples went through DNA digestion by DNase for 15 minutes at 37º C. The enzymatic activity of the digestive buffer was inhibited by adding 700 µL of R81 buffer and centrifugation (1 minute at 10 000 rpm at room temperature [RT]). The filtrate was collected and loaded again onto the column. The membranes were rinsed twice with 700 µL and 200 µL of A2W buffer, centrifuged as described above, and transferred into new Eppendorf tubes. Then, 40 µL of sterile water was added, and after 3-minute incubation at RT the tubes with the membranes were centrifuged as above. RNA quality and quantity were estimated using Implen NanoPhotometer (Eppendorf, Germany), and only the samples with a 260/280 nm absorbance coefficient between 1.8 and 2.1 were used for the final experiments. The TranScriba noGenome Kit (A&A Biotechnology, Poland) was used to perform reverse transcription, according to the manufacturer's recommendations in the MJ Research PTC-100 thermocycler (Marshall Scientific, USA). First, 1 µg of total RNA was mixed with the noGenome master mix. After 10-minute incubation at 42º C, 7 µL of the mentioned mix was added to the RT master mix. The RT master mix included 4 µL of TranScriba buffer, 0.5 µL of RNAse inhibitor, 2 µL of dNTP, 1 µL of starter oligo (dT), 4 µL of TranScriba reverse transcriptase and 1.5 µL of sterile water for one reaction. The reverse transcription protocol was as follows: the first step of 60 minutes at 42 ºC, the second step of 5 minutes at 70 ºC, and the final step of 5 minutes at 4 ºC. The obtained cDNA was stored at -20 ºC.

3.5.3. Gene expression analysis using real-time PCR

The real-time PCR gene expression analyses were performed in triplicate from three independent experiments. The reaction mix (per well) included 5 µL of RT PCR Mix

SYBR® (A&A Biotechnology, Poland), 0.5 µM of forward and reverse primers (Eurofins Genomics AT GmbH, Poland), and 1 µL of cDNA diluted with molecular biology water (16.65 ng cDNA per well). Real-time PCR was performed using the LightCycler 480 II (Roche Molecular Systems Inc., USA) instrument at the following conditions: pre-incubation at 95 °C for 10 minutes, 50 cycles of amplification: 10 seconds at 95 °C for denaturation, 30 seconds at 60 °C for annealing, and 15 seconds at 72 °C for elongation. The gene detection analyses and primer specificity were further improved by melting curve analysis. The gene expression was categorized using the following scale:

"0" lack of gene expression, Ct values above 35

"1" very low gene expression, Ct values between 30 and 35

"2" low gene expression, Ct values between 28 and 30

"3" regular gene expression, Ct values between 22 and 28

"4" high gene expression, Ct values between 15 and 22

"5" very high gene expression, Ct values below 15

3.6. DNA combing assay

1.6 million cells were cultured in 10 mL of media, then pulse-labeled with 5-iodo-2'-deoxyuridine (IdU) at 25 µM, followed by 5-chloro-2'-deoxyuridine (CldU) at 200 µM, for 15 minutes each. The cells were recovered by centrifugation at 300 g after each pulse, and the media were refreshed with each analog. Next, the cells were resuspended in cold PBS and warmed to 42 ºC, using $5x10^5$ cells per agarose plug. The cells were gently mixed with 1% agarose in PBS and divided into the casting mold to generate the plugs. Each plug was treated with proteinase K (in a buffer made of 1% Sarkosyl, 10 mM Tris pH 7.5, and 50 mM EDTA) at 50 ºC overnight. The following day the proteinase K treatment was stopped, and the plugs were incubated in TE50 buffer overnight. Later the DNA was stained with YOYO-1 (5 µM in TE solution for 2-5 minutes) to verify the integrity of the fibers. Agarose from plugs were melted in a MES buffer with β-agarose overnight at 42ºC and the extracted DNA was poured into a reservoir, where a coverslip was inserted to stretch the DNA fibers. The resulting fibers were visualized by immunofluorescence detecting the IdU and CldU analogs with red and green antibodies. The coverslips were incubated for 45 minutes with murine anti-BrdU (IdU, ref. 34780 Becton Dickinson, USA), and rat anti-BrdU (CIdU, Eurobio ref. ABC117-7513, France), as primary antibodies for the analogs, and for 30 minutes with goat anti-mouse IgG1 Alexa 564 (ref. A21123 Molecular Probes, Thermo Fisher Scientific, USA) and chicken anti-rat Alexa 488 (ref. A21470 Molecular Probes, Thermo Fisher Scientific, USA) as secondary antibodies for the analogues. The coverslips were incubated for 30 minutes with auto anti-ssDNA DSHB by Voss, E.W. (Hybridoma Product auto anti-ssDNA) (auto anti-ssDNA DSHB by Voss, E.W. (DSHB, USA) to detect whole DNA fibers, and then for 30 minutes with a secondary antibody goat anti-mouse IgG2a Alexa 647 (ref. A21241 Molecular Probes, Thermo Fisher Scientific, USA). Images were taken using a confocal microscope (DM6000; Leica). To obtain the fork velocity (FV) the length of the green track of the fiber in micrometers was multiplied by 2 to obtain Kb and dividing it by 15 minutes (time

of pulse). To obtain the fork velocity (FV) the length of the green track of the fiber in micrometers was multiplied by 2 to obtain Kb and dividing it by 15 minutes (time of pulse). To obtain the fork asymmetry (FA) the green long track was divided by the short green track in the detected initiation patterns. For this analysis, only the second analog incorporation tracks (green tracks) were considered.

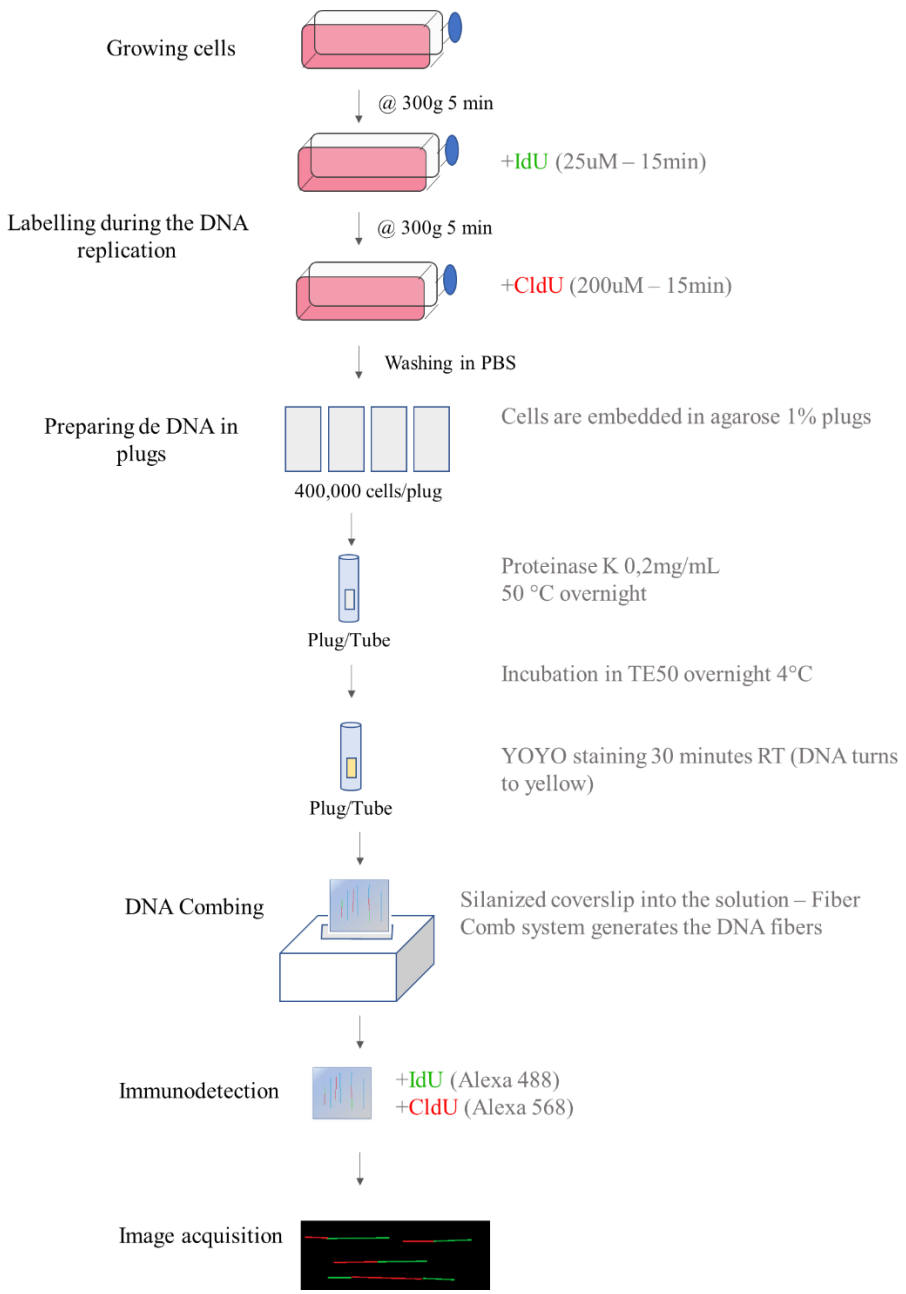

Figure 3. DNA combing assay protocol. 1.6 x 10^6 cells were centrifuged at 300 g for 5 minutes and resuspended in a medium with IdU (25 µM). After 15 minutes cells were centrifuged again and resuspended in a fresh media with CldU (25 µM) for another 15 minutes. Then, cells were washed in PBS and distributed in Eppendorf (400,000 cells). 1% agarose was added to embed the cells. After the plugs were formed, they were incubated overnight with proteinase K (0,2 mg/mL) at 50 ºC. The following day the cells were stained for 30 minutes with YOYO solution at room temperature. The agarose was

melted in MES buffer with β-agarose overnight. The DNA solution was poured into a reservoir and a silanized coverslip was inserted to comb the DNA. The coverslip containing the DNA fibers was then ready for immunodetection and were incubated with the antibodies which recognize the IdU and CldU analogs. After overnight incubation at room temperature in the dark, the sample was ready for visualization under the microscope.

3.7. Flow cytometry

The cells were treated with ER stress inducers at the concentration defined above prior to staining with annexin V FITC/PI or caspase 3/7 to study apoptosis. The cells were suspended in a binding buffer together with annexin V-FITC and PI (PI concentration, 1 μg/mL) for 10 minutes at room temperature. At the same time, another batch of cells was collected and stained with CellEventCaspase-3/7 Green Detection Reagent following the manufacturer's instructions and incubated at 37 ºC for 30 minutes. Finally, flow cytometry analysis was performed using a flow cytometer (FACS Calibur; Becton Dickinson, Biosciences, San Jose, USA). The software Weasel v.3.0.2 (Frank Battye) was used for data analysis.

3.8. Statistical analysis

For the combing assay analysis, the Mann-Whitney test was performed to compare the cell lines and analyze potential differences. Scatterplots were prepared to visually represent the differences between the two cell lines.

Statistical analysis was performed using TIBCO Software Inc. (2017) Statistica (data analysis software system), version 13. http://statistica.io.

4. Foreword to publication 1

The first publication in the series of articles forming the doctoral dissertation is a review article summarizing what is known about the DDR pathway in dogs.

The manuscript starts by describing the role of DNA damage response in cancer. It describes the cascade of proteins that are activated after DNA damage. First, activation of kinases ATR or ATM occurs, which next phosphorylates Checkpoint kinases 1 and 2. Those effector kinases by activating p53 or inhibiting CDKs are regulating the cell cycle allowing the repair mechanism to fix the damage or to induce apoptosis when it is not possible.

The review continues describing similarities between the DDR proteins in humans and dogs. A summary table (**table 1 in P1**) showed some of the most relevant DDR proteins such as TopBP1, p53, Rad51, BRCA1, BRCA2, Chk1, Chk2, PTEN, PCNA, p21, p27, and Cyclin A, showing the similarities and differences that are known between the human and canine protein.

A deeper explanation of what is known about some of those DDR proteins in dogs is described further in the text. Below there is a summary of the most relevant information on each of the proteins mentioned in the review.

1. BRCA1 and BRCA2

BRCA1 and *BRCA2* genes are linked to spontaneous cancer and metastasis in both humans and dogs [61].

The breed predisposition to mammary tumors (CMT) observed in dogs has the advantage of low levels of genetic variations that can be useful to study BRCA mutations [62], [63].

2. p53

The DNA binding region and the C- and N-termini are highly conserved between mammals and are the regions where more mutations have been described [64].

Overexpression of p53 and Bcl-2 proteins in human and canine tumors indicate apoptosis-resistant phenotype [65].

3. TopBP1

TopBP1 interacts with and regulates the activity of a wide range of different DDR proteins (e.g. BRCA1, 53BP1, p53, MDC1, ATR, BACH1 (BRCA1-associated C-terminal helicase), RPA/RAD51, RAD9 (Rad9 Checkpoint clamp component of 9-1-1 clamp: Rad9-Hus1-Rad1)) by binding through BRCT domains to phosphorylated sites within these partner proteins [66]–[69].

TopBP1 has been found to be expressed in both the nucleus and cytoplasm of malignant tissue in human and canine cancers [70]–[73].

4.Rad51

Rad51 expression has been found to be generally higher in human tumor cell lines and in primary tumors compared to normal tissues and it is associated with genomic instability and resistance to chemo- and radiotherapies [74].

The information about Rad51 available is mostly about CMT due to its strong relation with BRCA, some analyses suggest a relation of Rad51 with genomic instability but so far, mutations in Rad51 have not been proven to be the principal cause of cancer in dogs [75], [76].

Finally, the review ends with a reflection on the clinical aspects of the DDR pathway in cancer in dogs. First, it was mentioned how breed predisposition to diseases like lymphoma has a correlation with DNA damage defects [77], [78]. Subsequently, different possible therapeutic approaches such as the supplementation of fish oil or the selenium-rich diet for prostate cancer protection [79] were discussed. An interesting point regarding human and dogs' co-habitation and their influence on both health, is the fact that both species share the same environment and so the same chemical exposures. To finish, a remark about the possibility of using therapies such as PARP inhibitors that are in use for humans is mentioned as a real possibility also in veterinary medicine [80], [81].

5. Publication 1

P1 - DNA Damage Response (DDR) proteins in canine cancer as potential research targets in comparative oncology

Received: 8 December 2021 | Revised: 15 December 2021 | Accepted: 16 December 2021

DOI: 10.1111/vco.12795

REVIEW

Veterinary and Comparative Oncology WILEY

DNA damage response proteins in canine cancer as potential research targets in comparative oncology

Beatriz Hernández-Suárez[1] | David A. Gillespie[2] | Aleksandra Pawlak[1]

[1]Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences, Wroclaw, Poland

[2]Instituto de Tecnologías Biomédicas, Facultad de Medicina, Campus Ciencias de la Salud, Universidad de La Laguna, San Cristóbal de La Laguna, Spain

Correspondence
Beatriz Hernández-Suárez, Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences, Norwida 31, Wroclaw 50-375, Poland.
Email: beatriz.hernandez-suarez@upwr.edu.pl

Funding information
European Social Fund under the Operational Program Knowledge Education Development, Grant/Award Number: POWR.03.05.00-00-Z062/18; Polish National Agency for Academic Exchange, Grant/Award Number: PPI/APM/2019/1/00044/U/00001; UPWR 2.0: International and Interdisciplinary Programme of Development of Wrocław University of Environmental and Life Sciences

Abstract

The DNA damage response (DDR) is a complex signal transduction network that is activated when endogenous or exogenous genotoxins damage or interfere with the replication of genomic DNA. Under such conditions, the DDR promotes DNA repair and ensures accurate replication and division of the genome. High levels of genomic instability are frequently observed in cancers and can stem from germline loss-of-function mutations in certain DDR genes, such as BRCA1, BRCA2, and p53, that form the basis of human cancer predisposition syndromes. In addition, mutation and/or aberrant expression of multiple DDR genes are frequently observed in sporadic human cancers. As a result, the DDR is considered to represent a viable target for cancer therapy in humans and a variety of strategies are under investigation. Cancer is also a significant cause of mortality in dogs, a species that offers certain advantages for experimental oncology. Domestic dogs present numerous inbred lines, many of which display predisposition to specific forms of cancer and the study of which may provide insight into the biological basis of this susceptibility. In addition, clinical trials are possible in dogs and may lead to therapeutic insights that could ultimately be extended to humans. Here we review what is known specifically about the DDR in dogs and discuss how this knowledge could be extended and exploited to advance experimental oncology in this species.

KEYWORDS
BRCA1, BRCA2, canine cancer, p53, Rad51, TopBP1

Abbreviations: 53BP1, p53-binding protein 1; 9-1-1, Rad9-Rad1-Hus1; ATM, autosomal-recessive ataxia-telangiectasia mutated; ATR, autosomal-recessive ataxia-telangiectasia mutated and Rad3-related; ATRIP, ATR interacting protein; BACH1, BRCA1-associated C-terminal helicase; BCRT, BRCA1 carboxyl terminal; BRC, BRCA2 repeats; BRCA1, breast cancer 1; BRCA2, breast cancer 2; CC3, cleaved caspase 3; CDKs, cyclin-dependent kinases; cDNA, complementary DNA; Chk1, check point kinase 1; Chk2, check point kinase 2; CML, chronic myelogenous leukaemia; CMT, canine mammary tumour; DBD, DNA binding domain in p53; DNA, deoxyribonucleic acid; DNA-PKcs, DNA dependent protein kinases; DDR, DNA damage response; EDD, E3 identified by differential display; GST, specific glutathione-S-transferase; γH2AX, phosphorylated-gamma histone H2AX; H2AX, histone H2AX; HCC, hepatocellular carcinoma; HR, homologous recombination; IR, irradiation; MDC1, mediator of DNA damage protein 1; MDM2, murine double minute 2; MMR, mismatch repair; MRN, Mre11-Rad50-NBS1; MVC, minute virus of canine; NHEJ, non-homologous end joining; NOS, nitrogen species; p21, cyclin-dependent kinase inhibitor p21; p27, cyclin-dependent kinase inhibitor 1B; p53, tumour protein p53; PALB2, partner and localizer Of BRCA2; PCNA, proliferating cell nuclear antigen; PIKK, phosphatidyl inositol 3′ kinase-related kinases; PTEN, phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase; Rad51, Rad51 recombinase; RAD9, Rad9 checkpoint clamp component of 9-1-1 clamp; ROS, reactive oxygen species; RPA, replication protein A; SNP, single nucleotide polymorphisms; SSPC, single-strand conformational polymorphism; TopBP1, topoisomerase IIβ binding protein 1; tp53, gen of tumour protein p53; UV, ultraviolet light.

1 | INTRODUCTION

1.1 | DNA damage response and its role in cancer

The DNA damage response (DDR) is a signalling and effector pathway designed to ensure the genetic stability of eukaryotic cells. It is a complex, multi-faceted process that is activated in response to DNA damage inflicted by both endogenous sources, such as reactive oxygen species (ROS), reactive nitrogen species (NOS), or exogenous sources, such as ultraviolet light (UV) or irradiation (IR).[1] A variety of different mechanisms can contribute to genomic instability.[2] One is enzymatic deamination, which converts 5-methylcytidine to a thymidine residue through removal of the amine group. Replication of the resulting T/ G mismatch is mutagenic, and breast and other cancers have been found to exhibit mutation or aberrant expression of cytosine deaminases of the AID/ APOBEC families.[3] Another mechanism is microsatellite instability, in which expansions of repeated sequences of DNA occur during replication. Microsatellite instability is linked to mutations in the mismatch repair (MMR) system[4] and are found in hereditable cancers such as Lynch syndrome.[5] Macroscopic chromosome translocations, an abnormal rearrangement of one chromosome segment to another, are frequently observed in lymphoma.[6] Sporadic DNA damage lesions can also arise as a result of errors during DNA replication.[7] Somatically acquired mutations affecting DNA repair and genome stability genes are frequently observed in cancers, and it is considered that they play an important role in the development of the corresponding sporadic cancers.[8,9]

After a DNA damage lesion occurs, a cascade of highly coordinated signalling events begins which activate cell cycle checkpoints, promote DNA repair, and eliminate cells with irreparable lesions via programmed cell death or apoptosis.[10] This cascade (Figure 1) starts with the recognition of damage by the Mre11-Rad50-NBS1 (MRN) complex[11] and Rad9-Rad1-Hus1 (9-1-1) complexes, which act as damage sensors and help in the recruitment of additional DDR proteins to the damage site. These sensor molecules send signals to recruit the phosphatidyl inositol 3′ kinase-related kinases (PIKK), ataxia-telangiectasia mutated (ATM), ataxia-telangiectasia mutated and Rad3-related (ATR) and/or DNA dependent protein kinases (DNA-PKcs), which then phosphorylate and activate other transducers and effector proteins. One important target of ATM kinase is histone H2AX, which in its phosphorylated form (γH2AX) serves as a platform for the recruitment of DDR proteins at the damage site.[12] As a result, γH2AX is a well-known DNA damage marker widely used in DDR research that can be used to monitor and

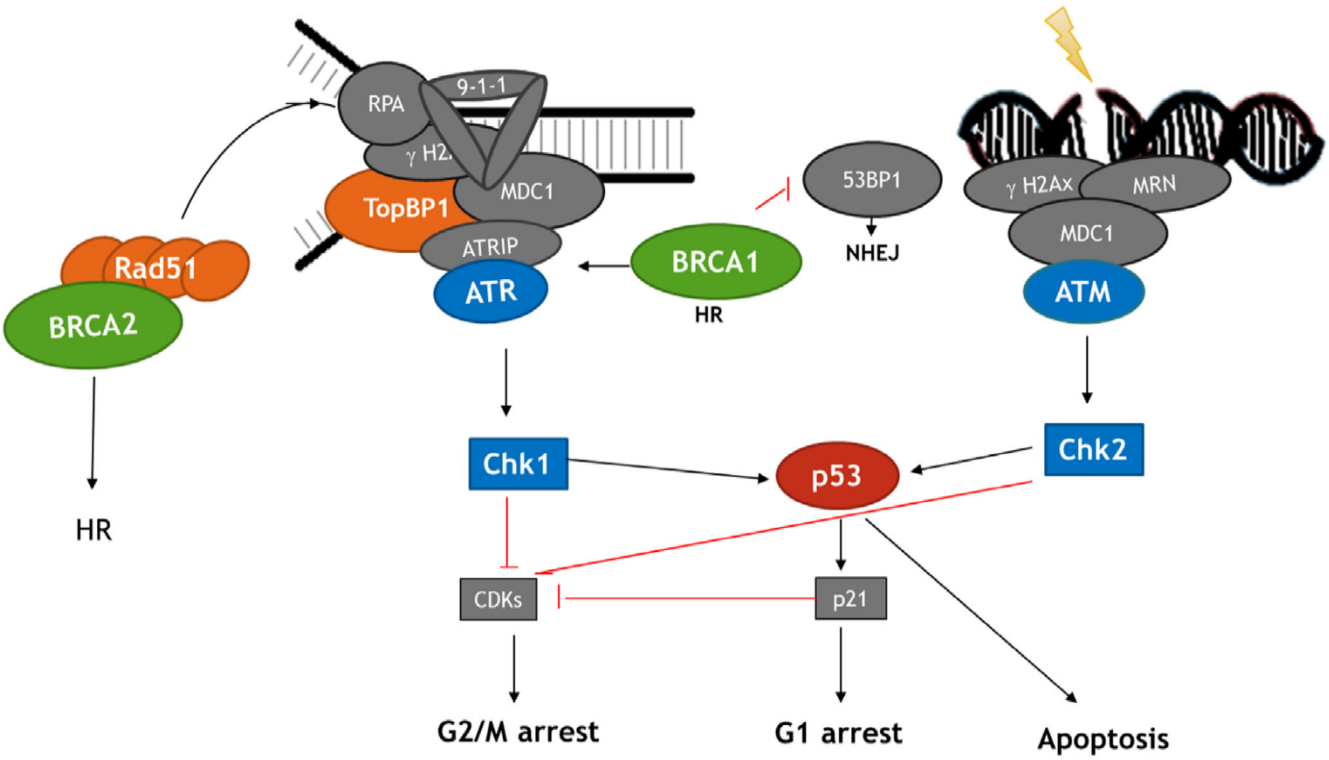

FIGURE 1 DDR signalling cascade. When DNA damage occurs, the cascade of DDR proteins starts. First, sensor proteins (MRN and 9-1-1 complexes) recognize the damage and RPA binds the single stranded DNA (ssDNA). ATM/ATR kinases are recruited to DNA damage site by interacting with the sensor proteins. Those kinases immediately phosphorylate histone H2AX at serine 139 (γH2AX), which helps to attract repair factors. Then, the mediator proteins (BRCA1, MDC1 and TopBP1) stabilize the protein interactions and increase the damage signalling. First, the transducer kinases ATM and ATR activate the effector kinases, Chk1/Chk2, by phosphorylation, which by activation of p53 or inhibition of CDKs induce cell cycle arrest or apoptosis if the damage is irreparable. The mediators BRCA1 and 53BP1 compete to promote different pathways: BRCA1 promotes HR repair pathways, while 53BP1 favours NHEJ pathway. Note: The principal kinases of the DDR are coloured blue. Other DDR proteins discussed at length in this review are depicted in green (BRCA1, BRCA2) or orange (Rad51, TopBP1), while functional components not discussed in detail are grey. Black arrows symbolize activation, and red lines symbolize inhibition

quantify damage lesions and also to detect DNA fragmentation arising from programmed cell death or apoptosis.[13]

During this initial step other important proteins such as p53-binding protein 1 (53BP1), Mediator of DNA Damage Checkpoint protein 1 (MDC1), Topoisomerase IIβ binding protein 1 (TopBP1) and Breast Cancer 1 (BRCA1) amplify the chromatin modifications and help in activation of the checkpoint effector kinases: Checkpoint kinase 1 (Chk1) and Checkpoint kinase 2 (Chk2). These kinases control multiple cell cycle checkpoints and promote DNA repair by modulating the activity of various effectors, such as cyclin-dependent kinases (CDKs), tumour suppressor protein 53 (p53), and Rad51.[14] Chk1 and Chk2 function to induce cell cycle arrest, DNA repair, chromatin assembly, transcriptional and posttranscriptional regulation of gene expression and cell death through apoptosis.[15–21] Other relevant proteins that participate in this cascade are Breast Cancer Associated 2 (BRCA2), whose principal role is to promote DNA repair by homologous recombination (HR),[22] and cyclin/CDK complexes and CDK inhibitors, such as p21 CIP1, whose role is to arrest the damaged cell at different points of the cell cycle.[23,24] Collectively, the DDR/checkpoint system acts to prevent cell division with damaged or partially replicated DNA and to promote accurate repair to enable the damaged cell to survive without permanent genetic damage or mutation. Evidence suggests that these processes play an important role in preventing neoplastic transformation and cell death under conditions of genomic or oncogenic stress.[25] A simplified diagram of the DDR signalling cascade is shown in Figure 1.

