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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

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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.

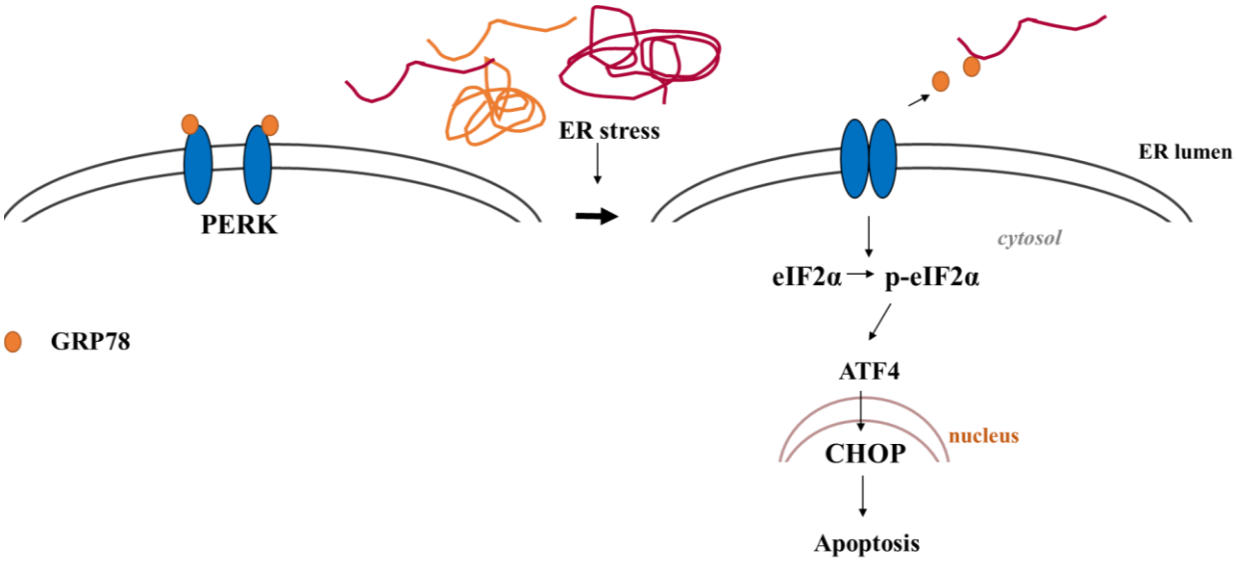

Fig. 1. Unfolding protein response pathway scheme. ER – endoplasmic reticulum; PERK – protein kinase RNA-like ER kinase; eIF2α – eukaryotic translation initiation factor 2α; p-eIF2α – phosphorylated eukaryotic translation initiation factor 2α; GRP78 – glucose-regulated protein 78; ATF4 – activating transcription 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°C 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.

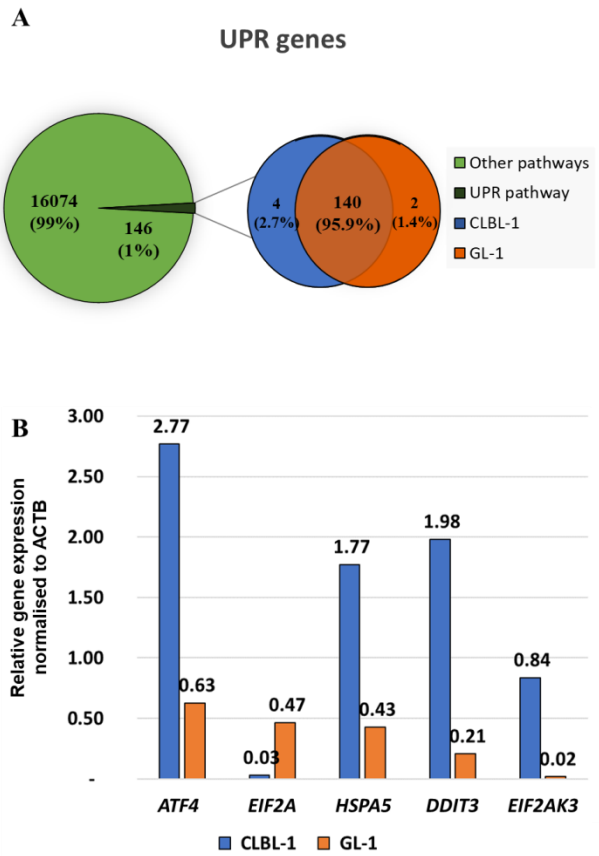

Fig. 2. Relative gene contents of the unfolded protein response (UPR) pathway and other pathways among genes expressed in the CLBL-1 B-cell lymphoma and GL-1 B-cell leukaemia canine cell lines (A) and relative expression of principal genetic components of the UPR pathway normalised to *ACTB* gene expression (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).