1.2 | DNA damage response in canine cancers

The DDR system is highly conserved and its organization and functional components are very similar in all mammalian species.[20,21] The similarity between dogs and humans in this context is special because certain important proteins respond in the same way to DNA damage compared to other species.[26] One example is shown in a study where the expression of p53 protein was monitored at various times after DNA damage in different mammalian species.[27] The authors generated human, mouse, dog, monkey, and rat kidney cell lines expressing a fluorescent p53. After irradiation, they observed p53 protein levels to increase and decrease in an oscillatory pattern over a period of 10 h in all species. The oscillatory profile of response of p53 protein was however found to be much slower and more similar between dogs and humans than in rodents.[27]

The literature lacks extensive information on the course of DDR in dogs and details on the role of its individual components. A number of papers describe selected proteins of the DDR pathway in dogs, however there are no studies that document the overall DNA repair process. In addition, although some studies have focused on the description of selected DDR proteins in relation to mutations, changes in the expression level, and their use in comparative oncology, in general they do not consider how knowledge of the canine DDR system could be used to develop new anticancer therapies.[28,29] One study on canine cell lines infected with a canine minute virus (MVC)[30] provides molecular insights into how the DDR functions in dogs. In the case of the DDR induced by MCV, ATM is known to be necessary to induce the G2/M arrest signal.[30] However, the proximal effector kinase(s) that mediate the cell cycle arrest remains unclear. Another recent study examining MMR deficiency in canine tumours, has shown that oral malignant melanoma and hepatocellular carcinoma (HCC) lack expression of one or more MMR proteins more frequently than other cancer types in dogs.[31] An interesting study showed differential rates of DNA repair rate in embryonic fibroblasts from humans, dogs, mice and rats.[32] As predicted, the DNA repair rate increased in the species with longer life span, suggesting a positive relationship between DNA repair proficiency and longevity. Non-Homologous End Joining (NHEJ) activity in humans and dogs was the highest both in vitro and in vivo, as longer life span may require a more functional DDR to allow cells extended replicative potential.[32]

Some studies investigating DDR proteins in dogs show important similarities in function and expression with their human homologues. A comparison in Table 1 clearly shows that many of the most important proteins involved in the DDR, such as TopBP1, p53, Rad51, BRCA1, BRCA2, Chk1[a], Chk2[a], PTEN, PCNA, p21, p27 and Cyclin A, are highly conserved between humans and dogs, indicating that the dogs can be research models for the investigation of DDR phenomena. Combining these discoveries with the fact that humans and dogs have the highest DNA repair rates, emphasizes again the potential of using of dogs as a research model to uncover biological insights that could be translated to humans.

An additional advantage of the dog model is the fact that in both species, humans and dogs, development of tumours is spontaneous, showing higher incidence with age. Heterogeneous course of disease in different patients with an analogous metastatic behaviour, comparable response to antineoplastic therapies, and alteration in DDR pathway are further similarities.[28,63,64] Additionally, over 360 genetic disorders related to cancer were described in dogs, and this constitutes the largest set of natural genetic disorders in a non-human species.[65] These findings clearly justify the need for close cooperation between veterinary and human oncologists in the field of genetic instability and particularly DDR disorders. The increasing availability of a wide panel of various canine cancer cell lines hugely facilitates such studies.[66–69]

Due to significance of DDR disorders in the cancer development in dogs, this review discusses the key components of the DDR, presents their physiological role and the abnormalities associated with these proteins found in canine cancers. We consider findings on the potential causes of genetic instability in canine cancers and indicate potential new research directions. These directions may contribute to the development of new anti-cancer therapies in veterinary medicine, and in view of the potential role of the dog as a model for the study of human diseases, also in human medicine.

2 | SELECTED DDR PROTEINS AND THEIR POTENTIAL ROLE IN CANINE RESEARCH

2.1 | BRCA1 and BRCA2

Inherited germ-line mutations affecting the BRCA1 and BRCA2 genes are associated with the development of familial cancer in women.

TABLE 1 Summary of similarities and differences in DDR proteins between humans and dogs

Protein	Dogs	Humans
TopBP1	• Similar function in both species • Overexpressed in malignancy • Cytoplasmic localization in malignancy[33–35] • Validated antibodies[33–35]	• Inhibition of p53 pathways when TopBP1 is overexpressed[36]
p53	• 78.4% shared protein identity • Similar function in both species • Role in malignant transformation when mutated • Highly conserved DNA binding region and C- and N-termini • Alteration present in different types of cancers • Low-frequency inherited mutations in cancer[37–45] • Validated antibodies[44,45]	—
Rad51	• 99% gene homology • Interaction with BRCA2 and BRCT domains • Overexpressed in cancer • Presence of polymorphisms in tumour[24,46–49] • Interaction with BRCA2 C-terminal domain[50] • Validated primers[48,49]	—
BRCA1	• 84% of gene identity • BCR conserved regions share 77% homology • Mutations increased risk of mammary tumour • Increased malignancy when expression decrease[46,51–53] • Validated primers[46,53]	—
BRCA2	• 68% of protein homology • Mutations increased risk of mammary tumour • Increased malignancy when expression increased[46,48,52,54,55] • Validated primers[46]	• Its loss triggers p53 mutations[56]
Chk1[a]	• Inhibition suppresses proliferation[57]	
Chk2[a]	• Mutation in cancer[58]	
PTEN	• Lack of or reduced expression correlated with malignancy[29,59]	
PCNA	• Increased in proliferating tumours[60]	
p21	• Overexpressed in tumour[24,61]	
p27	• Loss is related to malignancy[24,61,62]	
Cyclin A	• Overexpression correlated with carcinogenesis[23]	

[a]These proteins have not been deeply studied in dogs. For TopBP1, p53, Rad51, BRCA1, and BRCA2, studies that have validated antibody reagents for protein detection, or PCR primers for mRNA quantification, with appropriate positive and negative controls are indicated.

BRCA1 and BRCA2 are tumour suppressors discovered in the early 1990s as genes conferring breast cancer susceptibility.[70,71] Their principal role is to maintain genomic stability by promoting the error-free repair of DNA double strand breaks (DSBs) by HR.[22] Although BRCA1 and BRCA2 were initially described as breast cancer predisposition genes, and germ-line mutations in either gene markedly increase the risk of this type of cancer in affected individuals, it is known that inherited or sporadic mutations in BRCA1 and BRCA2 can also contribute to the development of other cancers, for example, ovarian cancer, pancreatic cancer, melanoma, or even prostate and breast cancer in men.[72] BRCA1 and BRCA2 were also reported to play a role in some other genetic syndromes such as Fanconi anaemia.[73]

The important role of these proteins in genomic stability is related to their influence on DNA repair, replication, and transcription.[56] BRCA1 is a multi-functional protein that plays multiple regulatory roles through interactions with other important components involved in each of these processes. Thus, BRCA1 interacts with different DDR proteins such as TopBP1[33,74] or the MRN complex to impede its binding to DNA, it co-localizes with γH2AX after DNA damage, controls the activity of c-Abl (a protein kinase involved in apoptosis), and is hyperphosphorylated by DDR kinases in response to DNA damage.[22,56] Interestingly, it was shown to have a role in G2/M checkpoint by activating Chk1.[75] BRCA1 possess a highly conserved RING domain in its structure,[53] through which it gains its E3 ubiquitin ligase activity.[76] By means of its C-terminus, BRCA1 is capable of binding some elements of the transcriptional machinery (RNA polymerase II or helicase A), which explains its role in transcription control.[56]

BRCA2 is a large protein that contains eight clustered BRC motifs that are highly conserved in mammalian species. In fact, the BRC motifs constitute the regions of BRCA2 that are the most highly conserved between humans and dogs, with 70% identity.[77] In addition, sequence analysis of germline BRCA2 mutations associated with familial breast and ovarian cancer showed that these occur most frequently in exon 11 (where the BRC motifs are located).[78]

BRCA1 has two roles in HR repair (Figure 2). First, it promotes end resection at the damage site by inhibiting the activity of 53BP1 and consequently the NHEJ pathway.[74,79] Second, it promotes Rad51 to replace RPA bound to ssDNA generated by strand resection. To do this, BRCA1 acts in conjunction with BRCA2, which binds Rad51 and promotes its translocation from the cytoplasm to the nucleus.[80] BRCA1 forms a complex with PALB2 that acts as a link between BRCA1 and BRCA2, forming the BRCA1-PALB2-BRCA2-Rad51 foci at the sites of DNA damage that are needed to start HR repair.[79]

BRCA2's primary role in HR is through its interaction with Rad51 (more information about this interaction is provided in the section on Rad51), to regulate the subcellular localization of Rad51 (Figure 2).[46,50,80] BRCA2 binds Rad51 from its BRC repeats in exon 11 and from its COOH-terminal domain encoded by exon 27 (both exons are highly conserved regions). The importance of the BRCA2-Rad51 interaction for genomic stability was supported by a study of mutant mice bearing a deletion of BRCA2 in exon 27, which exhibited a high incidence of spontaneous cancer.[81] HR also seems to be controlled by p53 interaction with BRCA2, where p53 binds to the BRCA2-Rad51 complex to repress HR. It is not clear yet if this suppression happens because p53 disrupts formation of the BRCA2-Rad51 complex or because p53 prevents binding of the complex to DNA.[82] It is known that BRCA2 deficiency results in chromosome instability, which is

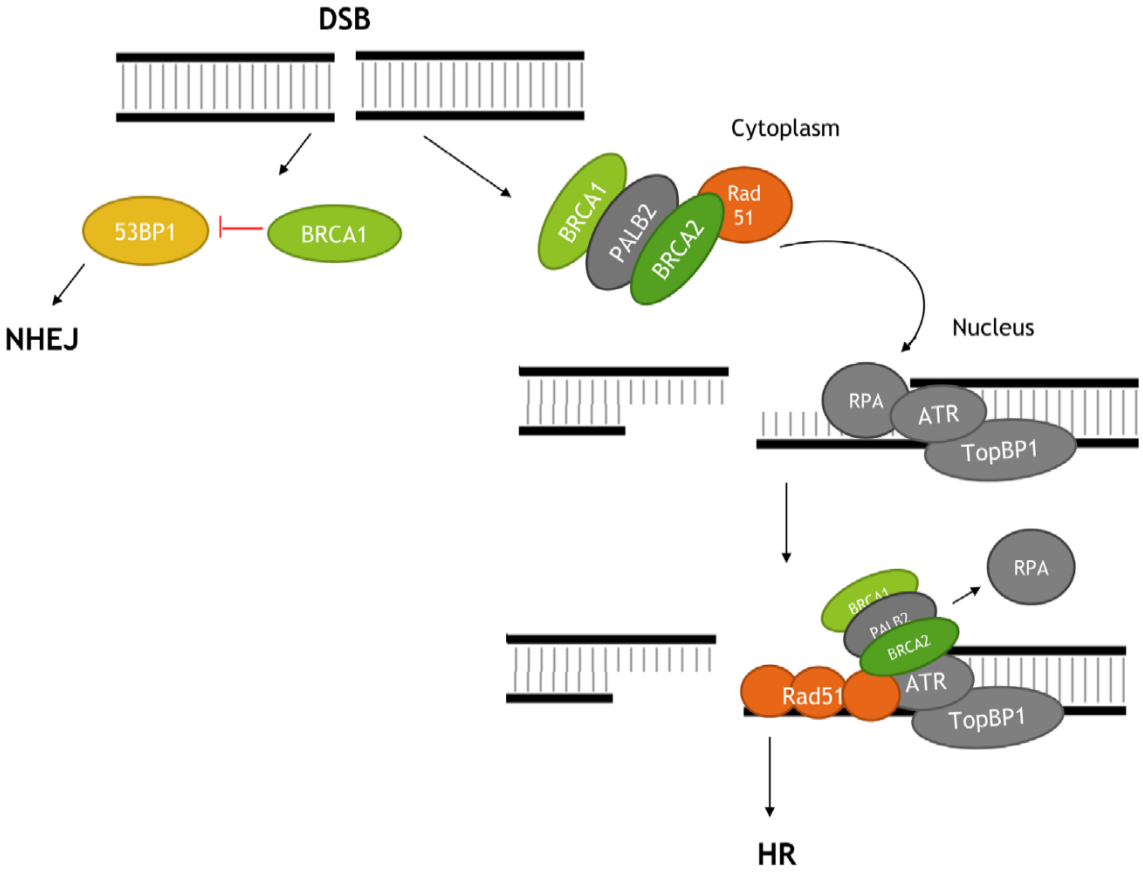

FIGURE 2 The role of BRCA1/2 in DNA repair via homologous recombination. After a DNA lesion occurs, sensor proteins attract 53BP1 to start the NHEJ. However, when BRCA1 is recruited to the DNA lesion sites, it inhibits 53BP1-TopBP1 interactions and promotes HR pathway instead. BCRA1 helps in the recruitment of Rad51. Then Rad51 forms a complex with PALB2 and BRCA2, and the complex is relocated from the cytoplasm to the nucleus. There, Rad51 binds DNA lesion site by interacting with RPA and replacing it. Finally, Rad51 forms nucleoprotein filaments on ssDNA that create a more stable platform for the repair machinery

related to the poorly understood role of BRCA2 in stabilization of stalled replication forks.[83]

Studies in which BRCA genes were depleted or produced defective proteins, identified the most important roles of these proteins in DNA repair system. The loss of BRCA1 not only results in defective repair, but it also boosts apoptosis, genetic instability, and tumorigenesis.[84] BRCA2 deficiency results in elevated levels of chromosome breaks, probably due to its role in stabilization of stalled replication forks, but more interesting is that its loss triggers tp53 mutations, which can contribute to cancer progression.[56]

The canine BRCA1 gene was described for the first time in a comparative study in 1996,[53] when BRCA1 genes from human, mouse and dog were sequenced and compared. This revealed an 84% identity of the canine gene with its human homologue.[53] Subsequent sequencing of the canine BRCA2 gene showed a greater homology between humans and dogs than between humans and mice, with a 76% cDNA sequence homology and 68% BRCA2 protein homology.[48] Due to the important role of BRCA1 and BRCA2 in breast cancer in humans, most of the studies in dogs focused on mammary tumours. A study on gene expression showed that decreased BRCA2 levels are related to tumour development.[85] These experiments identified different splicing variants of BRCA2, one of which could contribute to a reduction in BRCA2 protein levels. This particular splice variant encodes a form of BRCA2 that is unable to interact with a stabilizer protein, DSS1 (deleted in split hand/split foot). Thus, this transcript encodes an unstable and dysfunctional isoform of BRCA2 protein.[85] Further studies of single nucleotide polymorphisms (SNPs) in canine BRCA genes have shown that variants of both BRCA1 and BRCA2 genes can be associated with canine mammary tumour (CMT) development as occurs in humans, whilst other SNPs appear only in tumour samples and never in control normal tissue.[52,86,87]

The canine homologues of BRCA genes are also linked to spontaneous cancer and metastases.[88] Nieto and colleagues[51] analysed BRCA1 expression levels in mammary dysplasia and tumours. They showed that in normal mammary gland the expression of BRCA1 was exclusively nuclear, whereas in a neoplastic gland BRCA1 protein was also observed in the cytoplasm. The relationship between BRCA1 expression and malignancy was also analysed and, as expected, malignancy increased when the levels of BRCA1 decreased.[51] In contrast, another study demonstrated that although BRCA1 levels did not correlate with malignancy, overexpression of RAD51 and BRCA2 was observed in lymph node metastases.[46] The association of high

BRCA2-Rad51 expression with malignancy likely relates to their function in DNA repair by HR.

The clinical and molecular similarities existing between the mammary tumours in canines and humans, makes the dog a useful model to study mammary cancer.[89] The observed similarities also apply to the relationship between mammary tumours and DNA damage because mammary tumour development in dogs and women has been associated with deregulation of BRCA1/2 gene function.[85,90,91] As dogs have a long history of inbreeding with low levels of genetic variation, it has been suggested that canine mammary tumour (CMT) development in a single breed should have a more defined homogenous origin compared with human, which could permit identification of breed specific CMT risk factors.[92] Therefore, the breed predisposition to CMT has been used to study BRCA2 mutations.[80,93] One study conducted in Sweden regarding the English Springer Spaniel breed, which has the highest incidence of CMT in that country, showed that mutations in BRCA1 or BRCA2 increased the risk of the cancer development fourfold. In addition, whereas the frequency of BRCA1 mutations rate seemed to be higher in malignant samples, the frequency of BRCA2 mutations was similar in malignant and benign tumour samples.[52] However, this study did not assess the functional effects of BRCA1/2 gene mutations and further investigations are required to understand the molecular mechanisms responsible for the observed phenomenon in this breed. When considering the aetiology and pathogenesis of CMT, or even genetic/breed predispositions, it must not be forgotten that steroid hormones (oestrogen and progesterone) and their receptors play significant roles in mammary tumour development.[94] As with early pregnancy/oophorectomy in women, early spaying in dogs is linked with lower disease incidence,[95] therefore, any research describing the incidence of CMT must be considered in relation to the current ovariohysterectomy trends in a given country.

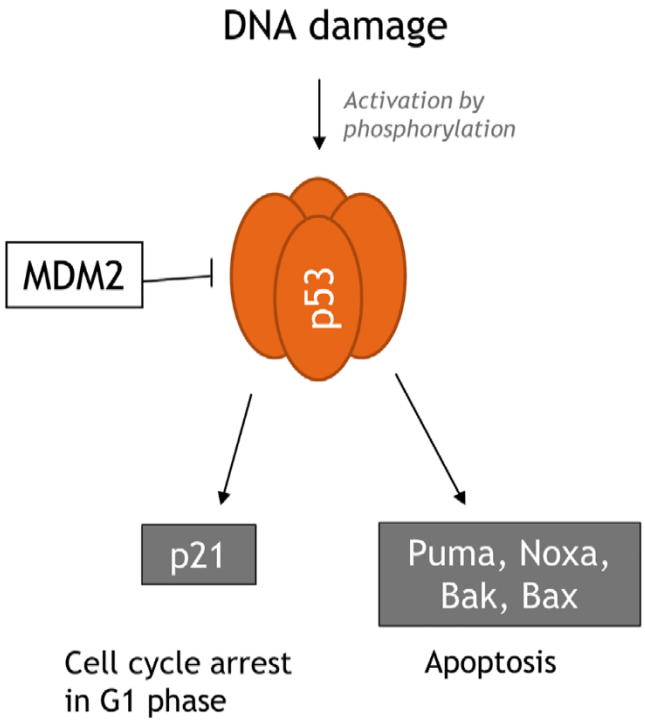

FIGURE 3 Function of p53 in response to DNA damage. After DNA damage p53 is phosphorylated and consequently activated. p53 triggers the cell cycle arrest by inducing expression of p21, which binds and inhibits the activity of cyclin/CDK complexes, such as cyclin E/CDK2 and cyclin A/CDK2 that are necessary for the transition from G1 into S-phase of the cell cycle. p53 can also trigger apoptosis by inducing the expression of other target proteins such as Puma, Noxa, Bax, and Bak, which are pro-apoptotic proteins.[101] A major regulator of p53 activity is a ubiquitin ligase MDM2, which ubiquitinates and targets p53 for rapid degradation via the proteasome in the absence of cellular stress

2.2 | p53

p53 was discovered in 1979 through studies of SV40 antigen T oncoproteins and is widely known as the "guardian of the genome" due to its function in maintaining genomic integrity.[96,97] In its different roles the protein: (a) Acts as a transcription factor for different genes to regulate the cell cycle (Figure 3); (b) Triggers apoptosis in response to oncogene activation or cell proliferation under stress conditions; (c) Silences repetitive sequences of DNA that could be cancer promoters; and (d) Helps in prevention of bacterial and viral infections acting as a part of the innate immune system.[14] It is also involved in anti-angiogenesis and autophagy.[98–101]

The activity of p53 is regulated by multiple post-translational modifications. Site-specific phosphorylation catalysed by multiple protein kinases, including ATM and Chk2, can modulate p53 stability and gene-regulatory functions in diverse ways, depending on the site of modification. p53 is degraded by proteasomes after being ubiquitinated by MDM2, a ubiquitin ligase.[102,103]

After more than 40 years of research on p53, its importance in cancer is clear and a loss of wild type tp53 expression or tumour suppressor function is frequently linked to malignant transformation.[41] In addition, when tp53 is mutated, it can gain oncogenic functions and facilitate cell transformation and tumorigenesis.[38] "Gain of function" mutations can alter p53 molecular functions in complex ways: (a) Through a change of protein function by disruption of protein–protein interactions (e.g., disruption of ATM-MRN complex formation by capturing MRN through interaction with MRE11); (b) By increasing or inhibiting the activity of certain transcription factors (e.g., mutated p53 can bind to p53-related p63/p73 proteins, forming aggregates and preventing their activation in response to DNA damage and consequently suppressing p63/p73-dependent transcription of apoptotic or growth-inhibitory genes such as Bax or p21); (c) Changes in binding properties to other DNA regions not related to gene expression (e.g., matrix elements that participate in chromatin remodelling).[104] Aberrations of p53 expression or function appear in more than 50% of human cancers, and 90% of p53 mutations occur in so called hot-spots, often in the DNA binding domain encoded by exons 5–8.[37]

In veterinary medicine tp53 is an important research topic, and as in humans, it has a role in malignant transformation when mutated.[105]

Veldhoen and Milner isolated the canine version of p53 in 1998 and presented its homology analysis with other mammalian species. Canine p53 protein shares 86.3% homology with feline, 72.3% with murine, and 78.4% with human protein. The central core of the protein, the DNA binding region, together with regions at the C- and N-termini are highly conserved between species[106] and these are also the regions prone to mutations, in both humans and dogs.[39]

Several studies have documented altered p53 expression in different types of veterinary cancers. Takeda et al. analysed mammary tumours, squamous carcinomas and basal cell tumours in dogs and cats documenting p53 protein expression levels by immunohistochemistry.[23,107] Detectable expression of p53 protein by this method was found in 24.6% and 16.3% of mammary tissue samples in dogs and cats respectively, with a higher percentage of expression in the malignant versus benign samples, indicating that p53 overexpression can be related to malignancy. In the case of squamous carcinoma samples, the percentage of cells that expressed p53 was high, 37.5% in dogs and 40% in cats, suggesting an association between high levels of the protein and tumorigenesis.[107] However, no p53 overexpression was detected in the basal cell tumour samples.[23,107]

Another study analysed 170 samples of CMT which were classified into three different groups depending on the malignancy grade.[44] The first analysis was a histological classification. The authors compared the histological grade with the malignancy level of the tumour, observing that higher histological grade was significantly correlated with higher malignancy. Then, sections of each CMT sample were stained with a validated antibody against p53. They observed that p53 was detectably expressed only in a minority of samples, 8 out of 170. A correlation between p53 expression and higher histological grade was observed; but there was no correlation between p53 and malignancy. The conclusion was that p53 expression is related to proliferation of the tumour.[44]

In a more recent study, 35 samples of intestinal cancer were analysed by immunohistochemistry. Histopathology analysis showed that 20 of them were malignant tumours, and the remaining 15 were benign. Interestingly, the malignant samples expressed higher levels of p53 compared to the benign ones. Again, in this study, a validated antibody was used to detect p53, and we can add that we have tested the same antibody and observed p53 expression by western blot in canine cell extracts (BHS and AP, unpublished results). Furthermore, sequencing analysis revealed that 3 of the malignant tissues analysed carried a tp53 mutation.[108]

Another immunohistochemistry analysis performed by Kumaraguruparan and colleagues involved 30 samples of mammary tumours of dogs and humans.[60] The study compared the expression level of p53 and Bcl-2 (an antiapoptotic protein) in tumour and adjacent non-tumour tissues. Both proteins were found to be overexpressed in the tumour as compared with the adjacent normal tissue, 78% and 75% of the samples presented elevated levels of Bcl-2 expression in tumour tissue in humans and dogs respectively, and 75% and 73% of the samples in the case of p53.[60] The authors concluded that the overexpression of both proteins denoted an apoptosis-resistant phenotype in canine and human cells. This finding paved a path for a concept in which CMT can be used as a model for breast cancer study in humans. A more specific analysis on the mutational status of canine p53 protein and mutations in exons 5–8 of tp53 in 20 mammary canine tumours (12 malignant and 8 benign)[109] confirmed mutations in 33% of the examined tumour samples. The malignant tumours contained four missense and two nonsense mutations, while in the benign tissues two missense and one silent mutation were detected. Five out of six missense mutations were located in the highly conserved regions corresponding to the DNA-binding domain in humans. These regions are often called "hot-spots". Researchers did a follow-up of the dogs and found that four of them suffered from tumour recurrence after surgery and died. Interestingly, three out of the four deceased dogs exhibited mutant tp53.[109]

Another study examined p53 in multicentric lymphoma in 28 dogs.[110] A total of 19 B-cell and 9 T-cell type lymphomas were assessed, and found to show differential expression of p53, with higher levels in T-cell than B-cell lymphoma as has also been observed in humans. Perhaps surprisingly, levels of p53 expression were similar in all samples within each tumour type, although elevated p53 expression is also rare in human lymphoma.[110] Even though tp53 seems not to be overexpressed in lymphoma, it can be used as a prognostic factor as demonstrated by the genetic study of Koshino et al.[111] They performed a PCR SSPC (single-strand conformational polymorphism) to analyse tp53 mutation in 43 dogs with high-grade lymphoma before and after treatment. They found that only 16% (7 out of 43 dogs) presented a tp53 mutation. After the chemotherapy, 88% of the non-mutation cases responded well to the treatment, versus only 33% of the mutated cases, meaning that tp53 mutations had a negative prognostic significance.

tp53 mutation can also be inherited as in the human multi-cancer susceptibility Li-Fraumeni syndrome.[40] Veldhoen and colleagues investigated tp53 mutations in eight canine lymphomas, comparing tumour and non-tumour tissue. Out of eight dogs only three expressed a mutant tp53, and interestingly one of them presented a heterozygous mutation (tp53+/−) both in the tumour and in normal somatic tissue, indicative of germline transmission. This study was the first evidence of heritable tp53 mutations in dogs.[42] Another study focusing on germline mutations of tp53 involved 10 dogs with cancer. Three of the animals presented a tp53 mutation, including one with heterozygous mutation in normal somatic tissues, confirming heritability of tp53 mutations.[43] Germline mutations of tp53 were also found in multicancer-like syndrome and were suspected to be the cause of the tumour.[58] The mutations analysed in the study were those of tp53 and Chk2, where the latter were associated with failure in G2 arrest and genome instability.

A recent innovative study reanalyzed all the data from the 684 whole genome and exome sequences of canine tumours available to date, in order to quantify tumour mutational burden.[112] The authors performed a comparison between tumour and normal samples from each tumour type analysed, a breed validation in order to detect germline mutations associated to a specific breed, and finally, a human-dog comparison. They observed that there is a relation between somatic mutations and the type of the tumour; T-cell

lymphoma, osteosarcoma, oral melanoma and hemangiosarcoma presented the highest tumour mutational burden. The analysis also showed that the mutation burden was similar among breeds for a given tumour type, meaning that variation is primarily tumour type-specific rather than related to breed. The overall tumour mutational burden values were slightly lower in dogs compared to humans. Another interesting fact is that the tumour mutational burden is correlated with tp53 mutations. tp53 mutation have been found in 16.7% of the analysed canine tumours, and it was shown that tp53 mutations were preferentially present in tumours bearing a relatively higher tumour mutational burden. In the analysis comparing breeds, tp53 mutations seems to be more frequent in Golden Retrievers, Maltese or Rottweiler, compared to others. However similar oncogenic pathway alterations were observed in canine and human cancers of a given type, suggesting that the evolution of cancer is similar in dogs and humans.[112]

Many immunohistochemistry analyses have been done to study p53, but there is the potential for conflict regarding the reliability of this technique when it comes to study of mutations of tp53. Strong immuno-expression of p53 could represent accumulation of non-functional but stable mutant protein, whereas absence of detectable expression could be considered incorrectly as the normal, vanishingly low levels of wildtype tp53, when actually p53 expression is truly absent because of missense mutations.[44] Despite this caveat, the results obtained by immunohistochemistry to detect tp53 mutations discussed in the following studies,[44,60,110] correlate with results obtained by PCR[108,111] and sequencing analysis.[112] All come to the same conclusion: p53 mutation can be used as a prognostic factor that its related to proliferation and malignancy of tumours. The novel technique of CRISPR/Cas9 has been used to developed a tp53 knockout canine cell line,[113] which will mark a milestone in p53 study in canine cancer. In sum, the results of various studies clearly indicate that p53 plays a key role in human and animal carcinogenesis. Both the structure and function of this protein (also in its mutated form) showed considerable inter-species homology.

2.3 | TopBP1

TopBP1 is a protein with multiple roles in the DDR. It encompasses eight hydrophobic multiple protein–protein interaction domains similar to BRCT (BRCA1 carboxyl terminal), which make it interesting due to the structural similarities to BRCA1. In fact, TopBP1 and BRCA1 share 35% of sequence homology.[33,35] To achieve its diverse functions, TopBP1 interacts with and regulates the activity of a wide range of different DDR proteins (e.g., BRCA1, 53BP1, p53, MDC1, ATR, BACH1 (BRCA1-associated C-terminal helicase), RPA/RAD51, RAD9 (Rad9 Checkpoint clamp component of 9-1-1 clamp: Rad9-Hus1-Rad1) by binding through BRCT domains to phosphorylated sites within these partner proteins (Figure 4).[25,36,114,115] The domain of TopBP1 which interacts with and activates ATR (the ATR Activation Domain, AAD) is distinct from the eight BRCTs domains.[116] Importantly, TopBP1 binds not only to multiple interacting partner proteins but also to DNA.[25]

TopBP1 is also involved in HR activity. BRCA2 recruits Rad51 to the RPA-coated ssDNA, but TopBP1 is needed for the formation of Rad51 foci. The mechanism is not clear but available data suggest that BCRT 7/8 domains are essential for filament formation.[117] Another way of HR regulation is through the interaction of TopBP1 with 53BP1 and BRCA1. When both proteins bind to TopBP1, BRCA1 inhibits 53BP1 and resection of DNA ends can occur. In cancer cells without BRCA1, the TopBP1-53BP1 interaction is stabilized and impairs HR, which is likely to result in genomic instability.[74] The authors suggest that the interaction between BRCA1 and TopBP1 is promoted by ATR, which promotes HR under replication stress conditions.

Another interesting function of TopBP1 is the regulation of p53 protein. The interaction between BRCT 7/8 of TopBP1 with DBD of p53 inhibits p53 activation. In this way, p53 cannot induce cell cycle arrest or apoptosis, and damaged cells continue to survive and replicate with potential for malignant transformation.[36]

Expression of TopBP1 is expected in nucleus, as it is a protein involved in replication and DDR signalling. However, in breast cancer, TopBP1 expression has also been observed in the cytoplasm compared to normal breast tissue.[33,118] A histological study on the expression level of TopBP1 in breast samples from 12 healthy human patients and 61 carcinomas[33] showed that both overexpression and downregulation of the protein were related to cancer. TopBP1 occurred not only in nucleus but also in cytoplasm in cancer cells, suggesting that this mis-localization could be related to malignancy. TopBP1 overexpression also affects p53 function. When TopBP1 is overexpressed, the G1/S phase checkpoint and apoptosis control mediated by p53 is inhibited, what promotes cancer development.[36] TopBP1 overexpression could be caused by EDD (E3 identified by differential display) protein, a ubiquitin conjugating the enzyme that controls the localization of TopBP1, which is frequently altered in different types of cancers.[119,120] A schematic description of TopBP1 protein interactions is shown in Figure 4.

The importance of TopBP1 in veterinary medicine is a contemporary and interesting research topic. Immunochemistry studies of TopBP1 in dogs and cats with mammary cancer,[34,35] confirmed its expression in all samples but the reaction with the polyclonal antibody against the human protein was much greater in the malignant tissues.[34] As already mentioned, TopBP1 appears in the cytoplasm of malignant but not of normal cells. The pattern of TopBP1 staining in samples for both humans and dogs was similar. In both cases, normal and benign tissues showed nuclear TopBP1, whereas in malignant tissue both nuclear and cytoplasmic staining was observed. In both studies, the incidence of aberrant expression of TopBP1 was found to be statistically significant in malignant samples compared to normal and benign tissue.[33,34] Based on these results, it can be concluded that TopBP1 protein is similar in structure in humans, dogs and cats (cross reaction with human antibody) and probably performs similar functions and undergoes similar changes in canine, feline and human cancers: its overexpression and cytoplasmic localization is related to increased malignancy of mammary tumours.

The role of TopBP1 in canine cancer is probably as significant as in human but thus far relatively little research has been performed on

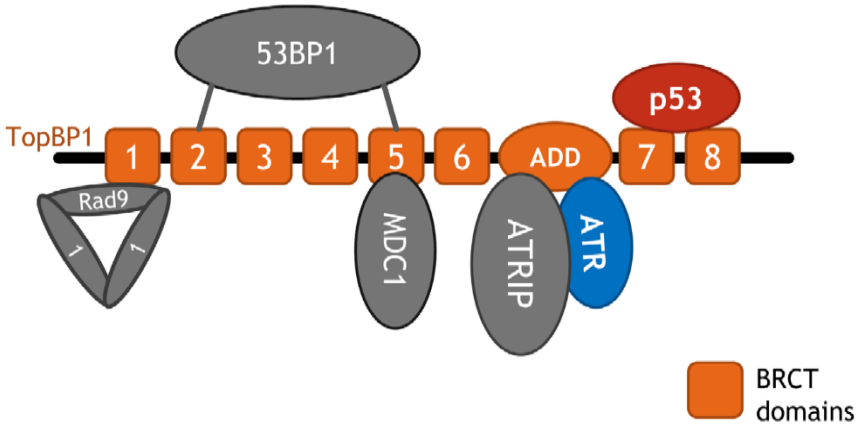

FIGURE 4 TopBP1 interactions. TopBP1 contains 8 BRCT domains through which it binds different DDR components, and one AAD through which it interacts with ATR kinase. After DNA damage, MDC1 is recruited to the damage site.[114] It binds γH2AX at serine 139 and helps in the recruitment of MRN complex.[121] It also binds the 5th BRCT domain of TopBP1, which is recruited to this DNA damage site by interaction with MRN and Rad9.[122] ATR is recruited to the RPA-coated ssDNA, but it is not enough to be activated. TopBP1 interacts with ATR through its ADD domain helping ATR activation in a ATRIP dependent manner.[121,123] This is how TopBP1 participates in the activation of ATR-Chk1 pathway as a response to DNA damage.[124,125] Interestingly, it was shown that 53BP1 can bind TopBP1 along with Rad9, and cooperate in the activation of ATR to control G1/S checkpoint[126]

this subject. Even though TopBP1 has been proved to be related with malignancy, it is not simply a proliferation marker. The previous cited studies compare TopBP1 expression with Ki67,[33,34] which is a nuclear protein frequently used as a marker of proliferation in cancer.[127] Immunohistochemistry staining showed that TopBP1 was expressed in all benign and malignant samples, but only samples expressing Ki67 were proliferating tumours.[33,34] More research is needed both on the structure and function of TopBP1 in animal cancer.

2.4 | Rad51

Rad51 is a recombinase of the RecA family of highly conserved proteins that share a common protein fold in their catalytic domain. All members are DNA-dependent ATPases and can create nucleoprotein filaments on DNA that activate the catalytic activity of the DNA bound proteins.[128] Simultaneously, as a component of the DDR, Rad51 plays a central role in HR pathway. The principal function of Rad51 is first to stabilize a DNA chain broken due to direct damage or replication errors, and second, to recognize homologous DNA present in a sister chromatid to start pairing and strand exchange.[128] The requirement for a sister chromatid for DSB repair via HR probably explains why high levels of Rad51 are expressed in cells in the S and S/G2 phases. Rad51 is further stabilized upon formation of the nucleoprotein filament, which can show two conformations (open and closed), making it flexible and dynamic. The regulation of Rad51 activity in DDR is mediated by its interaction with other important proteins of this system. p53, by binding to Rad51, can inhibit strand DNA exchanges and regression of stalled replication forks.[47] BRCA2 is directly related to the regulation and coordination of HR repair mediated by binding Rad51 (Figure 2). Specifically, BRCA2 binds Rad51 through the repeated BRC domains. This way it helps Rad51 both to relocate from the cytoplasm to the nucleus under the conditions of damage and to load Rad51 onto DNA to form the nucleoprotein filament. During nucleoprotein filament formation BRCA2 helps Rad51 to displace RPA from single stranded DNA.[129,130] Other important interactions of Rad51 involve protein kinases. For example, ATM activates c-Abl which then regulates Rad51 activity. In this context, it is interesting that the oncogenic version of c-Abl, BCR-ABL fusion kinase (expressed in chronic myelogenous leukaemia [CML]) also phosphorylates Rad51.

The level of Rad51 expression is generally higher in human tumour cell lines and in primary tumours than in normal tissues and it is associated with genomic instability and resistance to chemo- and radiotherapies.[131] Also, Rad51 shows sequence polymorphism in different tumours.[132] There are two possible explanations for the elevated Rad51 levels observed in tumour cells. The first is that increased Rad51 expression contributes to malignant transformation, and the second that it reflects the higher proliferation rates of malignant cells, since Rad51 levels are maximal during S and S/G2 under normal conditions.[47]

The canine RAD51 gene cloned and sequenced in 2001 showed a very high homology with human and murine genes.[48] As murine Rad51 was known to interact with BRCA2 through its C-terminus and not only through BRC domains as in humans, the canine Rad51 was tested for its capacity to bind both, the BRC domains and BRCA2 C-terminus. It was found that a deletion of BRCA2 C-terminal domain could increase genomic instability and predispose to cancers in dogs (see BRCA2).[50] This study also demonstrated that Rad51 bound strongly to BRC 1, 2 and 4, moderately strongly to BRC 8, and weakly to BRC 3, 5 and 7, while no interaction was observed with BRC 6 domain. Tests were also performed to check the importance of C-terminus in the strength of Rad51-BRCA2 interaction, where surprisingly, BRC 3, 5 and 8 deletions resulted in stronger binding. Based on these experiments, the authors hypothesized that the strength and correct arrangement of those domains could be related to HR repair

proficiency. In other experiments, the effects of BRC3 and BRC4 polymorphisms on the interaction with Rad51 were investigated. To that end, the BRC domains of BRCA2 gene were sequenced from 236 dogs of five different breeds, three with a high risk of CMT, one with a low risk, and one previously not researched. Two polymorphisms were detected, T1425P and K1435R (described before). The first polymorphism did not appear in Labrador retriever breed, which is interesting, as this breed was the one with low risk of CMT. However, it appeared in 26 other dogs (two were homozygotes). The second polymorphism appeared in all breeds, in 96 dogs (26 homozygotes), but was predominant in Chihuahuas, one of the breed with high risk of CMT.[55,133] Another study analysed the relationship between RAD51 mutations (A209S and T225S) and PALB2 protein. A two-hybrid assay indicated that the interaction of mutant type Rad51 with PALB2 was weaker than that of wild type Rad51. The conclusion was that RAD51 mutations affected oligomerization of the protein and that this could be the reason for its attenuated interaction with PALB2, which could promote cancer in dogs.[49]

Ozmen et al. investigated genetic variations in exons 11 and 27 of BRCA2 gene (a region corresponding to Rad51 binding site), and variations in exons 6 to 9 from RAD51 gene (corresponding to PALB2 and BRCA2 interaction regions) in canine mammary gland. The most prevalent polymorphism found was T1425P in BRC3. This polymorphism was also found by Ochiai et al. and suggested to be associated with CMT.[55] In the second part of the study, a total of nine variations of canine RAD51 gene in exon 7, exon 8, intron 7 and intron 8 were identified. Four of them were non-synonymous and altered the protein sequence. Interestingly, all these potentially significant variations were located in the region binding PALB2 and none in that binding BRCA2.[80,134]

Other genetic variations associated with CMT consist of three single nucleotide polymorphisms (SNP), two in RAD51 gene and one in STK11 (serine/threonine kinase involved in cell growth control). This analysis included 373 dogs of which 212 suffered from CMT. The incidence of the SNPs was as follows: 50.9% in dogs with CMT and 35.4% in healthy individuals for RAD51-SNP1, 42% in CMT and 31.9% in healthy individuals for RAD51-SNP2, and 40.3% in CMT and 26.7% in healthy individuals for STK11. The SNPs in both genes were located in the intronic regions, but neither association was significant enough to provide strong evidence for a causal role in tumour development.[135]

Due to the important functional connections between BRCA2 and Rad51, all studies concerning Rad51 in dogs are carried out in animals suffering from CMT. Some data suggest a possible relation between RAD51 alterations, genomic instability, and predisposition to CMT, but so far RAD51 variations have not been confirmed as a primary cause of cancer.

3 | CLINICAL ASPECTS OF DNA DAMAGE RESEARCH IN CANCER IN DOGS

Research on this issue is important both because of the role of DNA damage in the pathogenesis of various types of cancer and because DDR disorders are a potential therapeutic target for cancer therapy. Examples of how the assessment of DNA damage can help in the clinical treatment of cancer in dogs is shown by the recent results of a number of scientific studies.

It is well known that some dogs breeds are predisposed to lymphoma[136–139] and genetic disease studies in dogs are especially powerful, due to dogs' relative inbreeding and the associated lack of genetic heterogeneity.[140,141] It was hypothesized that lymphoma susceptibility may be associated to breed-related increase in DNA damage and a study to evaluate this hypothesis is the research done by Thamm et al. seeking to explain the cause of the higher incidence of lymphoma in Golden retrievers.[142] The subject of the article is a pilot study on 21 Golden retrievers with lymphoma, 20 age-matched healthy Golden retrievers and 20 age-matched healthy mixed-breed dogs, evaluating DNA repair capability following exposure to either ionizing radiation (IR) or the chemical mutagen bleomycin. The research shows inter-individual variation in DNA repair capacity, evaluated in stimulated canine lymphocytes exposed in vitro utilizing the G2 chromosomal radiosensitivity assay to quantify chromatid-type aberrations (gaps and breaks).[143] Surprisingly, the results of the study point to more individual (rather than breed) susceptibility, but at the same time suggests that deficiencies in heritable factors related to DNA repair capabilities may be involved in the development of canine lymphoma. These studies set the stage for larger confirmatory studies, as well as candidate-based approaches to probe specific genetic susceptibility factors.

Another study trying to explain the same genetic/breed predisposition to lymphoma was carried out on boxers.[144] Research aimed to evaluate whether boxer dogs have more endogenous DNA damage in peripheral leukocytes, than age-matched non-boxers, and whether DNA damage is associated with specific Glutathione-S-transferase (GST) alleles. Authors found no difference in leukocyte DNA damage, as measured by the comet assay, between boxers and age-matched non-boxer dogs, nor did they see an association with DNA damage and advancing age within the boxer breed. Observed lack of correlation may be explained by the fact that the experimental set-up was different from that used in the Golden retriever study and relied only on an assessment of spontaneous DNA damage. If the response to induced DNA damage ex vivo in boxers versus non-boxers was measured it might possibly uncover DNA repair defects that are masked in a population with heterogeneous exposures.

Another aspect of the clinical use of DNA damage research is shown in the article describing how fish oil supplementation (1000 mg; containing 232 mg EPA and 136 mg DHA), could affect DNA damage in PBMC of healthy dogs.[145] In this study, also no DNA damage was induced, and the percentage of cells bearing spontaneous DNA damage was assessed using comet assay. The study showed that fish oil supplementation not only does not induce DNA damage in PBMC, but actually reduces it. An interesting continuation of this study seems to be to investigate whether a similar effect of fish oil may be observed in conditions of induction of DNA damage in the previously mentioned Golden Retrievers and Boxers. This could be of key importance for an introduction of fish oils as an adjuvant therapy in the treatment of lymphoma in those breeds where the defective

response to DNA damage is considered to be one of the predisposing causes.

The role of diet in the prevention of certain types of cancer has also been shown in a study using the dog as a model for prostate cancer research.[146] Authors presented the first evidence that prostatic DNA damage measured by comet assay may serve as a functional marker of selenium's anticarcinogenic effect on the prostate. Results suggest that measurement of selenium concentration can provide a non-invasive method for titrating and individualizing optimal selenium intake required for prostate cancer protection.

Another example of the potential clinical usefulness of DNA damage assessment may be a study which aimed to determine whether healthy people and dogs in the same households share urinary exposures to potentially mutagenic chemical carcinogens which can lead to the development of urothelial carcinoma.[147] Although voided urothelial cell yields were inadequate to quantify DNA damage, research showed that healthy humans and pet dogs have shared urinary exposures to known mutagenic chemicals, with significantly higher levels in dogs. Correlation studies between mutagenic chemicals found in urine and their effects on DNA damage induction may provide key information on the pathogenesis of urothelial carcinoma in humans and dogs.

Assessment of the DNA damage may also be used in clinical trials to elucidate the mechanism of action of tested agents. Such an application is presented by Dull et al. in their article describing an immunofluorescence assay that distinguishes between apoptosis and drug-induced DSBs by measuring coexpression of γH2AX and membrane blebbing-associated cleaved caspase 3 (CC3) to indicate apoptosis, and γH2AX in the absence of CC3 blebbing to indicate drug-induced DNA damage.[13] Because the primary pharmacodynamic endpoint for genotoxic agents is induction of markers of DNA damage repair such assays may have broad clinical and preclinical applicability and be of fundamental importance in the development of new therapies in veterinary oncology.

In veterinary medicine, we have more and more reports on preclinical and clinical trials using DNA damage determinations.[148–153] It seems that it is only a matter of time until knowledge about disorders of DNA damage repair in canine cancer cells will be introduced into clinical use to target specific defects with maximally effective therapies. In human medicine, an excellent example of such an approach is the introduction of PARP inhibitors to the treatment of BRCA-dependent breast and ovarian cancer in women.[154–156] Thanks to the dissemination of knowledge about the possibilities of DDR research in dogs, similar targeted therapies could be also introduced in veterinary medicine.

4 | CONCLUSIONS AND FUTURE DIRECTIONS

Cancer is a genetic disease linked to genomic instability in the incipient cancer cell. Failures in DDR systems are likely to contribute to cancer development, which makes them an interesting and important topic in cancer research. The findings we discuss here clearly show similarities between canine and human DDR proteins and justify the use of dogs as valuable models for DDR study. Further studies on the role and significance of key DDR components: BRCA1, BRCA2, p53, TopBP1 and Rad51 proteins in canine tumours will provide the missing information. The importance of such research is significant: if the DDR could be targeted for cancer therapy in dogs, this could aid development of analogous treatments in human oncology.

ACKNOWLEDGEMENTS

The publication financed by the project "UPWR 2.0:international and interdisciplinary programme of development of Wrocław University of Environmental and Life Sciences", co-financed by the European Social Fund under the Operational Program Knowledge Education Development, under contract No. POWR.03.05.00-00-Z062/18 of 4 June 2019 and by the Polish National Agency for Academic Exchange under Grant No. PPI/APM/2019/1/00044/U/00001. David A Gillespie is an Augustín de Betancourt Investigador of the Universidad de La Laguna.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Conceptualization, Aleksandra Pawlak; writing—original draft preparation, Beatriz Hernández-Suárez and Aleksandra Pawlak; writing—review and editing, Aleksandra Pawlak and D.G.; supervision, Aleksandra Pawlak and David A. Gillespie; All authors have read and agreed to the published version of the manuscript.

ORCID

Beatriz Hernández-Suárez https://orcid.org/0000-0002-5377-6609
David A. Gillespie https://orcid.org/0000-0002-6338-0544
Aleksandra Pawlak https://orcid.org/0000-0002-7449-1661

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How to cite this article: Hernández-Suárez B, Gillespie DA, Pawlak A. DNA damage response proteins in canine cancer as potential research targets in comparative oncology. *Vet Comp Oncol*. 2022;1-15. doi:10.1111/vco.12795

6. Foreword to publication 2

As we have previously reviewed in **P1**, besides the importance of DDR in cancer, little is known about it in canine cancer. Especially, there is an important lack of information on one of the most important routes: the ATR-Chk1 pathway.

In the following chapter, we present several results connected with the DDR in a panel of hematopoietic canine cancer cell lines (**P2**), which gives a sneak peek into the important role of those proteins in cancer.

1. The rationale of our research objectives and hypotheses.

Because humans and dogs shared a high percentage of DDR protein homology [26] (protein sequences human-dog alignments attached as **supplementary data in P2**), we consider that the still unknown ATR-Chk1 pathway in dogs may function similarly as it does in humans. However, validation of methodologies and reagents is required to allow the investigation of molecular features of DDR in canine cancer.

The aim of this research was to facilitate the molecular research of DDR in canine cancer by validating different techniques and reagents.

2. The methodology and techniques used in the study.

- Panel of canine lymphoma and leukemia cell lines
- RNA-sequencing of two selected cell lines to check the expression of the DDR mRNA levels
- *In vitro* treatments with 20 µM of etoposide for 2 hours
- Western blot technique to analyze DDR protein levels
- qPCR to analyze mRNA levels
- DNA combing assay to study replication dynamics in the two selected canine cell lines

More details about the techniques are available in Chapter 3.

3. The results obtained in the study

In **P2**, we have done an initial characterization of some of the DDR components (ATR, Claspin, Chk1, Rad51) in canine lymphoma and leukemia cell lines.

The first result presented is a sequencing analysis that showed that the CLBL-1 cell line has highly expressed DDR genes compared to the GL-1 cell line in basal conditions (**figure 2 of P2**).

In our analysis, we focused on the ATR, Claspin, Chk1, and Rad51proteins, due to their important role in DDR and replication. The analysis of those four targets by western blot and qPCR confirmed the obtained information in the sequencing analysis (**figure 3 of P2**). An interesting higher expression of Rad51 was found in CLBL-1 compared to CLB70 and GL-1 cell lines.

The next experiment consisted in treating the cells with etoposide, a chemotherapeutic drug that is used to treat several cancer types [82], and it is known to induce DNA damage

in the cell. Due to its mechanism of action, it was expected to observe an increase in the activated form of Chk1 protein (phosphorylated Chk1) [83], [84]. In our results, we observed that after only 2 hours of 20 μM of etoposide treatment, an increase in phosphorylation levels of Chk1 was observed in the three cell lines tested, being higher in CLBL-1 and CLB70 compared to GL-1 (**figure 4 of P2**). The presence of DNA damage in the cell was confirmed by the increase of the signal of γH2AX.

The intriguing observations of elevated expression of Chk1 and Rad51 after short etoposide treatment led us to the conclusion that those cells may have a kind of replication problem. It is well known that when a cell suffers from replication stress, DDR is activated in order to induce cell cycle arrest to lead replication to finish [85]. Replication stress may cause fork asymmetry which usually ends in fork stalling and collapse that promotes genetic instability [86]. In cancer cells, a decrease in fork speed has been found under conditions of replication or oxidative stress [87]. In order to check this new hypothesis, a DNA combing assay was performed in CLBL-1 and GL-1 cell lines. The results showed that GL-1 replication speed is faster than CLBL-1 (**figure 6 in P2**), which was interesting given the fact that GL-1 duplication rate is slower than the CLBL-1 rate.

The alterations at the protein level described in **P2** signalized the possibility of using ATR, Claspin, Chk1, or Rad51 as possible targets to develop new anticancer therapies for dogs.

4. Conclusion on P2

The validation of the techniques and reagents presented in P2, and the preliminary data obtained in this research, signalized the importance of DDR in canine cancer. These results have demonstrated that DDR components in dogs are similar to humans both morphologically and functionally. Although more research is needed, those results are the first clue to propose the canine lymphoma and leukemia cell lines as models to study the DDR pathway in cancer.

7. Publication 2

P2 - Studying the DNA Damage Response pathway in hematopoietic canine cancer cell lines – a necessary step for finding targets to generate new therapies to treat cancer in dogs

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 16 August 2023
DOI 10.3389/fvets.2023.1227683

OPEN ACCESS

EDITED BY

Monica Sforna,

University of Perugia, Italy

REVIEWED BY

Joy Archer,

University of Cambridge, United Kingdom
Maurice Zandvliet,

Utrecht University, Netherlands

*CORRESPONDENCE

Beatriz Hernández-Suárez
beatriz.hernandez-suarez@upwr.edu.pl

RECEIVED 23 May 2023

ACCEPTED 31 July 2023

PUBLISHED 16 August 2023

CITATION

Hernández-Suárez B, Gillespie DA, Dejnaka E, Kupczyk P, Obmińska-Mrukowicz B and Pawlak A (2023) Studying the DNA damage response pathway in hematopoietic canine cancer cell lines, a necessary step for finding targets to generate new therapies to treat cancer in dogs.

Front. Vet. Sci. 10:1227683.

doi: 10.3389/fvets.2023.1227683

Studying the DNA damage response pathway in hematopoietic canine cancer cell lines, a necessary step for finding targets to generate new therapies to treat cancer in dogs

Beatriz Hernández-Suárez[1]*, David A. Gillespie[2], Ewa Dejnaka[1], Piotr Kupczyk[3], Bożena Obmińska-Mrukowicz[1] and Aleksandra Pawlak[1]

[1]Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences, Wrocław, Poland, [2]Facultad de Medicina, Instituto de Tecnologías Biomédicas, Universidad de La Laguna, Tenerife, Spain, [3]Division of General and Experimental Pathology, Department of Clinical and Experimental Pathology, Faculty of Medicine, Wroclaw Medical University, Wroclaw, Poland

Background: Dogs present a significant opportunity for studies in comparative oncology. However, the study of cancer biology phenomena in canine cells is currently limited by restricted availability of validated antibody reagents and techniques. Here, we provide an initial characterization of the expression and activity of key components of the DNA Damage Response (DDR) in a panel of hematopoietic canine cancer cell lines, with the use of commercially available antibody reagents.

Materials and methods: The techniques used for this validation analysis were western blot, qPCR, and DNA combing assay.

Results: Substantial variations in both the basal expression (ATR, Claspin, Chk1, and Rad51) and agonist-induced activation (p-Chk1) of DDR components were observed in canine cancer cell lines. The expression was stronger in the CLBL-1 (B-cell lymphoma) and CLB70 (B-cell chronic lymphocytic leukemia) cell lines than in the GL-1 (B-cell leukemia) cell line, but the biological significance of these differences requires further investigation. We also validated methodologies for quantifying DNA replication dynamics in hematopoietic canine cancer cell lines, and found that the GL-1 cell line presented a higher replication fork speed than the CLBL-1 cell line, but that both showed a tendency to replication fork asymmetry.

Conclusion: These findings will inform future studies on cancer biology, which will facilitate progress in developing novel anticancer therapies for canine patients. They can also provide new knowledge in human oncology.

KEYWORDS

Chk1, Rad51, ATR, Claspin, lymphoma, leukemia

1. Introduction

Comparative oncology studies cancer across a range of animal species. Thanks to that, it can provide new insights into cancer development and risk factors that may also affect humans. According to the American Veterinary Medical Association, about half of the dogs aged over 10 years will suffer from cancer (1) and in the United States, around 4.2 million dogs are diagnosed with cancer eachyear (2). With this huge number of patients and a shorter life span than humans, the possibility of completing a clinical trial testing new therapies in canine patients is really promising. Due to biological similarities between cancers in humans and dogs, the results of such trials could potentially be extended to human medicine (3).

Several fundamental regulatory cellular processes are frequently altered in cancer. Disturbances in the functioning of the DNA Damage Response (DDR) pathway are often connected with carcinogenesis (4–11) and resistance to genotoxic stress (12–15), but they also present an opportunity to be used as target for anticancer therapies. Such a therapeutic approach includes the use of DDR inhibitors to overcome cell resistance to genotoxic therapies, or documenting variations in the expression of DDR proteins as potential markers of sensitivity to specific therapies in oncological patients (7, 16–18). Thus, there is a need to validate reagents and molecular techniques for use in canine cells, which will facilitate comparative oncology research.

The DDR is one of the pathways whose dysfunction can lead to cancer. ATR and Chk1 comprise the principal DDR pathway available to most cancer cells that lack functional p53, which is found altered in almost 50% of human cancers and has also been reported in a variety of canine cancers (19, 20). The ATR-Chk1-Claspin pathway has been found to be upregulated in cancer cells, as compared with non-cancerous cells in humans (6), therefore its inhibition presents an attractive target for new-generation cancer therapies (18, 21). In normal circumstances, the DDR plays a fundamental role in the regulation of cell cycle progression and DNA replication regulation (Figure 1) (22). For example, after DNA damage or during replication stress, thanks to the activation of various DDR components, it is possible to prevent defective cells from dividing by inducing cell cycle arrest. To this end, ataxia telangiectasia mutated and Rad3-related (ATR) kinase phosphorylates and activates checkpoint kinase 1 (Chk1), which induces cell cycle arrest (23). During this complex process, an important mediator protein called Claspin helps to activate Chk1 (13, 24). While the cell cycle arrest continues, the DDR system cooperates to recruit the repair machinery, including proteins involved in homologous recombination (HR), to repair any DNA damage that has occurred. An important component here is the BRCA1-PALB2-BRCA2 complex, which recruits the recombinase Rad51 to form filaments and bind damaged DNA to form a D-loop structure (two strands of a double strand DNA that are separated by a third strand) (25–27). Rad51 responds to replication stress in three ways: (1) by helping promote fork reversal when DNA polymerase progression on a single-stranded DNA (ssDNA) template is blocked (e.g., by DNA breaks), (2) by protecting the ssDNA ends from being degraded by endonucleases, and (3) by promoting restart of replication fork progression (28).

To facilitate research into the significance of DDR pathway disturbances in cancer, as well as to inform studies on the development of new therapies targeting the DDR in dogs, we conducted a series of experiments on canine lymphoma/leukemia cell lines to assess (1): the expression of transcripts of DDR components by RNA sequencing in two selected canine cancer cell lines (2), the basal expression levels of key proteins involved in the DDR (ATR, Claspin, Chk1, Rad51), together with checking the feasibility of using commercially available antibodies, and (3) the functionality of the DDR pathway in canine model cells by assessing the DDR pathway activation after DNA damage, using the DNA damaging agent etoposide (detection of γH2AX and p-Chk1). Finally, we performed DNA combing assays to assess DNA replication dynamics in canine lymphoma/leukemia cells by directly visualizing replication fork progression rates and replication origin firing.

2. Materials and methods

2.1. Cells and cell culture

A panel of canine lymphoma/leukemia cell lines: CLBL-1 (B-cell lymphoma), CLB70 (B-cell chronic lymphocytic leukemia), and GL-1 (B-cell leukemia) was used in this study. The CLBL-1 cell line was a gift from Barbara Rütgen from the Institute of Immunology, Department of Pathobiology from the University of Vienna (29), the GL-1 cell line was received from Yasuhito Fujino and Hajime Tsujimoto from the Department of Veterinary Internal Medicine at the University of Tokyo (30), and the CLB70 cell line (31) was established with the participation of researchers from our laboratory; the studies involving animals participants were reviewed and approved by the New York Academy of Sciences *Ad Hoc* Committee on Animal Research and were approved by the First Local Committee for Experiments with the Use of Laboratory Animals, Wroclaw, Poland (approval no. 24/2014).

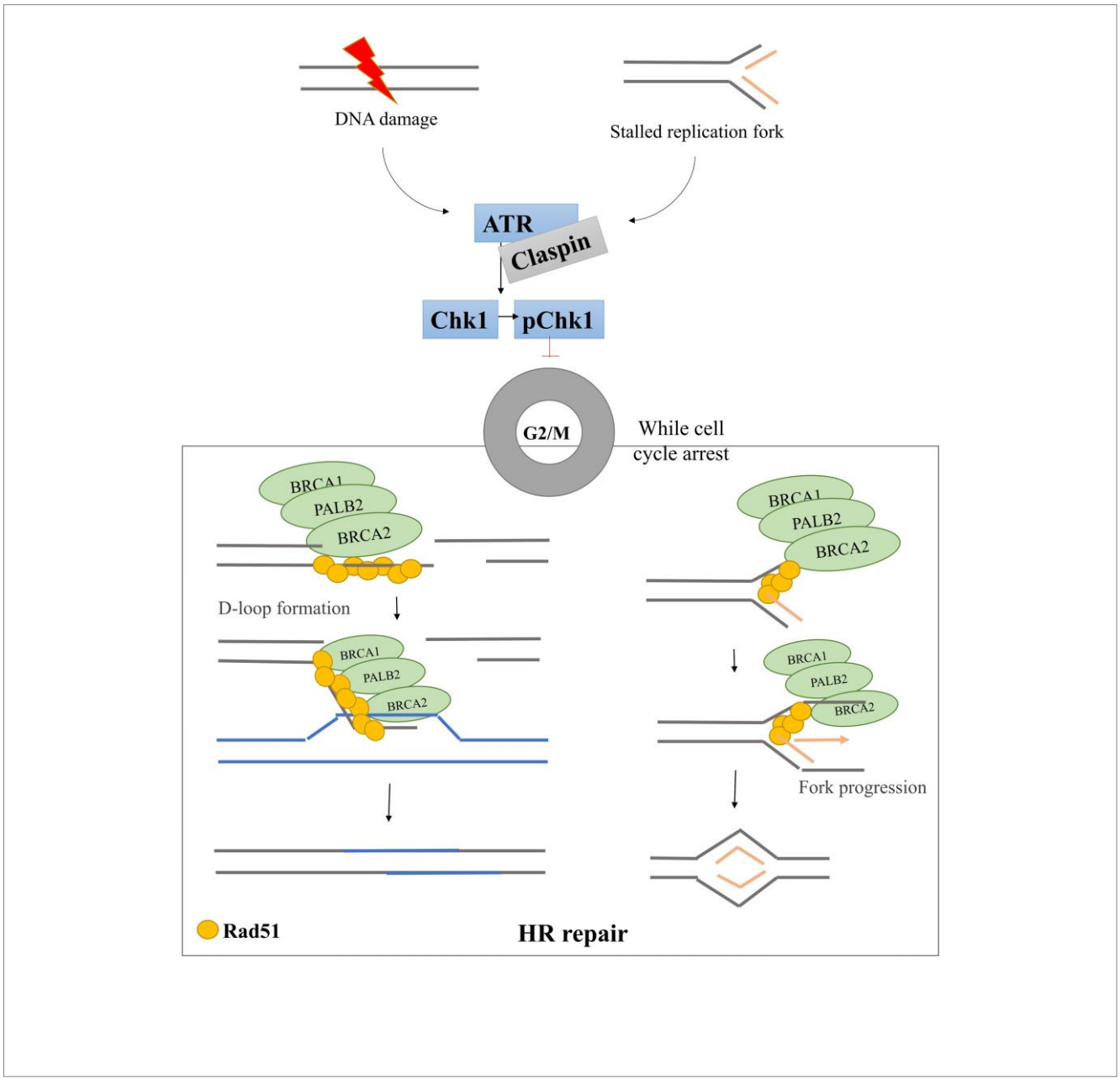
The culture medium RPMI 1640 (Institute of Immunology and Experimental Therapy, Polish Academy of Science, Wrocław, Poland) was used for the CLBL-1 and GL-1 cell lines, and Advanced RPMI (Gibco, Grand Island, NY, United States) for the CLB70 cell line. The culture media were supplemented with 2 mM L-glutamine (Sigma Aldrich, Steinheim, Germany), 100 U/mL of penicillin, 100 μg/mL of streptomycin (Sigma Aldrich, Steinheim, Germany), and 10–20% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, United States). The cells were cultured in an atmosphere of 5% CO_2 and 95% humidified air, at 37°C in 25 cm^2 cell culture flasks (Corning, New York).

2.2. RNA sequencing

RNA was obtained from cultures of the selected cell lines CLBL-1 and GL-1 during unperturbed growth and sequenced by Novogene (United Kingdom). The expected number of Fragments Per Kilobase of transcript sequence per Millions of base pairs sequenced (FPKM) was used to calculate relative gene expression (32). The DDR related GO lists used for the intersection analysis were those presented in Table 1, the gene set information was obtained from the GSEA database (33, 34).

2.3. Treatments

DNA damage was induced by treatment with etoposide (Sigma Aldrich, United States) (a topoisomerase II inhibitor), at 20 μM for 2 h.



Treatment conditions were selected based on literature (35) and previous preliminary (Supplementary Figure S5) analysis.

2.4. Western blot

3 x 10^5cells/mL were cultured in 10 mL of media in a 25 cm^2 culture flask per condition. After 48 h of incubation, the samples were lysed in urea/ SDS buffer (composed of 900 µL of 7 M urea, 25 µL of 5 M NaCl, 25 µL 2 M Tris–HCl (pH = 8), 50 µL 20% SDS), and run in 8–12% bis-tris acrylamide gels prepared using a BioRad Mini-PROTEAN Tetra Vertical Electrophoresis Cell system. The samples were transferred to nitrocellulose membrane using BioRad Mini Trans-Blot® Cell for wet transfer and BioRad Trans-Blot® Turbo™ Transfer System device for semi-dry transfer.

The antibodies used in the study were selected based either on available literature data on reactivity with canine cells (Table 2) or comparison of protein sequence homology, and preliminary test results involving a comparison of the observed bands with the predicted molecular mass (kDa) of the protein of interest. Goat Anti-Mouse Immunoglobulins/HRP (#P0447 at 1:20000 concentration in TBS-T solution) and Goat Anti-Rabbit Immunoglobulins/HRP (#P0448 at 1:10000 concentration in TBS-T solution) were used as secondary antibodies. Both secondary antibodies were from Dako, now part of Agilent (United States, Santa Clara).

2.5. qPCR

2.5.1. Bioinformatic sequence analysis and primer design

The *Canis lupus familiaris* nucleotide accession number sequences for mRNA of the target genes (TGs): Atr, Claspin, and six housekeeping genes (HKGs): Actb, Ppia, and Rplp0 were taken from

TABLE 1 DDR related GO lists.

Gene set	Species
AMUNDSON_DNA_DAMAGE_RESPONSE_TP53	Human
GOBP_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR	Human
GOBP_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR	Mouse
GOBP_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR_RESULTING_IN_CELL_CYCLE_ARREST	Human
GOBP_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR_RESULTING_IN_CELL_CYCLE_ARREST	Mouse
GOBP_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_RESULTING_IN_TRANSCRIPTION	Human
GOBP_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_RESULTING_IN_TRANSCRIPTION	Mouse
GOBP_NEGATIVE_REGULATION_OF_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR	Human
GOBP_NEGATIVE_REGULATION_OF_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR	Mouse
GOBP_POSITIVE_REGULATION_OF_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR	Human
GOBP_POSITIVE_REGULATION_OF_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR	Mouse
GOBP_POSITIVE_REGULATION_OF_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR_RESULTING_IN_TRANSCRIPTION_OF_P21_CLASS_MEDIATOR	Human
GOBP_REGULATION_OF_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR	Human
GOBP_REGULATION_OF_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR	Mouse
GOBP_REGULATION_OF_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR_RESULTING_IN_TRANSCRIPTION_OF_P21_CLASS_MEDIATOR	Human
GOBP_REGULATION_OF_DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR_RESULTING_IN_TRANSCRIPTION_OF_P21_CLASS_MEDIATOR	Mouse
REACTOME_P53_DEPENDENT_G1_DNA_DAMAGE_RESPONSE	Human
REACTOME_SUMOYLATION_OF_DNA_DAMAGE_RESPONSE_AND_REPAIR_PROTEINS	Human
WP_DNA_DAMAGE_RESPONSE	Human
WP_DNA_DAMAGE_RESPONSE_ONLY_ATM_DEPENDENT	Human
WP_MIRNA_REGULATION_OF_DNA_DAMAGE_RESPONSE	Human
WP_MIRNAS_INVOLVED_IN_DNA_DAMAGE_RESPONSE	Human

the Nucleotide Center for Biotechnology Information (NCBI) database (NCBI, United States). The sequences were transferred into the Universal Probe Library. The designed primers and their amplified sequences were additionally verified for their specificity in the Nucleotide Basic Local Alignment Search Tool - Nucleotide-BLAST (NCBI, USA). Gene names, primer sequences for TGs and HKGs, amplicon size, as well their respective gene accession numbers are summarized in Table 3.

2.5.2. RNA isolation and reverse transcription

A total of 1×10^7 cells cultured in 10 mL from the CLBL-1, CLB70, and GL-1 lymphoma cell lines were centrifuged at 300 g, 4° C, and resuspended in 500 µL of TRIzol reagent (Invitrogen, United States). The cells were immediately transferred to a low-temperature freezer and stored in Eppendorf tubes at −80° C for further analysis. Total RNA isolation was performed using Total RNA Zol-Out™ D (A&A Biotechnology, Poland) according to the protocol provided in the isolation kit. Briefly, the cells were removed from the low-temperature freezer and thawed on ice for 30 min. After that, 167 µL of ultra pure molecular biology water (A&A Biotechnology, Poland) were added, and the sample was mixed by inversion. Next, the cells were spun for 10 min at 10000 rpm. The supernatant was mixed with 1 volume of 96–100% ethanol (Stanlab, Poland) and gently agitated until a homogenous solution was obtained. The supernatants from each tube were transferred into new tubes with an RNA membrane binding column and were centrifuged through the column for 1 min at 10000 rpm and 4° C. The columns were rinsed with 700 µL washing A2W buffer for 2 min at 10000 rpm. DNA digestion for 15 min at 37° C in a thermoblock was performed using DNAse according to the manufacturer's protocol. The enzymatic activity of the digestive buffer was inhibited by adding 700 µL of R81 buffer and centrifugation [1 min at 10000 rpm at room temperature (RT)]. The filtrate was collected and loaded again onto the column. The membranes were rinsed twice with 700 µL and 200 µL of A2W buffer, centrifuged as described above, and transferred into new Eppendorf tubes. Then, 40 µL of sterile water were added, and after 3-min incubation at RT the tubes with the membranes were centrifuged as above. RNA quality and quantity were estimated using Implen NanoPhotometer (Eppendorf, Germany), and only the samples with a 260/280 nm absorbance coefficient between 1.8 and 2.1 were used for the final experiments. The TranScriba noGenome Kit (A&A Biotechnology, Poland) was used to perform reverse transcription, according to the manufacturer's recommendations in the MJ Research PTC-100 thermocycler (Marshall Scientific, United States). First, 1 µg of total RNA was mixed with the noGenome master mix. After a 10-min incubation at 42° C, 7 µL of the mentioned mix were added to the RT master

mix. The RT master mix included 4 µL of TranScriba buffer, 0.5 µL of RNAse inhibitor, 2 µL of dNTP, 1 µL of starter oligo (dT), 4 µL of TranScriba reverse transcriptase and 1.5 µL of sterile water for one reaction. The reverse transcription protocol was as follows: the first step of 60 min at 42°C, the second step of 5 min at 70°C, and the final step of 5 min at 4°C. The obtained cDNA was stored at −20°C.

2.5.3. Gene expression analysis using real-time PCR

The real-time PCR gene expression analyzes were performed in triplicate from three independent cell cultures. The reaction mix (per well) included 5 µL of RT PCR Mix SYBR® (A&A Biotechnology, Poland), 0.5 µM of forward and reverse primers (Eurofins Genomics AT GmbH, Poland), and 1 µL of cDNA diluted with molecular biology water (16.65 ng cDNA per well). Real-time PCR was performed using the LightCycler 480 II (Roche Molecular Systems Inc., United States) instrument under the following conditions: pre-incubation at 95°C for 10 min, 50 cycles of amplification: 10 s at 95°C for denaturation, 30 s at 60°C for annealing, and 15 s at 72°C for elongation. The gene detection analyzes and primer specificity were further improved by melting curve analysis. The gene expression was categorized using the following scale:

"0" lack of gene expression, Ct values above 35.
"1" very low gene expression, Ct values between 30 and 35.
"2" low gene expression, Ct values between 28 and 30.
"3" regular gene expression, Ct values between 22 and 28.
"4" high gene expression, Ct values between 15 and 22.
"5" very high gene expression, Ct values below 15.

2.6. DNA combing assay

A total of 1.6×10^6 cells were cultured in 10 mL of media, then pulse-labeled with 5-iodo-2′-deoxyuridine (IdU) at 25 µM, followed by 5-chloro-2′-deoxyuridine (CldU) at 200 µM, for 15 min each. The cells were recovered by centrifugation at 300 g after each pulse, and the media were refreshed with each analog. Next, the cells were resuspended in cold PBS and warmed to 42°C, using 5×10^5 cells per agarose plug. The cells were gently mixed with 1% agarose in PBS and divided into the casting mold to generate the plugs. After treatment with proteinase K (in a buffer made of 1% Sarkosyl, 10 mM Tris pH 7.5, 50 mM EDTA) at 50°C overnight, the DNA was stained with YOYO-1 (5 µM in TE solution for 2–5 min) to check the quality of the fibers. After melting the agarose, the extracted DNA was poured into a reservoir, where a coverslip was inserted, and the DNA fibers were stretched. The resulting fibers were visualized by immunofluorescence detecting the IdU and CldU analogs with red and green antibodies. The coverslips were incubated for 45 min with murine anti-BrdU (IdU, ref. 34,780 Becton Dickinson, United States), and rat anti-BrdU (CIdU, Eurobio ref. ABC117-7513, France), as primary antibodies for the analogs, and for 30 min with goat anti-mouse IgG1 Alexa 564 (ref. A21123 Molecular Probes, Thermo Fisher Scientifics, United States) and chicken anti-rat Alexa 488 (ref. A21470 Molecular Probes, Thermo Fisher Scientifics, United States) as secondary antibodies for the analogs. The coverslips were incubated for 30 min with autoanti-ssDNA DSHB by Voss, E.W. (Hybridoma Product autoanti-ssDNA) autoanti-ssDNA DSHB by Voss, E.W. (DSHB, United States) to detect whole DNA fibers, and then for 30 min with a secondary antibody goat anti-mouse IgG2a Alexa 647 (ref. A21241 Molecular Probes, Thermo Fisher Scientifics, United States). Image acquisition was performed with a 40x objective using a confocal microscope (DM6000; Leica). The fork velocity (FV) was calculated by multiplying the length of the green track of the fiber in micrometers by 2 to obtain Kb, and dividing it by 15 min (time of pulse). Fork asymmetry (FA) was calculated by dividing the long track by the short track. For this analysis, only the first analog incorporation tracks (green tracks) were considered.

2.7. Statistical analysis

For the combing assay analysis, the Mann–Whitney test was performed to compare the cell lines and analyze potential differences. Scatterplots were prepared to visually represent the differences between the two cell lines.

Statistical analysis was performed using TIBCO Software Inc. (2017) Statistica (data analysis software system), version 13 http://statistica.io.

TABLE 2 Antibody list showing percentage protein identity between human and dog DDR components.

Protein	Clone	Ref. catalog	Dilution used in the study	% homology*		Literature
				Total protein	Epitope region	
Chk1	G-4	sc-8,408	1:1000 in 3% BSA in TBS-T	96.2		(36–39)
Phospho-Chk1 (SER345)	133D3	#2348	1:1000 in 3% BSA in TBS-T	96.2	100	(36, 38–41)
β-Actin	C4	sc-47,778	1:1000 in 3% milk in TBS-T	97.22	100	(42–47)
ATR	C-1	sc-515,173	1:800 in 3% BSA in TBS-T	94.75	100	(39, 40, 48)
Rad51	G-9	sc-377,467	1:600 in 3% BSA in TBS-T	99.12	100	(41, 49, 50)
Claspin	B-6	sc-376,773	1:800 in 3% BSA in TBS-T	84.47	88	(40)
Anti-gamma H2AX	9F3	ab26350	1:1000 in 3% BSA in TBS-T	99.17	100	(43, 44, 48, 51, 52)

*Basic Local Alignment Search Tool (BLAST) of protein sequences. Antibodies immunogen sequences were analyzed in BLAST® from National Center for Biotechnology Information (NCBI) (www2) (53).

Values are given for both total protein and, where known, for the specific polypeptide region used as immunogen to generate each antibody.

TABLE 3 Gene names, forward (F) and reverse (R) primer sequences, amplicon nucleotide (nt) sizes with their respective gene accession numbers.

Gene name	Forward (F) and Reverse (R) primer sequences	Amplicon size (nt)	Gene accesion number
Target Genes (TGs):			
ATR	F: ACCAGACAGCCTACAATGCT R: CCACTTTGCCCTCTCCACAT	77	XM_038432561.1
CLSPN	F: CGCACAAAGCCAGGTGAAAA R: CGTTCCTCATGCCTACGGAG	80	XM_539598.6
Housekeeping genes (HKGs):			
ACTB	F: CGCAAGGACCTCTATGCCAA R: CTTCTGCATCCTGTCAGCGA	78	NM_001195845.3
PPIA	F: TTTGGCAAGGTCAAGGAGGG R: TGGTCTTGCCATTCCTGGAC	73	XM_038689274.1
RPLP0	F: ACATGCTGAACATCTCCCCC R: CAGGGTTGTAGATGCTGCCA	80	XM_038436104.1

3. Results

3.1. RNA-sequencing analysis revealed the presence of principal components of the DDR pathway in canine cell lines

The canine lymphoma cell line CLBL-1 and the canine leukemia cell line GL-1 expressed a total of 16,220 genes, from which 271 (~2%) are DDR pathway members. Specifically, the CLBL-1 cell line expressed 260 DDR genes, and the GL-1 cell line 266, with 255 genes in common (Figure 2A). The relative expression of the most important genes with a role in the ATR- and HR-repair pathways was analyzed for both cell lines. Higher expression of all the genes was found in the CLBL-1 than in the GL-1 cell line, except for RAD51 which showed slightly higher expression in the GL-1 cell line (Figure 2B).

3.2. Expression and activation of the DDR pathway components in canine cancer cells

Considering that ATR, Claspin, Chk1, and Rad51 are among the most important proteins of the DDR pathway, they are still quite uncharacterized in dogs. Thanks to the progress made in recent years in veterinary medicine, and in particular in veterinary oncology, currently there are some tools available for their study in dogs (for example antibodies and siRNAs).Our initial aim was to identify commercial antibodies against key components (ATR, Claspin, Chk1, Rad51) of the DDR that would be suitable for use in canine cells. Western blot screening was performed to analyze the basal protein expression levels, and to determine whether the pathway activation in response to DNA damage occurs in canine cells (detection of γH2AX and p-Chk1; Figure 3A; Supplementary Figure S4). All the antibodies used in the study were monoclonal antibodies generated using human epitopes as immunogens. The BLAST alignment comparing human and canine protein sequences demonstrated high homology overall and, where known, within the polypeptide region used as immunogen (Table 2; alignments in Supplementary material).

As detecting high-molecular-weight proteins by means of a western blot can be technically challenging (54), a qPCR was performed for the genes encoding ATR and Claspin, in order to obtain more information about the expression of these DDR components in the panel of the analyzed cell lines.

3.2.1. ATR

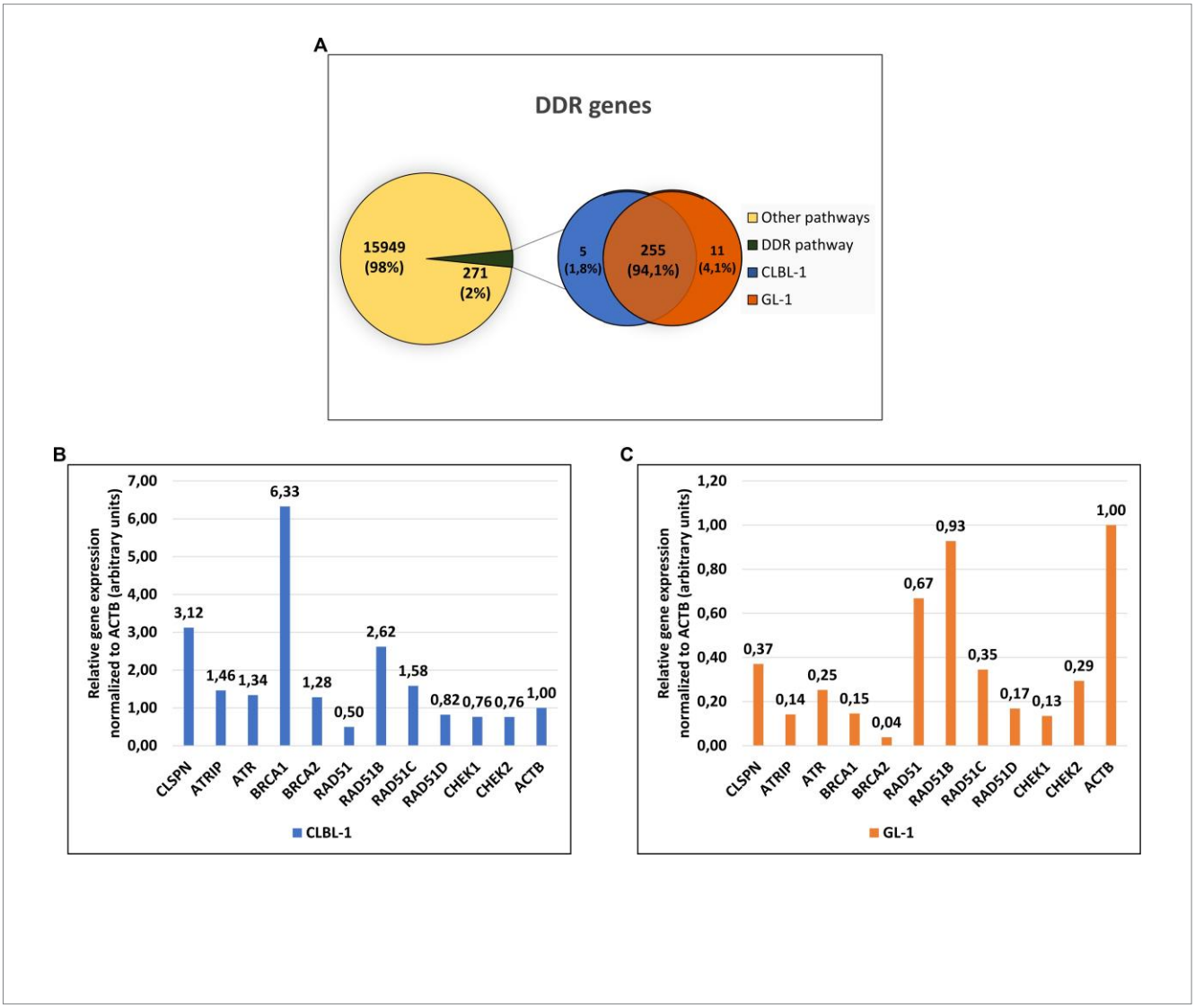
BLAST alignment demonstrated 94.75% identity between the human and canine ATR protein sequences, which justified the assumption that an antibody directed against a human protein would cross-react with the canine protein. Indeed, the band detected with the antibody ATR C-1 (Santa Cruz sc-515,173) corresponded with the ~220 kDa molecular mass expected for this protein. ATR was readily detected in only two of the three cell lines analyzed, with expression of ATR being higher in CLB70 cells than CLBL-1 and undetectable in GL-1 cells (Figure 3A). As ATR is considered essential for cell proliferation/ survival, and ATR mRNA expression was readily detected in the GL-1 cell line (see below), we assume that the level of ATR protein expression in this cell line is below the limit of detection using this particular method of cell extraction/ WB.

qPCR analysis confirmed that ATR mRNA is expressed in the three cell lines tested, with mean threshold cycle (Ct) values of 25.21 ± 0.27 for the CLBL-1 cell line, 28.15 ± 3.29 for the CLB70 cell line, and 25.72 ± 0.61 for the GL-1 cell line. Although the expression of ATR was substantially lower than the expression of the HKGs (Figure 3B), Ct values below 29 indicate that ATR mRNA is relatively abundant in these cells.

3.2.2. Claspin

In the case of Claspin, BLAST alignment demonstrated 84.47% identity between the human and canine proteins, again supporting the possibility of cross-reactivity of human antibodies with canine proteins. The expression of Claspin was detectable using the Claspin B-6 (Santa Cruz sc-376,773) antibody. The antibody recognized a protein of the expected molecular mass of ~180 kD, thus confirming cross-reactivity with canine Claspin. Claspin expression was observed in all three cell lines, although the expression levels varied. Claspin expression levels were substantially higher in the CLBL-1 and CLB70 cell lines than in the GL-1 cell line, similar to the expression of ATR (Figure 3A).

qPCR analysis showed that the Claspin mRNA was also expressed in the three cell lines, with Ct values of 24.95 ± 0.17 for the CLBL-1 cell



line, 27.63 ± 2.70 for the CLB70 cell line, and 26.19 ± 0.74 for the GL-1 cell line. Similar to ATR, the expression of the Claspin mRNA in each case was substantially lower than that of the HKGs (Figure 3B).

3.2.3. Chk1 and p-Chk1

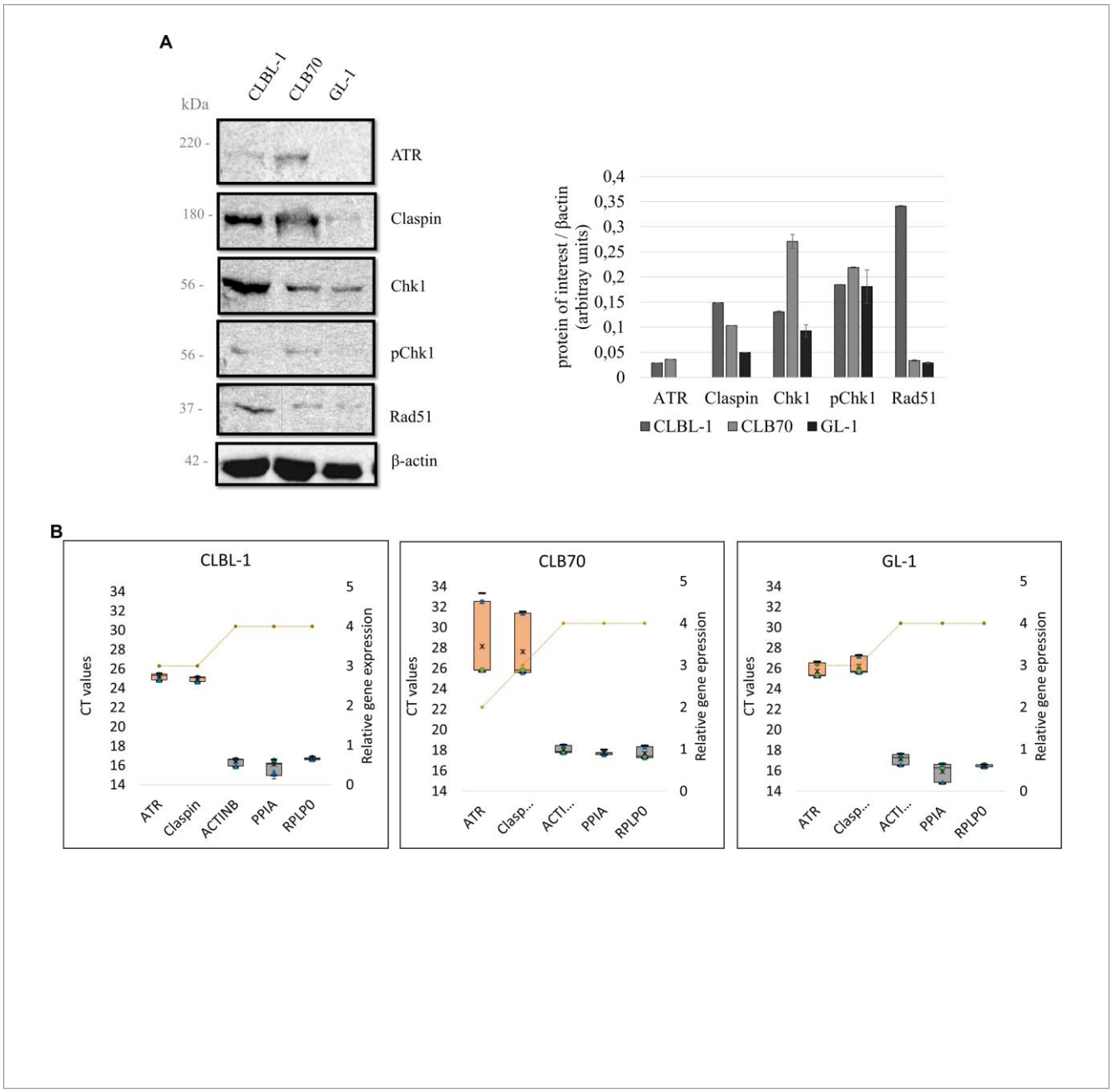
BLAST alignment for Chk1 protein sequences between humans and dogs showed a high 96.2% identity. This analysis also confirmed that canine Chk1 contains the key regulatory site, serine 345 (S345), that is phosphorylated by ATR to activate Chk1 in response to genotoxic stress. This means that with the use of the tested antibodies Chk1 G-4 (Santa Cruz sc-8,408) and Phospho-Chk1 (SER345) 133D3 (Cell Signaling #2348) directed against human epitopes, both the basal level expression of Chk1 kinase and its activation after DNA damage can be tested in canine cells. Indeed, both antibodies detected proteins of the expected molecular mass (~56 KD) in all three cell lines (Figure 3A). Interestingly, however, the two cell lines with the highest basal expression of Chk1 were again CLBL-1 and CLB70, which also exhibited the highest expression of both ATR and Claspin. Basal levels of active, phospho-S345 Chk1 were also detected in all three cell lines, being highly expressed in CLB70 as compared with the other cell lines (Figure 4). The ability to monitor the level of Chk1 kinase, as well as its activation, will facilitate the development of new molecularly targeted therapies in canine oncology.

3.2.4. Rad51

BLAST alignment comparing Rad51 protein sequences demonstrated 99.12% identity between human and canine protein, indicating a high probability that an antibody directed at the human protein will detect the canine homolog. As expected, using Rad51 G-9 (Santa Cruz sc-377,467) antibody, we detected a band of ~37 kDa corresponding to the expected molecular mass of Rad51. Basal Rad51 recombinase expression was detected in all three cell lines with the highest level of expression in the CLBL-1 cell line (Figure 3A).

3.3. Activation of the DDR pathway after etoposide treatment observed as an increase in Chk1 kinase S345 phosphorylation

Next, we analyzed the expression levels and possible regulatory modifications of the canine DDR proteins afterinducing activation of the DDR pathway by treating the cells with

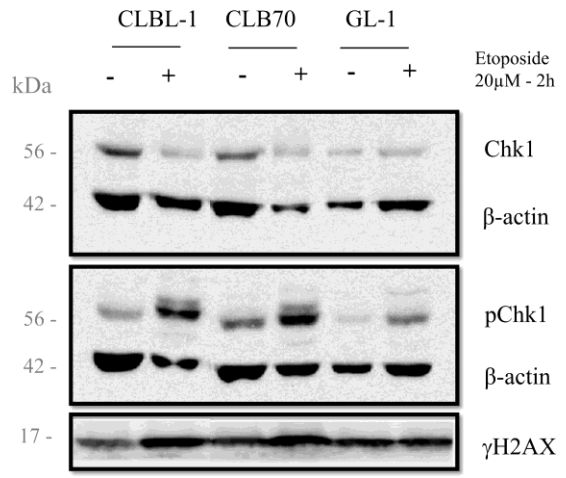


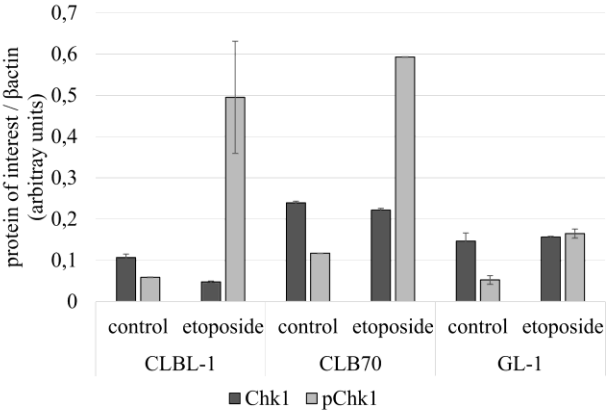
a classic DNA damaging agent, etoposide. Several studies have reported DDR activation through phosphorylation of Chk1 and an increase of Rad51 expression after treatment with the DNA damaging agent etoposide (55, 56). This information, togetherwith the fact that etoposide is a chemotherapeutic drug used as a treatment for several cancers (57, 58), are the reasons whywe decided to select it for our study to induce DNA damage. There were no major changes in the expression levels of totalChk1 after the treatment with etoposide, but the level of Chk1 phosphorylated at S345 varied considerably after exposure to this toxic agent (20 μM for 2 h). The CLBL-1 and CLB70 cell lines,which showed S345 phosphorylation of Chk1 in basal conditions, were also found to present a considerable increase in the phosphorylation levels after the treatment with the DNA damaging agent, while in the GL-1 cell line, this increase was more modest (Figure 4).

3.4. DNA combing assay in the canine cells

Advanced techniques, such as DNA combing, can provide much greater insight into DNA replication dynamics by directly visualizing replication fork progression rates and replication origin firing. Due to the important role of ATR-Chk1 in replication, we wished to evaluate the feasibility of performing DNA combing in canine cells.

The selected cell lines for this study were GL-1 and CLBL-1, since both present high expression level of Rad51 (Figure 3A; Supplementary Figures S1, S2), which may be related to replication stress. The protocol had to be slightly modified for use with suspension cells. Following the modified protocol, the cells were treated with proteinase K at 0.4 mg/mL. In the first experiment, many fibers were seen to be broken, so subsequently the cells were treated with a lower concentration of proteinase K (0.2 mg/mL), and the quality of the fiber integrity improved (Figure 5).





We then used the DNA combing assay to examine replication dynamics in two selected cell lines, CLBL-1 and GL-1. The data are summarized in Table 4 and Figure 6. The replication fork speed was significantly ($p = 8.86^{-21}$) faster in the GL-1 line (1.5 Kb/min) than in the CLBL-1 line (0.86 Kb/min; Figure 6A). When replication fork asymmetry was analyzed, both cell lines had similar means for the calculated ratios, 1.27 for the CLBL-1 and 1.32 for the GL-1 line (Figure 6B), indicating that both exhibited similar levels of replication fork asymmetry.

4. Discussion

4.1. RNA-sequencing analysis revealed the presence of principal components of the DDR pathway in canine cell lines

Due to the important role of the DDR in cancer and the paucity of information about it in canine cancer cells, an RNA-Seq analysis was initially performed in the selected cell lines, CLBL-1 and GL-1, under normal growth conditions. CLBL-1 and GL-1 cell lines were selected for this analysis as representative of common hematopoietic cancers - lymphoma (CLBL-1) and leukemia (GL-1). After sorting the genes by Gene Ontology terms (GO) related to the DDR (Table 1), we found that approximately 2% of expressed genes encode components of the DDR pathway (Figure 2A). Interestingly, the relative expression of most DDR genes in the CLBL-1 line was higher than in the GL-1 cell line, but the expression patterns changed as well. For the CLBL-1 cell line, the most highly expressed genes were *BRCA1*, *CLSPN*, and paralogue B of *RAD51* (*RAD51B*), while *RAD*51 exhibited the lowest relative expression (Figure 2B, in blue). In the case of the GL-1 line, the pattern was different, the highest expression was detected for *RAD51B* and *RAD51*, and the lowest for *BRCA2* (Figure 2C, in orange).

Thus, this RNA-Seq analysis revealed that the canine lymphoma and leukemia cells shared expression of a majority of DDR genes, but that the expression of certain key components differed substantially between the tumor types.

4.2. Expression and activation of the DDR pathway components in canine cancer cells

The first screening for the basal expression of DDR proteins (Supplementary Figure S1) indicated significant variations in the protein expression levels for different cancer cell lines. Based on these findings, together with the results of the RNA-Seq analysis (Figure 2), several lymphoma and leukemia cell lines were selected for further experiments. Validation of antibodies that recognize DDR proteins in dogs is needed, and selected commercial antibodies were tested in this study. The BLAST alignment analyzes were performed to check the homology between the human and canine protein sequences. With confirmed high homology, it was assumed that if an antibody detects a single band of correct molecular mass, there is a high probability that this corresponds to the protein of interest, particularly as all the tested antibodies were monoclonal (59, 60).

4.2.1. ATR

ATR was detected in all the cell lines at the mRNA level (Figure 3B). The obtained melting curves showed only one amplicon, which validated the identity of this qPCR product (Supplementary Figure S3). Ct values of 25.21 ± 0.27 for the CLBL-1cell line, 28.15 ± 3.29 for the CLB70 cell line, and 25.72 ± 0.61 for the GL-1 cell line were observed, indicating robust ATR mRNA expression in all tested cell lines (61).

The BLAST alignment for ATR protein showed a 94.75% protein identity in humans and dogs, meaning that the probability of an

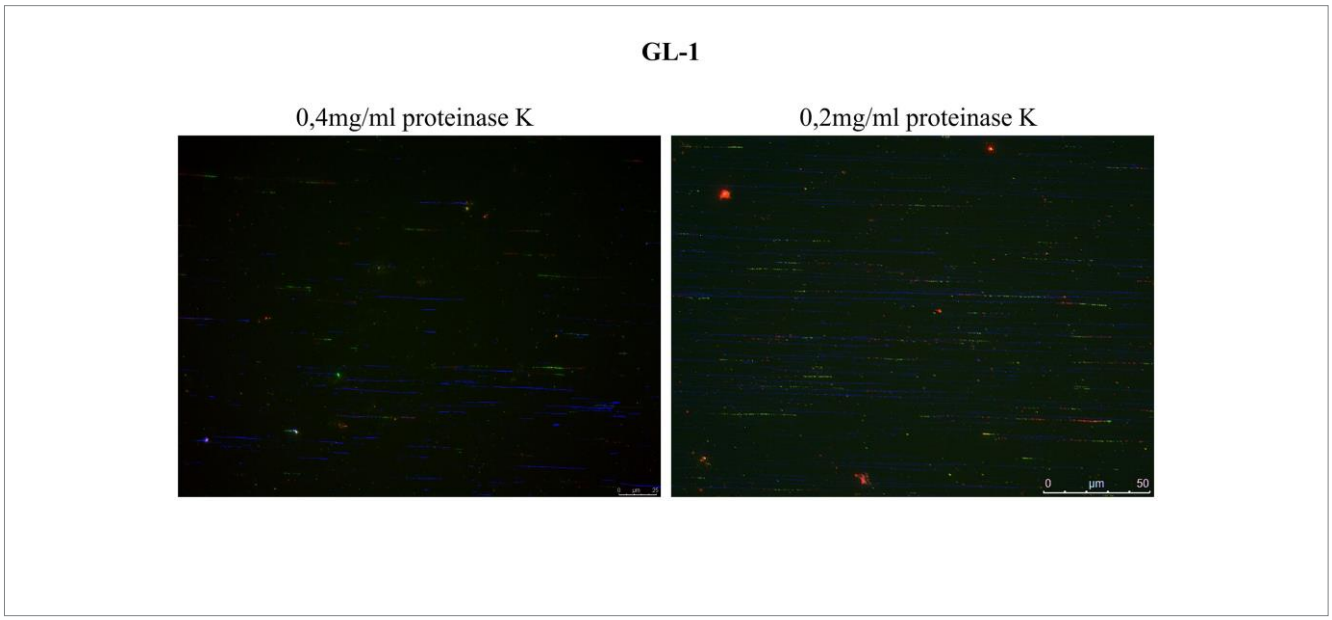


TABLE 4 Fiber patterns found in the analyzed cells.

Cell line	Progressing fiber	Initiation during the first pulse	Initiation before the first pulse	Termination	Cluster
CLBL-1	128	8	2	24	1
GL-1	124	14	5	21	10

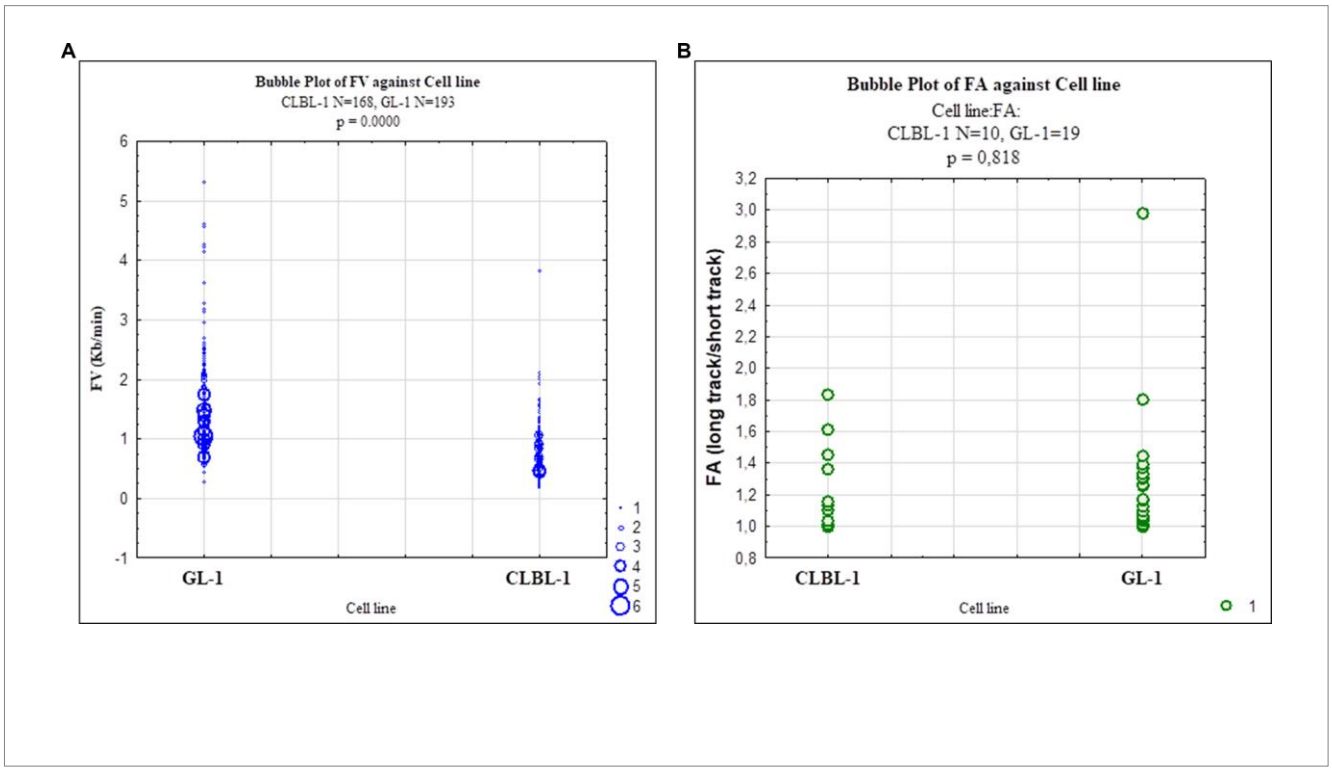
antibody designed to recognize the human protein cross-reacting with the dog protein is high. As presented in Figure 3A, the tested ATR antibody recognized the canine protein. To our knowledge, no previous studies on ATR at the protein level have been performed in dogs. Curiously, we found that ATR protein at the basal level was only detected in the CLBL-1 and CLB70 cell lines and not in GL-1, despite clear evidence for ATR mRNA expression in the latter. It is well known however that protein and mRNA levels do not always correlate; several studies demonstrated that the correlation can vary in adenocarcinoma samples (62), can be modulated after treatments with drugs such as rapamycin (63), and may vary during the cell cycle in synchronized cultures (64). What we can conclude from the presented results is that the *ATR* gene is widely expressed in the canine lymphoma/leukemia cell lines. However, as ATR has a high molecular weight of 220 kDa, and high-molecular-weight proteins are difficulted to transfer (54), it is likely that its expression was below the threshold of detection in the GL-1 cell line for unknown reasons. Nuclear extraction and/ or immunoprecipitation are options that could be used to increase the sensitivity of ATR protein detection in GL-1 cells and other canine cancers.

Variations in ATR expression are considered a marker of sensitivity and/or resistance to certain anticancer drugs. High expression of ATR protein has been proposed as a marker of cisplatin sensitivity in patients with bladder cancer (7). Also, in the case of a doxorubicin-resistant canine hemangiosarcoma cell line established to study drug resistance, it was found that the DDR pathway was attenuated, as the mRNAs for ATM, ATR, and Chk1 were significantly decreased, suggesting a possible role for ATR in doxorubicin resistance (15). Another study reported that ATR inhibitors in combination with pyrrolobenzodiazepine (PBD) increased the cytotoxicity of PBD as compared with the drug alone, and helped to overcome the resistance to PBD (65). In other work, human multiple myeloma (MM) cells were treated with MEDI2228, a ligand of the B-cell maturation antigens that induces ATM/ATR-Chk1/2 pathway activation, in combination with different inhibitors of the principal kinases of the DDR (ATM, ATR, and WEE1) (66). The results of that study showed an increase in the toxicity of this ligand when combined with the inhibitor, an interesting example of the use of ATR as a target to induce cell death in MM cells and to abrogate resistance to MEDI2228.

To our knowledge, this is the first time that ATR has been detected in canine lymphoma/leukemia cells providing a new opportunity to study this protein in veterinary oncology.

4.2.2. Claspin

A high percentage of protein identity between the human and canine Claspin proteins was confirmed by BLAST alignment (84.47%). Indeed, Claspin protein expression was detected in all the cell lines of our panel as a single band with a molecular mass of 180 kDa as seen in human cell lines (40). Claspin was highly expressed in the CLBL-1 cells compared to the other cell lines (Figure 3A). Interestingly, the mRNA of the Claspin gene was detected in all three cell lines although at a somewhat lower level in CLB70 cells (Ct value of 27) than in the other lines (Figure 3B). The melting curves showed only one peak, meaning that the primers designed for Claspin specifically amplify a single amplicon (Supplementary Figure S3). Contrary to what we observed in the CLB70 cell line for ATR, Claspin protein expressionand its mRNA level in the CLBL-1 cell line were higher than in theother cell lines of the panel. This corresponded with the observationfrom RNA-Seq that the *CLSPN* gene was among the most highly expressed genes in the CLBL-1 cell line (Figure 2B). This could be an



example of a regulated correlation between protein and mRNA expression, which is not as common as one might expect (64).

Claspin has been described to be highly expressed in prostate cancer cells in comparison with non-cancerous prostate cells (6). Many cancer cells present higher expression of the components of the ATR-Claspin-Chk1 pathway, as compared with non-cancerous cells, which can be related to resistance to radiotherapy (13, 14). To our knowledge, only one other study has analyzed Claspin in canine cells (67). In that experiment, a polyclonal antibody included in an apoptosis antibody array kit (Catalog # ARY009) was used. In our study, a monoclonal antibody for Claspin was validated in three different canine cell lines. This indicates the potential utility of this antibody in future veterinary research to test the effects of inhibiting Claspin, or to detect the protein expression level in different tumor samples.

4.2.3. Chk1 and p-Chk1

Chk1 showed a 96.7% identity between the human and canine protein sequences in the BLAST alignment (Supplementary Figure S1). Consistent with this, Chk1 protein was detected in all the cell lines at various levels, with the CLB70 and CLBL-1 lines showing higher expression than the GL-1 line (Figure 3). We also detected high Chk1 mRNA level in the CLBL-1 line (Figure 2B). Interestingly, Chk1 was found to be highly expressed in several tumors, as compared with non-malignant tissues (4, 5). It was described to be overexpressed in human leukemia cells, B-cell lymphomas, and highly expressed in hematopoietic cancers as compared with solid tumors (8, 9, 68), which is consistent with the results obtained in our canine lymphoma/leukemia cell lines. Interestingly, in the CLBL-1 and CLB70 cell lines, the basal level of kinase phosphorylation was much higher than in the GL-1 cell line, suggesting that the response to DNA damage in these two cell lines might be faster and stronger than in the latter.

Upregulation of Chk1 has been proposed as a target for anticancer therapies. Different studies have confirmed Chk1 inhibitors acting as apoptosis inducers in various human and canine tumor cells, indicating, for example, proliferation decrease in human neoplastic B-cells and mast tumor cell (MTC) canine cell lines (68, 69). Currently, there are several Chk1 inhibitors in phase II of clinical trials and the results in human cancers seem promising (8, 70). Knowing that Chk1 is overexpressed in human B-cell lymphomas, and that the antibodies have been validated in our canine cells, we propose the use of canine B-cell lymphoma/leukemia cell lines as a model to study the role of Chk1 in canine B-cell malignancies.

4.2.4. Rad51

The last protein we examined was Rad51. BLAST alignment showed 99.12% Rad51 sequence identity between humans and dogs. This suggests that antibodies designed to recognize human Rad51 will also recognize the canine homolog. In our study, the antibody employed recognized Rad51 protein in all the canine cell lines tested. Its expression was the highest in the CLBL-1 line, both at the protein and gene level (Figures 2B, 3). In the literature, high expression of Rad51 protein is related to genome instability (10, 11), which is a hallmark of cancer. Rad51 is overexpressed in mammary carcinomas, and this is related to metastases in lymph nodes in both humans and dogs (71–74). Rad51 is a protein which has been studied in canine tumors due to its connection with BRCA2, and several studies have documented Rad51 mutations in tumor canine cells (75–77) Bortezomib, a proteasome inhibitor that impairs HR and thus decreases the expression of Rad51, has been used to potentiate the effect of other drugs, such as inhibitors of poly (ADP-ribose) polymerase (iPARP) or MEDI2228. Such combinations can also downregulate Rad51 protein expression, increase cell death, and even help to eradicate tumors in *in vivo* mice models (66, 78). The effects of these drugs on Rad51 function and expression in dogs have not been studied yet. However, as bortezomib is a drug that can be safely administered in dogs (79), treatment with a combination of

bortezomib and other DNA damaging agents in canine cell lines could yield useful information to be potentially implemented in the veterinary clinic. Here, a new Rad51 antibody, clone G-9, has been validated in canine cells, and it was also recently validated in other hematopoietic human cell lines (49).

4.3. DNA replication dynamics in canine lymphoma/leukemia cell lines

Replication stress arises in cells with DDR defects during the replication of damaged DNA. Replication stress may cause fork asymmetry and consequently, fork stalling and collapse that promotes genetic instability (80). Cancer cells often seem to experience replication stress under conditions where normal cells do not, even when they replicate rapidly (18, 81). A reduction in fork speed has been described under conditions of replication or oxidative stress in cancer cells (82), while pronounced asymmetry of replication forks has been detected in medulloblastoma stem cells (83). Thus, measurement of fork speed and fork asymmetry could help to better understand and describe the phenotype of a cancer cell type, which may later be used in order to choose therapeutic approaches. The ATR-Chk1 pathway is a critical regulator of the replication stress, as its role is to regulate the replication fork progression and stability, presenting potential targets for combination therapies (84) Thus, the analysis of cellular replication in canine cancer cells could bring new opportunities to find targets for therapies. Here we presented for the first time the use of the DNA combing assay in canine cells.

The analysis was performed in two of the canine lymphoma/leukemia cell lines, CLBL-1 and GL-1. The CLBL-1 cell line presented a high basal level of Rad51, and the GL-1 cell line showed the highest expression of Rad51 after etoposide treatment. Both situations may indicate cell replication stress (Figure 2A; Supplementary Figure S2). The replication fork speed in human cells is approximately 2–3 Kb/min (85). In our study, the replication fork speed in the canine cells seemed to be lower, around 1.5 Kb/min for the GL-1 cell line, and 0.86 Kb/min for the CLBL-1 cell line (Figure 6; Supplementary Table S1). It can be concluded that GL-1 cells have a higher replication speed than CLBL-1, which is interesting as the GL-1 cell line's doubling time is 27.3 h, and for the CLBL-1 it is 19 h (30, 86). The mean values of fork asymmetry were higher than 1 in both cell lines (Supplementary Table S1), indicating a significant number of replication forks terminate asymmetrically in both cell lines (87).

Advanced and novel biomolecular techniques need to be applied in veterinary science in order to improve the quality of the research and stimulate progress in therapy and clinical discoveries. Basic research analysis studying the role of proteins and cellular responses to different treatments is a first step needed to generate a new therapy to treat cancer or any other disease. The structural and functional properties of the principal components of the DDR system are conserved in mammals, but little is known about them specifically in dogs (88, 89). This cellular pathway is under intense investigation in human medicine due to its effects on the clinical aspects of cancer and its potential use in new therapies (90–93). Even where there are effective therapies based on targeting DDR proteins, such as PARP inhibitors (94–96), further investigation is needed due to the fact that cancer cells develop resistance (97). The knowledge about the functioning of the proteins involved in tumor-related pathways, and specifically their behavior in cancer cells is fundamental to finding new targets to be used in therapies.

4.4. Importance of validation of techniques and reagents to improve veterinary medicine research

Comparative clinical trials showed analogous results in human and canine patients treated with iniparib and F14512 (topoisomerase II inhibitor), highlighting the similarity of both species in the way naturally occurring cancer and lymphoma respond to therapies (98, 99). Cell lines represent a predictive tool for developing therapies in both human and veterinary medicine (100–102), which means that canine cancer cells represent a tractable model to study cancer that can generate valuable information also for human medicine. We have presented here a set of techniques and reagents validated in selected canine lymphoma/leukemia cell lines, which will facilitate further research in this field. All the data obtained in this work make the selected canine cell lines attractive models to study molecular aspects of lymphoma and leukemia. Experiments on combinations of the tested drugs with inhibitors of the principal components of the studied pathways are planned in the near future.

5. Conclusion

To conclude, we propose the use of canine lymphoma/leukemia cells as a model to study DDR in cancer, with ATR, Claspin, Chk1, and Rad51 as promising targets for further analysis. Our results will facilitate further investigation on DDR in canine cancer by identifying validated antibodies for ATR, Claspin, Chk1, p-Chk1, and Rad51, and primers for ATR and Chk1, and bringing numerous opportunities to develop new targeted-anticancer therapies which later may be also implemented in human medicine.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

AP: conceptualization and project administration. BH-S and AP: methodology. BH-S: software, validation, formal analysis, data curation, writing–original draft preparation, visualization, and funding acquisition. BH-S, AP, PK, and ED: investigation. BO-M: resources. AP and DG: writing–review and editing. AP, DG, and BO-M: supervision. All authors have read and agreed to the published version of the manuscript.

Funding

The publication was financed by the project "UPWR 2.0:international and interdisciplinary program of development of

Wrocław University of Environmental and Life Sciences," co-financed by the European Social Fund under the Operational Program Knowledge Education Development, under contract No. POWR.03.05.00-00-Z062/18 of June 4, 2019 and by the Polish National Agency for Academic Exchange under Grant No. PPI/APM/2019/1/00044/U/00001. DG was an Agustín de Betancourt Investigador of the Universidad de La Laguna.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1227683/full#supplementary-material.

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8. Foreword to publication 3

As we mentioned in the introduction section (**Chapter 1**), there is some knowledge about how canine cells respond to ER stress, but little is known about the molecular basis of the UPR pathway in dogs and, specifically, about their role in cancer.

In the following chapter, we present several results on the UPR in a panel of hematopoietic canine cancer cell lines (**P3**). Our preliminary data shows the possibility of using those canine lymphoma and leukemia cells as a model to study UPR.

1. The rationale of research objectives and hypotheses.

Because humans and dogs shared a high percentage of UPR protein homology (**Table 2** in **P3**), UPR can lead to the initiation of the apoptosis process, and apoptosis and alterations in the UPR pathways are often described in cancer cells, we decided to start investigating this pathway.

The aim of this research was to facilitate the molecular research of UPR in canine cancer by validating different techniques and reagents necessary to conduct such research.

2. The methodology and techniques used in the study.

- Panel of canine lymphoma and leukemia cell lines
- RNA-sequencing of two selected cell lines to check the expression of the DDR mRNA levels
- In vitro treatments with: thapsigargin 2 μM for 2 hours or 1 μM for 5 hours, MG132 10 μM for 16 hours or 20 μM for 5 hours, and 20 μM of etoposide for 2 hours
- Western blot technique to analyze UPR protein levels
- Flow cytometry using annexin V FITC/PI or caspase 3/7 to study apoptosis

More details about the techniques are available in **chapter 3**.

3. The results obtained in the study.

In **P3**, we have done an initial characterization of some of the UPR components (eIF2α and CHOP) in canine lymphoma and leukemia cell lines.

The first result presented is a sequencing analysis that showed that in the two cell lines selected for this study, the UPR's most relevant components are expressed. Higher expression of target genes was found in the CLBL-1 cell line compared to GL-1, except for EIF2A, whose expression was higher in GL-1 (**figure 2 of P3**).

Activation of UPR was measured by checking the phosphorylation level of eIF2α and the expression of CHOP protein. An increase in the phosphorylation of eIF2α has been obtained after treating the cells with thapsigargin in a concentration of 2 μM for 2 hours (**figure 3A of P3**). It is important that the serine 51, which is the phosphorylation site of eIF2α in humans, is conserved between humans and dogs (**figure 3B of P3**).

Interestingly, no CHOP expression was detected on the first attempt (**figure 3A of P3**). In the next experiment, a decrease in the concentration of thapsigargin but longer incubation

time was tested (1 μM for 5 hours) and as a second ER stress inducer, the proteasome inhibitor MG132 in a 10 μM concentration for 16 hours was used. Also, a second antibody to detect CHOP protein was tested. Surprisingly, no CHOP detection was observed with the first antibody tested (CHOP (GADD153 (sc-7351)), but a clear signal in all the cell lines was observed with the newly applied antibody (CHOP (L637F #2895)). Finally, an increase in CHOP expression after ER stress induction was observed (**figure 4A of P3**).

The study showed that the first antibody (CHOP (GADD153 (sc-7351)) which is generated to recognize mouse CHOP protein, did not work in canine cells due to the low homology between CHOP protein (86%) between mouse and dog. Antibody (CHOP (L637F #2895)) which recognizes the human epitope, turned out to be better, probably because human and dog CHOP protein homology is 92% (**figure 4B of P3**).

Because CHOP is related to apoptosis, a flow cytometry analysis was performed in order to check if the increase of CHOP expression after ER stress inducers was correlated with an increase in apoptosis. In the annexin V and PI staining performed, no apoptosis was detected in any of the cell lines tested after inducing ER stress (**figure 5A of P3**). 40% of apoptotic cells were detected in CLBL-1 after thapsigargin treatment with the caspases 3/7 assay, and around 20% of apoptotic cells were observed in GL-1 and CNK-89 after MG132 (**figure 5B in P3**).

4. Conclusion on P3

The validation of the techniques and reagents presented in **P3,** and the preliminary data obtained regarding the molecular mechanisms of UPR in dogs, signalized the importance of doing research about this system in canine cancer. This research has shown the high similarity between the UPR components and mechanisms between human and dogs species, and they also present the first evidence that canine lymphoma and leukemia cell lines may be a suitable model to study UPR in cancer.

9. Publication 3

P3 - An initial characterization of the Unfolded Protein Response pathway in hematopoietic canine cancer cell lines -a necessary step for the introduction of new therapies in dogs with cancer

An initial characterisation of the Unfolded Protein Response pathway in haematopoietic canine cancer cell lines – a necessary step for the future development of new therapies in dogs with neoplasia

Beatriz Hernández-Suárez[1]✉, David A. Gillespie[2], Bożena Obmińska-Mrukowicz[1], Aleksandra Pawlak[1]

[1]Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Wrocław University of Environmental and Life Sciences, 50-375 Wrocław, Poland
[2]Instituto de Tecnologías Biomédicas, Facultad de Medicina, Campus Ciencias de la Salud, Universidad de La Laguna, La Laguna 38071, Tenerife, Spain
beatriz.hernandez-suarez@upwr.edu.pl

Received: March 28, 2023

Accepted: August 2, 2023

Abstract

Introduction: New and more effective therapies for canine cancer patients are urgently required and this necessitates advanced experimental research. Dogs are good models for studies in comparative oncology; however, canine cancer cell biology research is currently limited by low availability of validated antibody reagents and techniques. This study characterises the expression of key components of the unfolded protein response (UPR) in a panel of haematopoietic canine cancer cell lines using commercially available antibodies, and validates the methods used to study this pathway. **Material and Methods:** The CLBL-1 canine lymphoma cell line and the GL-1 canine leukaemia cell line sourced externally and two counterparts established in house (CNK-89 and CLB70) were used as models of different lymphoma and leukaemia canine cell lines for the study. The human U2OS cell line served as the control. Antibodies were selected for identifying UPR proteins according to known canine cell reactivity and canine–murine and canine–human homology. Endoplasmic reticulum stress was induced with thapsigargin and MG132 in the cell lines. Etoposide was used to induce DNA damage in the cells. The techniques used for this validation analysis were RNA sequencing to observe the expression of UPR components in canine cell lines, Western blot to observe changes of protein expression levels after inducing ER stress in the cells, and flow cytometry in order to study cell death. **Results:** Substantial variations in both the basic expression and agonist-induced activation of the UPR pathway were observed in canine cancer cell lines, although the biological significance of these differences requires further investigation. **Conclusion:** These findings will be a starting point for future studies on cancer biology in dogs. They will also contribute to developing novel anticancer therapies for canine patients and may provide new insights into human oncology.

Keywords: canine cancer, eukaryotic translation initiation factor 2α (eIF2α), CCAAT/enhancer binding protein homologous protein (CHOP), protein kinase RNA-like endoplasmic reticulum kinase (PERK).

Introduction

Cancer is the leading cause of death in dogs: nearly 50% of dogs will develop this disease by the age of 10 (2). It is known that cancer in humans and dogs is similar in the way that tumours develop and respond to therapies. Studies in dogs focused on the molecular pathways that are known to be fundamental for the development of cancer in humans will bring better understanding of the mechanisms of the disease and streamline the discovery of novel therapies for dogs.

Disturbances in the functioning of the unfolded protein response (UPR) can directly lead to carcinogenesis, but at the same time they could be an excellent therapeutic target in human and veterinary oncology. Therefore, there is a need to validate the reagents and molecular techniques analysing the UPR in

canine cells to improve their effectiveness in comparative oncological research.

The unfolded protein response pathway has a confirmed role in the neoplastic process. In the simplest terms, the UPR is triggered in response to endoplasmic reticulum (ER) stress to restore homeostasis. Under prolonged ER stress, the UPR pathway switches from being a homeostasis regulator to being a cell death–triggering pathway, inducing apoptosis (Fig. 1). The rapid and uncontrolled growth of cancer cells causes them to be frequently exposed to unfavourable conditions such as hypoxia or nutrient deprivation, which cause ER stress (23). When an increase in misfolded or unfolded proteins in the ER lumen is detected, the glucose-regulated protein 78 (GRP78) folding chaperone dissociates from protein kinase RNA-like ER kinase (PERK), which dimerises and autophosphorylates, leading to kinase activation. In the next step, activated PERK phosphorylates and inactivates the eukaryotic translation initiation factor 2α (eIF2α), leading to global suppression of translation. Subsequently, activated transcription factor 4 (ATF4), which is selectively translated in the presence of inactive eIF2α, stimulates the transcription of CCAAT/enhancer binding protein homologous protein (CHOP) (also known as growth-arrest and DNA-damage–inducible gene 153 (GADD153)) to completely stop protein synthesis in the cell and induce apoptosis (28).

The aims of the study were to determine whether there were variations in the UPR activity between canine cancer cell lines and to validate the methods used to study this pathway (RNA sequencing, Western blot and flow cytometry). Investigation was carried out of the expression level of the UPR genes involved in the PERK pathway by RNA sequencing and of the expression levels of the most important UPR proteins (CHOP, eIF2α and phosphorylated eIF2α (p-eIF2α)) after induction of ER stress with Ca^{2+} adenosine triphosphatase–inhibiting thapsigargin and proteasome-inhibiting MG132 by Western blot analysis. Simultaneously, the antibodies that recognise canine proteins were validated and the level of apoptosis in model cells due to ER stress activation was evaluated by Annexin V and propidium iodide (PI) staining and a caspase activation assay.

Material and Methods

Cells and cell culture. A panel of canine lymphoma/leukaemia cell lines were used in this study: CLBL-1 (B-cell lymphoma), CLB70 (B-cell chronic lymphocytic leukaemia), GL-1 (B-cell leukaemia) and CNK-89 (natural-killer-cell lymphoma). The U2OS human osteosarcoma cell line (obtained from ATCC) was used as a control. The CLBL-1 cell line was kindly provided by Dr. Barbara Rütgen from the Institute of Immunology, Department of Pathobiology at the University of Vienna (29), the GL-1 line was received from Dr. Yasuhito Fujino and Dr. Hajime Tsujimoto of the Department of Veterinary Internal Medicine at the University of Tokyo (22), and CLB70 (25) and CNK-89 (12) lines were established with the participation of researchers from our laboratory.

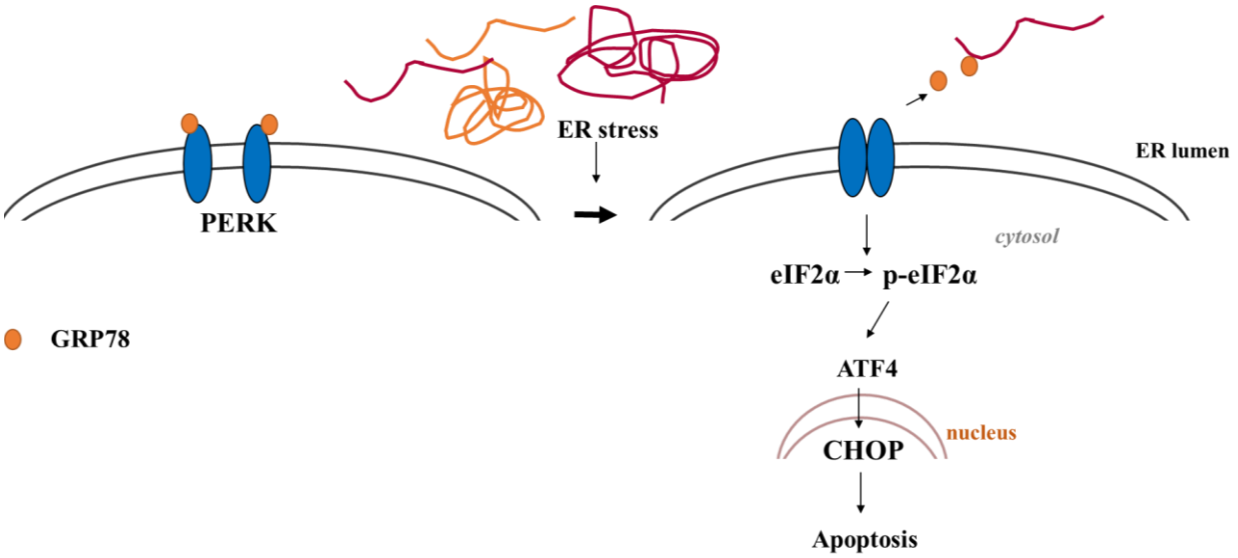


Fig. 1. Unfolding protein response pathway scheme. ER – endoplasmic reticulum; PERK – protein kinase RNA-like ER kinase; eIF2α – eukaryotic translation initiation factor 2α; p-eIF2α – phosphorylated eukaryotic translation initiation factor 2α; GRP78 – glucose-regulated protein 78; ATF4 – activating transcription factor 4; CHOP – CCAAT/enhancer binding protein homologous protein

Roswell Park Memorial Institute (RPMI) 1640 medium (Institute of Immunology and Experimental Therapy, Polish Academy of Science, Wrocław, Poland) was used for the CLBL-1 and GL-1 lines, and Advanced RPMI (Gibco, Grand Island, NY, USA) for the CLB70 and CNK-89 lines. The culture media were supplemented with 2 mM L-glutamine (Sigma-Aldrich, Steinheim, Germany), 100 U/mL of penicillin, 100 µg/mL of streptomycin (Sigma-Aldrich), and 10% to 20% heat-inactivated foetal bovine serum (FBS) (Gibco). Cells of the U2OS line were cultured in Dulbecco's modified Eagle's medium supplemented with 2mM glutamine and 10% FBS. The cells were cultured in an atmosphere of 5% CO_2 and 95% humidified air at 37℃ in 75 cm^2 cell-culture flasks (Corning, New York, NY, USA).

RNA sequencing. Cells of the CLBL-1 and GL-1 lines were sequenced in basal conditions by Novogene (Cambridge, UK). To estimate the relative gene expression, the expected number of fragments per kilobase of transcript sequence per million base pairs sequenced was used (19). The Gene Ontology (GO) knowledge base (9) lists selected for the intersection analysis of the UPR genes were the ones presented in Table 1, and the gene set information was obtained from the Gene Set Enrichment Analysis database (18, 31).

Treatments. The cells were treated with different drugs to induce ER stress and to test the activation of the pathway of interest. To induce ER stress, the cells were treated with thapsigargin at 2 µM for 2 h, and MG132 (both products of Sigma-Aldrich, St. Louis, MO, USA) at 10 µM for 16 h. For flow cytometry analysis after ER stress induction, thapsigargin was used at 1 µM for 5 h and MG132 at 20 µM for 5 h. In addition to ER stress, DNA damage was induced in the cells with the application of etoposide.

Western blot. The samples were lysed in urea/sodium dodecyl sulphate (SDS) buffer produced in the same laboratory where the blot was performed (900 µL of 7M urea, 25 µL of 5M NaCl, 25 µL of 2M Tris-HCl (pH = 8) and 50 µL of 20% SDS) and run in 8–12% bis-tris acrylamide gels also produced in the laboratory. A Mini-PROTEAN Tetra Vertical Electrophoresis Cell system was used. The samples were transferred to a nitrocellulose membrane using a Mini Trans-Blot Cell for wet transfer and a Trans-Blot Turbo Transfer System device for the semi-dry transfer method (all products of Bio-Rad, Hercules, CA, USA).

The antibodies that were used in the study (Table 2) were selected based on available literature data on reactivity with canine cells, comparison of sequence homology, and comparisons of obtained electrophoresis bands with the expected molecular weight. The antibodies' immunogen sequences were analysed in BLAST, the basic local alignment search tool from the National Center for Biotechnology Information (1).

Table 1. Gene Ontology knowledge base sets selected for the analysis

Gene set	Species
GOBP_REGULATION_OF_PERK_MEDIATED_UNFOLDED_PROTEIN_RESPONSE	human
GOBP_REGULATION_OF_PERK_MEDIATED_UNFOLDED_PROTEIN_RESPONSE	mouse
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	human
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	mouse
REACTOME_UNFOLDED_PROTEIN_RESPONSE_UPR	human
REACTOME_UNFOLDED_PROTEIN_RESPONSE_UPR	mouse
WP_PHOTODYNAMIC_THERAPYINDUCED_UNFOLDED_PROTEIN_RESPONSE	human
WP_UNFOLDED_PROTEIN_RESPONSE	human

Table 2. Antibody list showing the percentage of protein identity between humans and dogs

Protein	Clone	Supplier and catalogue No.	Dilution used in the study	% homology*
Anti-gamma H2AX	9F3	Abcam, Cambridge, UK, ab26350	1:1,000 in 3% BSA in TBS-T	99.17
Phospho-eIF2α (Ser51)	119A11	Cell Signaling Technologies, Danvers, MA, USA, 3597	1:1,000 in 3% BSA in TBS-T	96.07

eIF2α	D-3	Santa Cruz Biotechnology, Dallas, TX, USA, sc-133132	1 : 1,000 in 3% BSA in TBS-T	96.07
DDIT3/CHOP/GADD153	B-3	Santa Cruz Biotechnology, sc-7351	1 : 500 in 3% BSA in TBS-T	92.3
CHOP	L637F	Cell Signaling Technologies, 2895	1 : 1,000 in 3% BSA in TBS-T	92.3

* – homology according to the basic local alignment search tool for protein sequences; H2AX – histone family member X; eIF2α – eukaryotic translation initiation factor 2α; DDIT3 – DNA-damage–inducible transcript 3; CHOP – CCAAT/ enhancer binding protein homologous protein; GADD153 – growth-arrest and DNA-damage–inducible gene 153; BSA – bovine serum albumin; TBS-T – tris-buffered saline with Tween 20

A multiblast analysis was performed for the CHOP protein sequence to compare human, murine and canine genomes with the COBALT multi-alignment tool (24). Goat anti-mouse immunoglobulins conjugated with horseradish peroxidase (HRP) at 1 : 20,000 dilution in tris-buffered saline with Tween 20 (TBS-T) solution (cat. No. P0447; Dako, now part of Agilent Technologies, Santa Clara, CA, USA) and goat anti-rabbit immunoglobulins conjugated with HRP at 1:10,000 dilution in TBS-T solution (cat. No. P0448; Dako) were used as secondary antibodies.

Flow cytometry. The cells were treated with ER stress inducers at the concentrations defined above prior to staining with annexin V conjugated with fluorescein isothiocyanate (FITC) or staining with PI or caspase 3/7 (Invitrogen, Carlsbad, CA, USA) to study apoptosis. The cells were suspended in a binding buffer together with annexin V-FITC and PI (PI concentration 1 µg/mL) for 10 min at room temperature. At the same time, another batch of cells was collected and stained with CellEvent Caspase-3/7 Green Detection Reagent (Invitrogen) following the manufacturer's instructions, and then incubated at 37℃ for 30 min. Finally, flow cytometry analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA). Weasel v.3.0.2 flow cytometry software (https://www.frankbattye.com.au/contact.html) was used for data analysis.

Results

RNA sequencing (RNA-Seq) analysis of UPR pathway expression in the lymphoma and leukaemia cells. An RNA-Seq analysis was performed to verify the potential importance of the UPR members in the lymphoma and leukaemia cell lines. Cells of the CLBL-1 and GL-1 lines were selected for this analysis in order to have one lymphoma and one leukaemia sample to represent both malignancies. Of the 16,220 genes expressed in the CLBL-1 and GL-1 cell lines, 146 (approximately 1%) were members of the UPR pathway (Fig. 2A). Interestingly, 140 of these (95.9%) were expressed in both the CLBL-1 and GL-1 cell lines. The expression levels of the UPR genes that play a role in the UPR mediated by the PERK route were analysed (Fig. 2B). Higher expression levels of different UPR members were found in the CLBL-1 cell line, with the exception of *EIF2A*, the expression of which was greater in the GL-1 cell line.

Expression and activation of the UPR pathway components in canine cells in response to thapsigargin and MG132. All the antibodies used to detect UPR proteins were monoclonal and designed originally to recognise human or mouse epitopes. Alignments in the basic local alignment search tool (BLAST), the most widely used bioinformatics programme, showed a high degree of amino acid identity between the human and dog homologues of the UPR proteins of interest, making it likely that the antibody reagents selected would recognise the canine version.

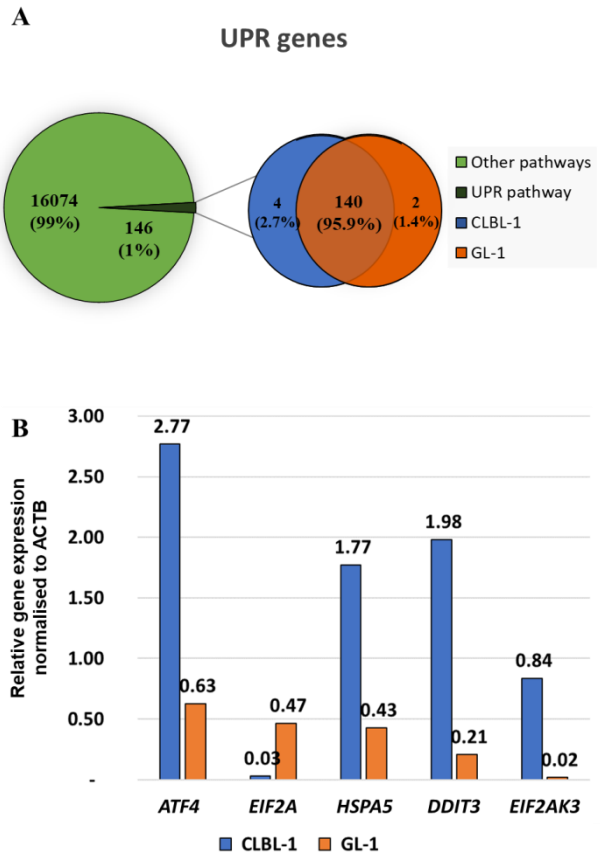
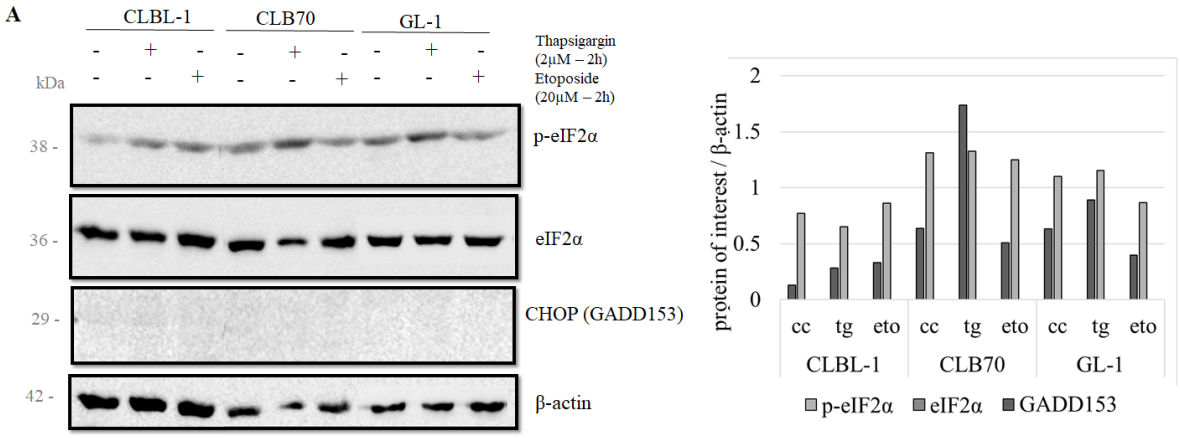


Fig. 2. Relative gene contents of the unfolded protein response (UPR) pathway and other pathways among genes expressed in the CLBL-1 B-cell lymphoma and GL-1 B-cell leukaemia canine cell lines (A) and relative expression of principal genetic components of the UPR pathway normalised to *ACTB* gene expression (B). *ATF4* – activated transcription factor 4; *EIF2A* – eukaryotic translation initiation factor 2α; *HSPA5* – heat-shock 70 kDa protein 5; *DDIT3* – DNA-damage–inducible transcript 3; *EIF2AK3* – eukaryotic translation initiation factor 2α kinase 3

Eukaryotic IF2α and its active phosphorylated form p-eIF2α. Alignment by BLAST comparing protein sequences demonstrated 96% identity between human and canine eIF2α. At the same time, the analysis confirmed that a key site of regulatory phosphorylation mediated by the UPR kinase PERK in the human and mouse homologues, serine at position 51 (S51), was also conserved in canine eIF2α (Fig. 3B) (alignments available in Table S1).

Antibodies against total eIF2α (clone D-3) and S51-phosphorylated eIF2α (clone 119A11) detected proteins of the expected molecular weights of ~36 and ~38 kDa, respectively. All cell lines clearly expressed eIF2α. The phosphorylation of eIF2α at S51 increased in all the analysed cell lines after thapsigargin treatment as compared with non-treated cells or cells with etoposide treatments (Fig. 3A).



B

Human EIF2A gene (1–180 bp)

CTCTTTCCGGGACAACATGGCGCCGTCCACGCCGCTCTTGACAGTCCGAGGATCAGAAGGACTGTACATGGTGAATGGACCACCACATTTT
ACAGAAAGCACAGTGTTTCCAAGGGAATCTGGGAAGAATTGCAAAGTCTGTATCTTTAGTAAGGATGGGACCTTGTTTGCCTGGGGCAA

Human eIF2α protein (1–60 aa)

----- MAPSTPLLTVRGSEGLYMVNGPPHFTESTVFPRESGKNCKVCIFSKDGTLFAWGNGEKVN

Dog EIF2A gene (101–280 bp)

CTCTTTCCGGGACAAGATGGCGCCGTCCACGCCGCTCCTGACAGTCCGAGGATCAGAAGGACTGTATATGGTAAATGGACCGCCACATTTT
ACAGAAAGCACTTTGTTTCCAAGGGAATCGGGGAAAAATTGCAAAGTCTATACCTTTAGTAAGGATGGCACCTTGTTTGCTTGGGGCAA

Dog eIF2α protein (1–60 aa)

----- MAPSTPLLTVRGSEGLYMVNGPPHFTESTLFPRESGKNCKVYTFSKDGTLFAWGNGEKIN

Fig. 3. Expression of unfolded protein response (UPR) proteins by canine lymphoma (CLBL-1 – B-cell lymphoma) and leukaemia (CLB70 – B-cell chronic lymphocytic leukaemia and GL-1 – B-cell leukaemia) cell lines treated with thapsigargin in order to induce endoplasmic reticulum stress (A). Expression levels were measured by Western blot and densitometry and the densitometric quantification of phosphorylated eukaryotic translation initiation factor 2α (p-eIF2α) was normalised to β-actin. Fragment of human and canine gene and eIF2α protein sequences surrounding residue 51, showing the conserved serine (phosphorylation site when activation of the protein takes place) (B). CHOP – CCAAT/EBP enhancer binding protein homologous protein; GADD153 – growth-arrest and DNA-damage–inducible gene 153; cc – control; tg – thapsigargin; eto – etoposide; bp – base pairs; aa – amino acids

CHOP (GADD153). A BLAST alignment comparing protein sequences demonstrated 92% identity between human and canine CHOP (Table S1). A multi-alignment analysis comparing canine, human and murine CHOP protein sequences was performed (Fig. 4B).

The monoclonal antibody generated against murine DNA-damage–inducible transcript 3 (DDIT3)/CHOP/GADD153 initially failed to detect CHOP expression in any of the tested cell lines (Fig. 3A). The test conditions were changed and a lower concentration of 1 μM of thapsigargin and longer incubation time of 5 h were investigated for their induction of the expression of CHOP in the cell lines. In this experiment, the cells were also treated with MG132, a proteasome inhibitor, in order to compare CHOP

expression levels in the cells after exposure to two different ER stress inducers. Again, CHOP expression was not detectable in any cell line using the murine antibody (Fig. 4A, first panel). It was decided to change the conditions of the experiment once again, but this time by changing the antibody to a human one. Surprisingly, this antibody was capable of detecting CHOP expression both before and after the treatment with ER stress inducers in all the cell lines. The detected protein migrated at 29 kDa, which is the expected molecular weight of CHOP (Fig. 4A, second panel). The induced levels of CHOP expression were higher after the treatment with MG132 than with thapsigargin, but were unaffected by etoposide. Different expression levels were observed for different cell lines, being higher for GL-1 than the others, including the human cell line (U2OS) serving as a positive control in this experiment. It is important to mention that no CHOP signal was detected with the first antibody, murine DDIT3/CHOP/GADD153, in any of the cell lines tested, even though it should have cross-reacted with different species' proteins (rat and human) as explained in the datasheet. In contrast, CHOP was detected in all the cell lines using the human antibody CHOP L637F.

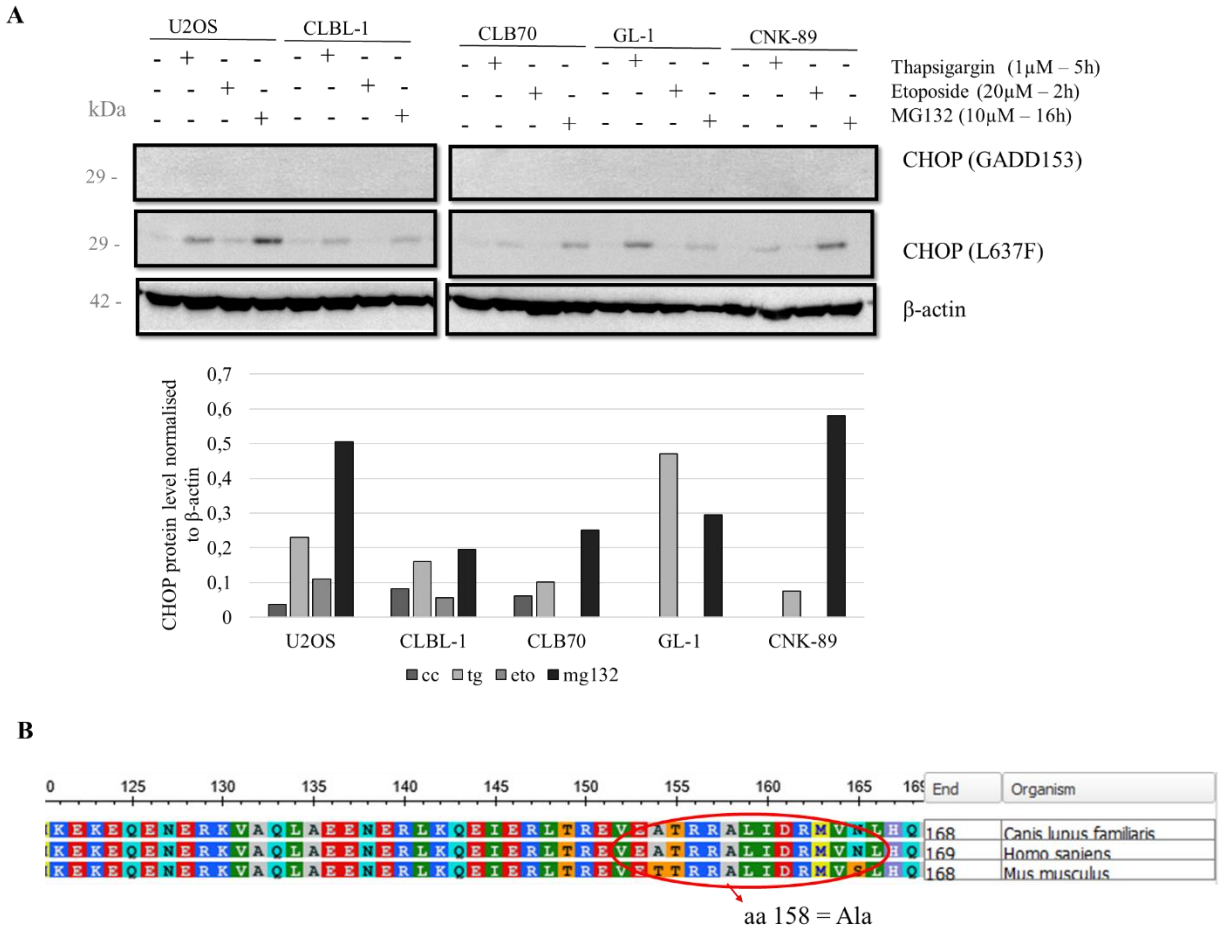


Fig. 4. Expression of CCAAT enhancer-binding protein homologous protein (CHOP) by canine lymphoma (CLBL-1 – B-cell lymphoma and CNK-89 – natural-killer-cell lymphoma), canine leukaemia (CLB70 – B-cell chronic lymphocytic leukaemia and GL-1 – B-cell leukaemia) and human osteosarcoma (U2OS) cell lines treated with thapsigargin and MG132 in order to induce endoplasmic reticulum stress; expression being detected by a murine (CHOP (growth-arrest and DNA-damage–inducible gene 153 (GADD153) clone B-3) and a human (CHOP clone L637F) antibody (A). Quantification was assessed by densitometry. (B) Murine–human species epitope differences between CHOP/GADD153 B-3 and CHOP L637F with indication of the greater homology at the protein level between dogs and humans than between dogs and mice. CHOP L637F antibody immunogen surrounds amino acid 158, circled in red. cc – control; tg – thapsigargin; eto – etoposide; aa – amino acid; Ala – alanine

Expression of CHOP in the GL-1 cell line and apoptosis levels. Since thapsigargin, the proteasome inhibitor MG132 and etoposide can cause apoptotic cell death, it was determined whether there were differences

in the incidence of apoptosis induced by these treatments between different cell lines, using annexin V and PI staining and caspase 3/7 activation. In all the studied canine cell lines, annexin V staining revealed that a majority of the treated cells were negative for annexin V and PI, indicating they were non-apoptotic, live cells (Fig. 5A). A small increase in apoptotic cells was observed in the GL-1 and CNK-89 cell lines after MG132 treatment, but not enough to constitute a major increase in apoptosis after induction of ER stress under the conditions described.

As observed in the annexin V assay, only minor increases in apoptosis were detected using the caspase 3/7 assay in all of the cell lines and conditions evaluated, although small differences between the cell lines were evident (Fig. 5B). Only the CLBL-1 cell line seemed to show a higher tendency to apoptosis after treatment with thapsigargin (40.2% ± 11.2%) than with MG132 (17.15% ± 3.64%), these CLBL-1 apoptosis rates comparing with 4.31% ± 1.49% in the control cell line (the pre-stress condition). This was in contrast to the GL-1 and CNK-89 cell lines, which seemed to present a greater tendency to apoptosis after MG132 treatment, with the level of apoptosis at 10.06% ± 2.14% with thapsigargin and 21.35% ± 5.9% with MG132 for the GL-1 line against 5.64% ± 2.14% for the pre-stress level, and at 4.86% ± 0.48% with thapsigargin and 22.25% ± 1.41% with MG132 for the CNK-89 line against 4.02% ± 0.37% for the pre-stress level. The CLB70 cell line showed no differences between the treatments except for slight but different increases after the ER stress induction, where 8.25% ± 0.9% was the apoptosis level after thapsigargin treatment and 9.45% ± 1.11% was the level after MG132 application, as compared with the pre-stress level of apoptosis of 7.77% ± 0.36%.

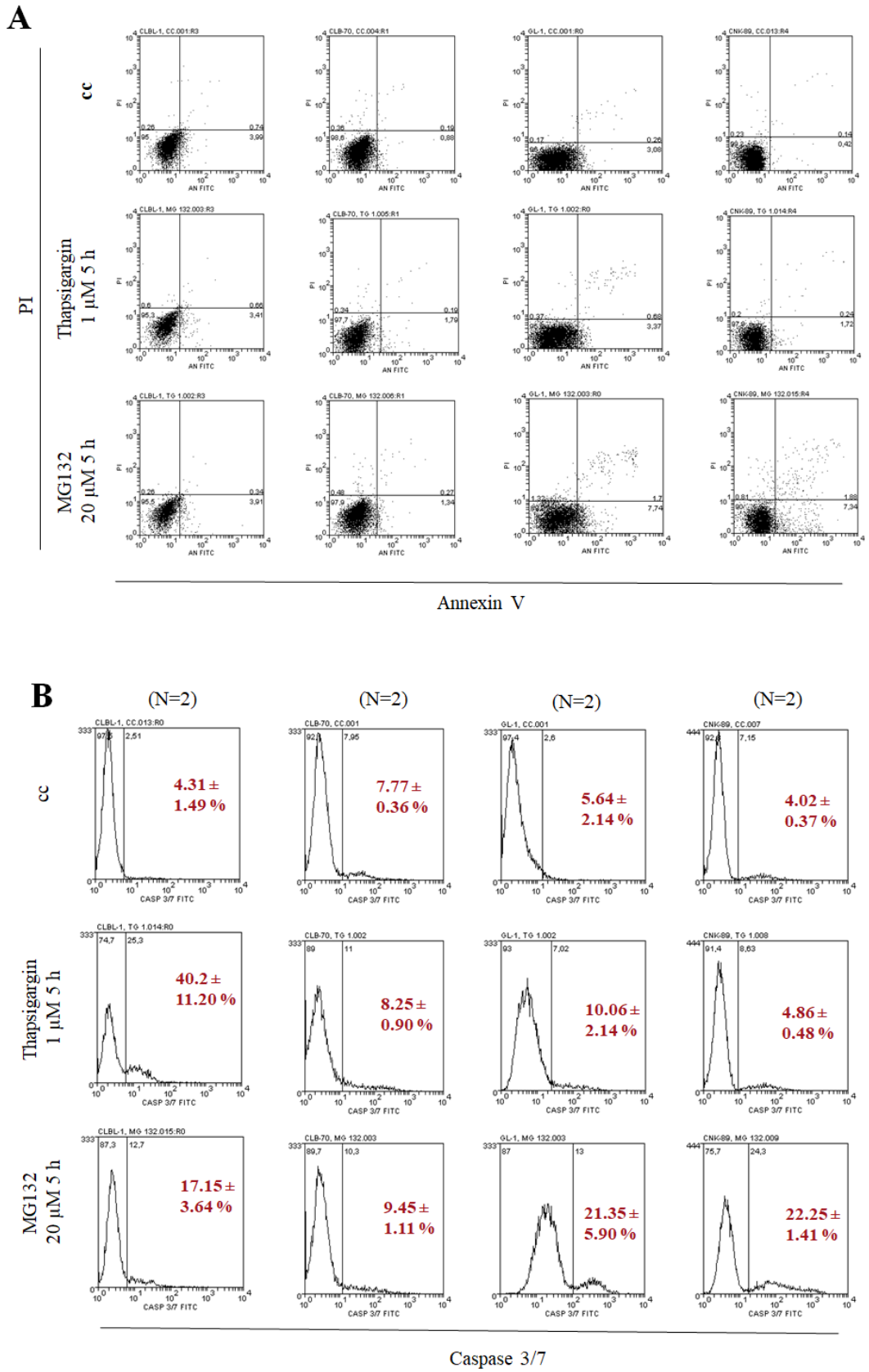


Fig. 5. Apoptosis in canine lymphoma (CLBL-1 – B-cell lymphoma and CNK-89 – natural-killer-cell lymphoma) and leukaemia (CLB70 – B-cell chronic lymphocytic leukaemia and GL-1 – B-cell leukaemia) cell lines treated with thapsigargin and MG132 to induce endoplasmic reticulum stress and stained with annexin V. The upper left quadrants represent necrotic cells, the bottom left quadrants the living cell population, the upper right quadrants late apoptotic events and the bottom right quadrants early apoptotic events (A). Apoptosis in the same cell lines after the same treatments indicated by caspase 3/7 activation, confirming no major increase in apoptosis (except for in the CLBL-1 cell line). The second peak corresponds to apoptotic cells which harbour

an active form of caspase 3/7. The red values represent means ± standard deviation (B). cc – control; FITC – fluorescein isothiocyanate

Discussion

Cells of the CLBL-1 and GL-1 lines were sequenced under basal conditions. The expressed genes were intersected with GO lists from the genes in humans and mice referring to ER stress and UPR terms. The RNA-seq analysis showed that approximately 1% of the genes expressed in canine lymphoma and leukaemia played a role in the UPR system, and that these cell lines shared 95.9% of the UPR genes. As the PERK signalling pathways have been described as cancer promotors (4), the relative expressions of the most important players were analysed in the canine cells. In the CLBL-1 cell line, the expressions of the *EIF2AK3* gene (encoding PERK), the *HSPA5* gene (encoding GRP78), the *DDIT3* gene (encoding CHOP) and the *ATF4* gene were higher than the expression of the *EIF2A* gene (encoding eIF2α). Looking at how this pathway works, it could indicate activation of the PERK route in the CLBL-1 line. The *ATF4* gene is the most intensively expressed gene in this cell line, followed by the *DDIT3* gene, which is typical for cells suffering from prolonged ER stress (30). In the GL-1 cell line, the expression of these genes was lower than in the CLBL-1 line, and no such differences between the genes were observed. Based on these results, it seems that the lymphoma cell line may have been in a chronic ER stress condition.

Commercial antibodies for CHOP, eIF2α and p-eIF2α were tested for validation. Analyses by BLAST were performed to assess the homology percentage between human and canine proteins, and the homology between the proteins from these two species being high at 92% was confirmed. This indicated the possibility of using human antibodies to detect canine proteins. The tested antibodies were monoclonal; therefore, it was considered that if the antibody detected a single band of the correct molecular weight, there was a high probability that it was the protein of interest (26).

The BLAST alignment for the eIF2α protein showed 96% homology in dogs and humans. As the homology was so high, the antibody against human protein was tested, and the expression of this protein was visible in all canine cell lines (Fig. 3A). The basal expression of eIF2α is not expected to vary much in the absence of stress, and only its phosphorylation should rise when the UPR is activated. The expression of eIF2α was more intense in the GL-1 than in the CLBL-1 line, which corresponded with the RNA-Seq analysis, where the *EIF2A* gene was more abundantly expressed in the GL-1 than in the CLBL-1 line. Unfolded protein response activation was observed in the cells undergoing ER stress, such as cells in patients suffering from cancer or other diseases, for example congestive heart failure (10). On the other hand, phosphorylation of eIF2α has been related to cancer survival and chemoresistance (13). A research study demonstrated that the PH domain leucine-rich repeat protein phosphatase (PHLPP) family regulates ER stress by dephosphorylating eIF2α, and its downregulation facilitates the survival of colon cancer cells under this stress (13). A similar study showed a contrary effect, where the TOR signalling pathway regulator-like (TIPRL) protein controlled ER stress by phosphorylation of eIF2α, thus contributing to cancer cells' ability to resist stress and to tumour development (15). In our study, a higher phosphorylation level of eIF2α was observed in ER stress conditions, as expected and as shown in a previous study (10).

Since the CHOP protein showed a 92% sequence identity in humans and dogs, and 86% in dogs and mice in the BLAST analysis, cross-reactivity of the murine antibody and the canine protein was expected. It was expected that CHOP would be expressed at a lower level in basal conditions (proper homeostasis) but be overexpressed under ER stress (6). The analysis by RNA-seq performed in this study revealed greater expression of CHOP in the CLBL-1 cell line than the GL-1 cell line, but as both lines expressed the gene, the protein was expected to be found in both cell lines. The increase in CHOP expression both on the transcriptional and translational levels has been described in different studies proving that this phenomenon occurs when the cell is under stress. The stress can be caused by a virus infection, cancer hyperosmolarity, or any other situation affecting ER homeostasis (5, 38). As the basal expression of CHOP was expected to increase following ER stress, we treated the cells with thapsigargin to induce UPR activation and investigated whether CHOP was expressed in the tested panel of the lymphoma and leukaemia cell lines. Surprisingly, no signal was detected with the murine CHOP (GADD153) antibody. A second experiment was performed in order to observe if prolonged ER stress induced CHOP protein expression in the cell lines. The cells were treated with 2 µM of thapsigargin for 2 and 4 h, but the results were the same and CHOP was not detected (Fig. S1B). This prompted us to try the second antibody and one of human origin, CHOP L637F. The conditions were changed, and the cells were treated with thapsigargin at a lower concentration but for a longer time (1 µM for 5 h) and with MG132 (a proteasome inhibitor) to induce ER stress in a different way. Both antibodies were tested under those conditions. Interestingly, the CHOP (GADD153) antibody still did not detect CHOP in any cell line, but the CHOP L637F antibody recognised the protein of interest in all the cell lines tested. While a molecular explanation of this phenomenon remains unclear, we recommend using CHOP L637F to detect CHOP in research on canine cell lines. The increase in CHOP expression after thapsigargin treatment was expected. It was shown in previous studies that CLBL-1 cells responded with increased expression of CHOP to 1 µM thapsigargin applied for 6 h (21), which was finally

reproduced in our setup. The difference between the two antibodies that recognised the same protein was the species of origin for the epitope used for immunisation, this being murine for the CHOP (GADD153) clone D-3 antibody and human for the CHOP clone L637F alternative.

A multi-alignment analysis showed that the CHOP L637F antibody binding region surrounding the amino acid (aa) 158 of the human protein is highly conserved in humans, dogs and mice. The immunogen for the CHOP (GADD153) antibody was a recombinant polypeptide spanning murine CHOP aa 1–168. However, one aa may be different in the murine sequence, and that is why even in the human U2OS cell line used as a control, the CHOP protein was not detected.

The CHOP L637F antibody was validated in human and murine cell lines (8, 16), but this was the first time it was used in canine lines. The importance of CHOP in the study of cancer and other diseases implies that the validation of this antibody in canine cell lines greatly assists future comparative research.

Cancer cells have adapted to survive under prolonged ER stress by altering the UPR system (30), which is why upregulated proteins in the UPR have been analysed in pharmacological trials in order to be used as therapeutic targets (3). This increase in CHOP expression is well-recognised as a marker of ER stress, and is being targeted to treat different diseases. A very good example of this is a comparative study in which a group of mice and dogs were treated with gentamicin, a potent antibiotic that may cause renal failure as a side effect, and with a combination of gentamicin and injection of mesenchymal stem cells (14). Kidney samples were harvested, and the cells were analysed. Following the antibiotic treatment, the level of CHOP increased, as the treatment potentiated apoptosis. However, the injection of mesenchymal cells inhibited the ER stress response and curbed the expression of CHOP and other UPR markers. Another interesting finding was made in a study on pulmonary adenocarcinoma in dogs (11). That research showed that the expression of UPR proteins was higher in tumour than non-tumour cells, and that in the metastatic cells the expression of UPR proteins was more abundant than in the primary tumour. Our GL-1 cell line showed greater expression of CHOP protein in basal conditions, whereas in the other cell lines it only increased after induction of ER stress, as expected. Overexpression of CHOP has been proposed as a target to overcome drug resistance. This proposal was tested in a study where salubrinal, a selective inhibitor of eIF2α dephosphorylation, was used in combination with tumour necrosis factor–related apoptosis-inducing ligand (TRAIL) in hepatoma cells (33). These cells are resistant to TRAIL, but a combination of these two drugs increased apoptosis by inducing overexpression of CHOP mediated by blocking dephosphorylation of eIF2α (33). Another example of treatment with induced CHOP overexpression was in killing non-small cell lung carcinoma cells resistant to cisplatin, where upregulation of CHOP made resistant cells susceptible to this medication (35).

Not only overexpression of CHOP is related to resistance, but a lower expression level of CHOP protein also seems to be connected to chemo- and radio-resistance in cancer cells. In a study analysing gastric cancer cells susceptible or resistant to cisplatin, ATF4 and CHOP were downregulated in the resistant cells (7). The first experiment overexpressing ATF4 showed how this raised CHOP levels and potentiated apoptosis in the resistant cells treated with cisplatin, while the second experiment showed how after depletion of CHOP the situation was reversed, and the susceptible cells became more resistant to cisplatin. This study proved the importance of CHOP in apoptosis, showing how cells became resistant to drugs when CHOP was not available to induce apoptosis under stress conditions. Another study analysed MG132, epoxomicin, and proteasome inhibitors I and BAY 11-7082 as antitumour agents in thyroid cancer cells. The model involved cells resistant and susceptible to the proteasome inhibitor, and the researchers found that the expression of CHOP did not increase in the resistant cells but was significantly elevated in the susceptible ones (34). With depletion of CHOP, sensitive cells became resistant to the proteasome inhibitor. This interesting relationship between drug resistance and the expression of CHOP highlights the importance of studying this protein in cancer patients. The mechanism by which CHOP induces ER-stress–related apoptosis is one of regulating the flux of pro- and anti-apoptotic proteins. The CHOP protein stimulates the expression of pro-apoptotic proteins, such as Bim, and decreases the expression of anti-apoptotic proteins, such as Bcl-2 (27). Thus, CHOP is the link between UPR and apoptosis, and it can be used as a target to induce apoptosis in cancer cells. It was found to be the apoptosis inducer after treatment with vernodalol (a lactone which in combination with TRAIL showed promising results and no side effects in the treatment of diffuse large B-cell lymphoma in an *in vivo* model (37)). It had the same inductive effect on apoptosis when administered with activin A (a glycoprotein proposed to treat multiple myelomas, as it induces apoptosis in an NS-1 myeloma cell line (8)).

Keeping this in mind, the next step of our study was to analyse apoptotic cell death after activation of the UPR by inducing ER stress in the tested cells. The same ER stressors – thapsigargin and MG132 – were used, but the incubation time for MG132 was changed to 5 h at a concentration of 20 μM, as it was found that a 16-hour incubation with 10 μM MG132 was toxic to the cells (Fig. S1A). Thapsigargin at 1 μM was concentrated enough for some apoptotic cells to be detected with annexin V/PI staining (36), but in our study only a small increase in the number of apoptotic cells was found after MG132 treatment. In the caspase 3/7 activation analysis, differences were observed between the investigated cell

lines, with CLBL-1 being more sensitive to thapsigargin, and GL-1 and CNK-89 being more sensitive to MG132, as compared with the CLB70 line that showed no difference between these treatments. When at a 10 μM concentration, MG132 is expected to reduce the cell survival rate and transcription of the anti-apoptotic proteins after 4 h, as shown in a study in β-pancreatic human and murine cell lines (17). The same study also showed a related increase in the expression of UPR activation markers (CHOP, ATF-4 or GRP78) (17). Our results of Western blot analysis were in accordance with those findings (Fig. S1A), as was the increase in caspase activation observed in the GL-1 and CNK-89 cell lines. Based on the presented results, the five-hour incubation with those ER-stress inducers was not enough to induce apoptosis in the investigated lymphoma and leukaemia cells, but it was enough to detect UPR activation. The FACS analyses performed were not sufficiently conclusive to support a link between the observed high expression of CHOP and apoptosis in the GL-1 cell line. Further research is needed to better understand this phenomenon. A good starting point could be to test whether CHOP is responsible for apoptosis induction in CLBL-1 and CLB70 lines when they are treated with proapoptotic compounds, as was proved with Licochalcone A, a flavonoid with anticancer properties that induces apoptosis *via* CHOP in non-small lung cancer cells (32).

Numerous studies on the role of UPR in human cancer have demonstrated an interesting opportunity of using the proteins involved in this pathway as novel therapeutic targets. Clinical trials are testing the drugs that activate the UPR, for example ABTL0812, which is a first-in-class small molecule that induces ER stress *via* ATF4-CHOP in the cell (20).

Human medical research has many reagents, methodologies and tools at its disposal, while veterinary sciences lack validation of resources. One of the aims of our study was to validate the applied reagents and methodologies in order to improve the quality of veterinary research on the UPR pathway. As humans and dogs present high protein homology, using the tools already described for humans seems valid, but they need to be tested. In this study, we validated several antibodies, which proved efficient in different canine cell lines and will be useful tools for future research on UPR. The activation of the UPR system can be determined by the increase in the phosphorylation levels of eIF2α, or by the expression of different UPR components such as CHOP or ATF4 proteins. The study clearly showed that Western blot determination of p-eIF2a is the best method to study UPR activation in canine cells in the tested model. Because of the noted importance of the UPR in cancer, the research concerning this pathway is fundamental when looking for targets of individualised therapy to treat cancer in dogs.

To conclude, we propose the use of canine lymphoma/leukaemia cells as a model to study the UPR in cancer. The use of the CHOP clone L637F and not the GADD153 clone D-3 antibody is recommended when conducting research in canine cells. Our findings will facilitate further investigation on the UPR in canine cancers, bringing numerous opportunities to develop new targeted anticancer therapies which later may be also implemented in human medicine.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: The publication was financed by the project titled "UPWR 2.0: international and interdisciplinary programme of development of Wrocław University of Environmental and Life Sciences", co-financed by the European Social Fund under the Operational Program Knowledge Education Development, under contract No. POWR.03.05.00-00-Z062/18 of June 4, 2019, and by the Polish National Agency for Academic Exchange under grant No. PPI/APM/2019/1/00044/U/00001. David A. Gillespie is the recipient of funding from the Agustín de Betancourt Foundation.

Animal Rights Statement: None required.

Acknowledgements: We would like to thank Barbara C. Rütgen at the Institute of Immunology, Department of Pathobiology of the University of Vienna for providing the CLBL-1 cell line, and Yasuhito Fujino and Hajime Tsujimoto at the Department of Veterinary Internal Medicine of the University of Tokyo for providing the GL-1 cell line.

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10. Summary

This doctoral dissertation has opened new possibilities for researchers interested in studying DNA damage response and Unfolded protein response in canine cancer. Little is known about the molecular mechanisms of those two cellular pathways in dogs, and one of the reasons for such a situation is the lack of proper and well-validated research tools. In the presented dissertation, validation of antibodies that recognize the most important proteins of both pathways, proper western blot conditions for DDR and UPR study, canine ATR and Claspin pair of primers, and the adapted protocol of DNA combing assay dedicated for canine floating cells is described. Mentioned tools will facilitate further research in the broadly understood field of veterinary and comparative oncology.

The information collected and presented in the form of a literature review in P1 raised awareness about the similarities of the DDR system in dogs and humans and about the importance of doing more research in the field of veterinary oncology. It is clear that there is a lack of information on such important components of the DDR pathway as ATR and Chk1 kinases in dogs. Because of p53 mutations, cancer cells rely on the ATR-Chk1 pathway, making both proteins essential in the development of innovative anticancer therapies. Several human clinical trials with Chk1 inhibitors are now underway, with promising results but still no Chk1 inhibitors have been tested in dogs. Based on the similarities between humans and dogs DDR systems, and the results presented in **P2**, we consider that such a therapeutic approach, using Chk1 or even ATR inhibitors could be a promising direction also in veterinary medicine. Thanks to the tools presented in **P2**, further research can be carried out efficiently in canine cancer cells, and the lymphoma and leukemia cell lines used in this dissertation, have been demonstrated to be a promising model to study DDR in cancer.

UPR is critical in cancer because the tumor environment produces hypoxia, which causes ER stress. Cancer cells learn how to survive under those conditions, relying on the UPR system to be able to survive. The fact that UPR components are highly expressed in several cancers and that this system is highly connected with apoptosis, makes it attractive from a therapeutical point of view. In the results presented in **P3**, the high similarity in the UPR components and their function between humans and dogs is revealed. The activation of UPR markers (p-eIF2α and CHOP) has been successfully detected in the panel of canine cancer cells thanks to the validation of new reagents.

The required initial step of verifying methodologies for studying DDR and UPR on canine cells has been completed, and the impact of this dissertation is to facilitate further research on the topic. Thanks to the validated methods, veterinary researchers know what tools can be used to identify potential targets in DDR and UPR. Once the targets are selected, clinical trials will test if the proposed therapies targeting DDR or UPR are the future of anticancer therapy in dogs. Another important impact of this research was the finding of significant similarity between the DDR and UPR in humans and dogs, which signaled the potential interest in clinical trials in dogs, as those analyses could bring some insights that may be later used in human medicine.

Conclusion

The research carried out in the presented thesis was methodological research with the aim of validating the different techniques and reagents needed in order to further explore the molecular mechanisms of the DDR and UPR pathways in canine cancer. The following are the most important conclusions drawn from the data analysis:

1. The DDR and UPR antibodies designed to recognize human epitopes, present cross-reactivity with canine proteins.
2. The murine antibody against CHOP protein (GADD153 (sc-7351)) is not suitable for canine cancer cells, because it does not show cross-reactivity with canine cells.
3. The high expression level of CHOP protein seems to not be related to an increase in apoptosis in canine cancer cell lines tested.
4. Novel techniques such as DNA combing assay work efficiently in canine lymphoma and leukemia cells.
5. Changes in the expression levels of principal proteins of the DDR and UPR pathways have been found in canine cells, which may indicate the potential use of elements of these pathways as new therapeutic targets.

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Scientific achievements

- **Publications**

2018

Gong E-Y, Hernández B, Hernández Nielsen J, Smits V.A.J, Freire R, Gillespie D.A.

Chk1 KA1 domain auto-phosphorylation stimulates biological activity and is linked to rapid proteasomal degradation.

Scientific reports

2018-12-03 | journal-article

doi: 10.1038/s41598-018-35616-9

2020

Pawlak A, Henklewska M, Hernández Suárez B, Luzny M, Kozlowska E, Obminska-Mrukowicz B, Janeczko T

Chalcone Methoxy Derivatives Exhibit Antiproliferative and Proapoptotic Activity on Canine Lymphoma and Leukemia Cells

Molecules

2020-09-23 | journal-article

doi: 10.3390/molecules25194362

Mazur M, Kudrynska A, Pawlak A, Hernandez-Suarez B, Obminska-Mrukowicz B, Gladkowski W

Biotechnological Approach for the Production of Enantiomeric Hydroxylactones Derived from Benzaldehyde and Evaluation of Their Cytotoxic Activity

Catalysts

2020-11-13 | journal-article

doi: 10.3390/catal10111313

2021

Pawlak A, Bajzert J, Bugiel K, Hernández Suárez B, Kutkowska J, Rapak A, Hildebrand W, Obmińska-Mrukowicz B, Freire R, Smits V.A.J.

Ubiquitin-specific protease 7 as a potential therapeutic target in dogs with hematopoietic malignancies

Journal of Veterinary Internal Medicine

2021-03-02 | journal-article

doi: 10.1111/jvim.16082

Pawlak A, Henklewska M, Hernández-Suárez B, Siepka M, Gładkowski W, Wawrzeńczyk C, Motykiewicz-Pers K, Obmińska-Mrukowicz B.

Methoxy-Substituted γ-Oxa-ε-Lactones Derived from Flavanones-Comparison of Their Anti-Tumor Activity In Vitro.

Molecules

2021-10-18 | journal-article

doi: 10.3390/molecules26206295

Hernández-Suárez B*, Gillespie D.A., Pawlak A.

DNA damage response proteins in canine cancer as potential research targets in comparative oncology.

Vet Comp Oncol.

2021-12-19 | review

doi: 10.1111/vco.12795.

2023

Hernández-Suárez B*, Gillespie D.A, Dejnaka E, Kupczyk P, Obmińska-Mrukowicz B, Pawlak A.

Studying the DNA Damage Response pathway in hematopoietic canine cancer cell lines – a necessary step for finding targets to generate new therapies to treat cancer in dogs.

Frontiers in Veterinary Science

2023-08-16 | journal-article

doi: 10.3389/fvets.2023.1227683

Hernández-Suárez B*, Gillespie D.A, Obmińska-Mrukowicz B, Pawlak A.

An initial characterization of the Unfolded Protein Response pathway in hematopoietic canine cancer cell lines -a necessary step for the introduction of new therapies in dogs with cancer.

Journal of Veterinary Research

2023-09 | journal-article

doi: 10.2478/jvetres-2023-0042

- **Congresses as a participant**

2020

EACR-ESOI Virtual Conference on Imaging Cancer held through Zoom webinar – 08.07.2020

- Attendance as participant

EACR Virtual Congress held online - 18-19.06.2020

- Attendance as participant

- **Congresses as an active participant**

2020

Nowe trendy w badaniach naukowych - wystąpienie młodego naukowca, Edycja II (Poland) – 20-22.11.2020

- Oral presentation – Influence of proper lysis buffer selection in western blot technique (Beatriz Hernandez Suarez and Aleksandra Pawlak)
- Oral presentation – Testing UPR system in canine cancer cells (Beatriz Hernandez Suarez and Aleksandra Pawlak)
- Poster - Dog as a model for DDR study (Beatriz Hernandez Suarez and Aleksandra Pawlak)

III Congreso de Jóvenes Investigadores/as de Canarias y I Congreso Internacional de Jóvenes por la Investigación (Tenerife) – 26-27.11.2020

- Oral presentation - Expression of DNA damage response proteins in canine cancer cells. (Beatriz Hernandez Suarez, Aleksandra Pawlak and David Gillespie)

2021

EACR 2021 Virtual Congress - Innovative Cancer Science: Better Outcomes Through Research – 09-12.06.2021

- Poster - CHOP overexpression in canine lymphoma cells (Beatriz Hernández Suárez and Aleksandra Pawlak)

Konferencja Młodych Naukowców nt.: Analiza Zagadnienia, Analiza Wyników – Wystąpienie Młodego Naukowca, Edycja III (Poland) – 12-13.06.2021

- Poster - Flavanone-derived γ-oxa-ε-lactones induce apoptosis in canine cancer (Beatriz Hernández Suárez, Marta Henklewska, Monika Siepka, Witold Gładkowski, Czesław Wawrzeńczyk, Karolina Motykiewicz-Pers, Bożena Obmińska-Mrukowicz and Aleksandra Pawlak)
- Poster – Importance of protein's quantification in Western Blot (Beatriz Hernández Suárez and Aleksandra Pawlak)

Jornadas de Ciencia Canaria y VI Encuentro de Jóvenes Científicos (Spain) – 13-14.12.2021

- Oral presentation - El cómo Polonia me descubrió mi nueva pasión: el cáncer canino (Beatriz Hernández Suárez)

2022

Nowe trendy w badaniach naukowych - wystąpienie młodego naukowca, Edycja V (Poland) – 21-23.10.2022

- Poster - The importance of antibody selection – analysis of C/EBP homologous protein (CHOP) protein in canine cancer cells (Beatriz Hernández Suárez, and Aleksandra Pawlak)

2023

EACR-AACR-SIC - Basic and translational research conference, Immune Responses & DNA Repair - Cancer Fields Converging (Italy) – 15-17.03.2023

- Poster - Canine Cancer: a perfect model to study the role of DDR pathways in tumorigenesis and therapy (Beatriz Hernández Suárez, David A. Gillespie, and Aleksandra Pawlak)

EACR 2023 Congress: Innovative Cancer Science, Annual Congress of the European Association for Cancer Research (EACR 2023) (Italy) – 12-15.06.2023

- Poster - The use of PhenDC3 to induce cell dead in canine lymphoma and leukemia cells by stabilizing G-quadruplexes. (Beatriz Hernández Suárez, and Aleksandra Pawlak)

The 15th International Congress of the European Association of Veterinary Pharmacology and Toxicology: Theme: Our Health, Our Animals, Our Environment! (Belgium) – 02-05.07.2023

- Oral presentation - CHK1 is an interesting target to defeat lymphoma/leukemia in dogs. (Beatriz Hernández Suárez, David A. Gillespie and Aleksandra Pawlak)

- **Scholarships**

10.2022 – 06.2023 PhD for academic excellence scholarship "STER - Doctoral Scholarships for Foreigners" nr PPI/STE/2018/1/00002/U/001.

02.2020 – 09.2020 PhD for academic excellence scholarship "STER - Doctoral Scholarships for Foreigners" nr PPI/STE/2018/1/00002/U/001.

2019 - current PhD student scholarship, Projekt „UPWr 2.0: międzynarodowy i interdyscyplinarny program rozwoju Uniwersytetu Przyrodniczego we Wrocławiu" - Umowa nr POWR.03.05.00-00-Z062/18.

- **Internships**

2022

Centre de Recherches en Cancérologie de Toulouse (Oncopole) (CRCT) - Toulouse (France) – 10.01.2022-09.01.2023

One-year internship in the team of Stefania Millevoi "RNAreg : RNA-Binding Proteins And Genotoxic Stress".

-CO-Immunoprecipitation

-RNA-Immunoprecipitation

-RT-qPCR

-Plasmids purification

-siRNA transfection

Centro Andaluz de Biología Molecular y Medicina Regenerativa (CABIMER) - Seville (Spain), Erasmus + internship – 06.09.2021-05.12.2021

Three months internship, in the Andres Aguilera's team, supervised by Sonia Barroso:

-DNA combing assay

- **Trainings**

2022

<<Les lignées cellulaires génétiquement modifiées (OGM) comme modèle d'étude (English: Genetic modified cell lines (GOM) as a model)>> certificate issued by CNRS Formation (France) – 17-18.11.2022

The objectives of this training are as follows: Know the regulatory framework for developing new genetically modified cell lines (GMOs), be able to choose the vectorization method to derive a new igneous cell, understand the CRISPR-Cas system applied to the genetic modification of cells, and know how to analyze what needs to be maintained, improved or changes in cellular culture practice

<< Image J>> certificate issued by Inserm DR Toulouse – Formation (France) – 28-30.03.2022

Onsite training course on Image J software. Discover the possibilities of Image J software. Acquire the basics of processing and analyzing images in biology.

<<Intro to Omics course>> (online), certificate issued by Glasgow Polyomics, University of Glasgow – 24-28.10.2022

A course held over 5 days, aimed at familiarizing participants with the basis and application of various omics disciplines: genomics, transcriptomics, metabolomics, lipidomics, proteomics, and bioinformatics. Each of the omics disciplines will be covered by a lecture and a practical bioinformatics session. By the end of the course users should understand, for each omics level: the basis of the discipline, the instrumentation used to generate high-throughput biological data, key applications, and how to visualize the resulting data using commonly used software packages. Participants will also be aware of how different large-scale data sets can be integrated in order to obtain better biological inference and appreciate the nature of other modern challenges in bioinformatics.

2021

<<European Grant Writing and Management Workshop>> (online), certificate issued by Prof Miklas Scholz, Lund University – 9.11.2021 and 14.12.2021

This practical workshop teaches graduates and PhD students to write publishable conference and journal articles as well as fundable substantial tender and grant applications such as large Horizon Europe grants in engineering and science. Moreover, the workshop gives practical insights in European grant management using the current WATERAGRI and RainSolutions grants as examples.

<<HMX Pro Immunology – Immuno-oncology>> (online), certificated issued by Harvard Medical School – 25.06.2021

Advances in our knowledge of the immune system are changing the ways in which we treat various types of cancer. Understanding how immune cells recognize and kill cancer cells, and what we can do to enhance their ability to fight cancer, is important for anyone working to develop new cancer treatments or apply them in the clinic.

<<HMX Pro Pharmacology – Essentials >> (online), certificated issued by Harvard Medical School – 25.06.2021

Understanding how drugs act in the body is vital for effectively treating patients. In HMX Fundamentals Pharmacology, you'll learn about the principles governing what the body does to a drug and in turn what a drug does to the body. Along the way you will see how this knowledge comes into play in real-world scenarios and clinical settings.

2020

<<Western Blot training>> (online), certificate issued by Abcam – 10.2020

From basic principles and essential steps of the western blot protocol to optimization and more advanced techniques. The training shows how to run western blot experiments and help troubleshoot them.

<<Antibody basics training>> (online), certificate issued by Abcam – 08.2020

A guide from choosing the right antibody for experiment, handling and storing antibodies through to validation, choosing the appropriate controls, optimizing your protocol, and troubleshooting. Description about the difference between antigen vs antibody and how to choose a high-quality antibody with minimal batch-to-batch variability.

- **Awards**

Audience award in the 5th edition of the polish selection in the conquest « Ma thèse en 180 secondes Europe Centrale », co-organise by CCFEF and l'association Plejada in coopertation with l'Institut français de Pologne. – 03.2023

Aim was to explain the subject of the doctoral thesis: "The role of tumor associated proteins in DNA damage and Unfolded protein responses and their use as target for the development of novel canine cancer therapy." in 3 minutes, and in French.

Publication authorship statement

Beatriz Hernández Suárez

Name and surname

Wroclaw August 18, 2023

(place and date)

Department of Pharmacology and Toxicology,

Wroclaw University of Environmental and Life Sciences,

Norwida 31,

50-375 Wroclaw, Poland

Affiliation

STATEMENT

I declare that in the paper:

> Hernández-Suárez B*, Gillespie DA, Pawlak A. DNA damage response proteins in canine cancer as potential research targets in comparative oncology. Vet Comp Oncol. 2022 Jun;20(2):347-361. doi: 10.1111/vco.12795. Epub 2022 Jan 10. PMID: 34923737; PMCID: PMC9304296.

my role was the leading one. My contribution as corresponding and first author was to collect the literature required to write this review, to establish the concept of the manuscript, to prepare the figures, write the first draft and the final version, submit it to the journal, and apply all the revisions requested by reviewers under the revision process prior to acceptance.

18/08/2023 Beatriz HS

Date and signature

Beatriz Hernández Suárez

Name and surname

Wroclaw August 18, 2023

(place and date)

Department of Pharmacology and Toxicology,

Wroclaw University of Environmental and Life Sciences,

Norwida 31,

50-375 Wroclaw, Poland

Affiliation

STATEMENT

I declare that in the paper:

> Hernández-Suárez B*, Gillespie D.A, Dejnaka E, Kupczyk P, Obmińska-Mrukowicz B, Pawlak A. Studying the DNA Damage Response pathway in hematopoietic canine cancer cell lines – a necessary step for finding targets to generate new therapies to treat cancer in dogs. Front. Vet. Sci. 2023 Aug; vol. 10 doi: 10.3389/fvets.2023.1227683

my role was the leading one. My contribution as corresponding and first author was to conduct the research, analyze and visualize the results, prepare the figures, write the first draft and the final version, submit it to the journal, and apply all the revisions requested by reviewers under the revision process prior to acceptance.

18/08/2023 Beatriz

Date and signature

Beatriz Hernández Suárez

Name and surname

Wroclaw August 18, 2023

(place and date)

Department of Pharmacology and Toxicology,

Wroclaw University of Environmental and Life Sciences,

Norwida 31,

50-375 Wroclaw, Poland

Affiliation

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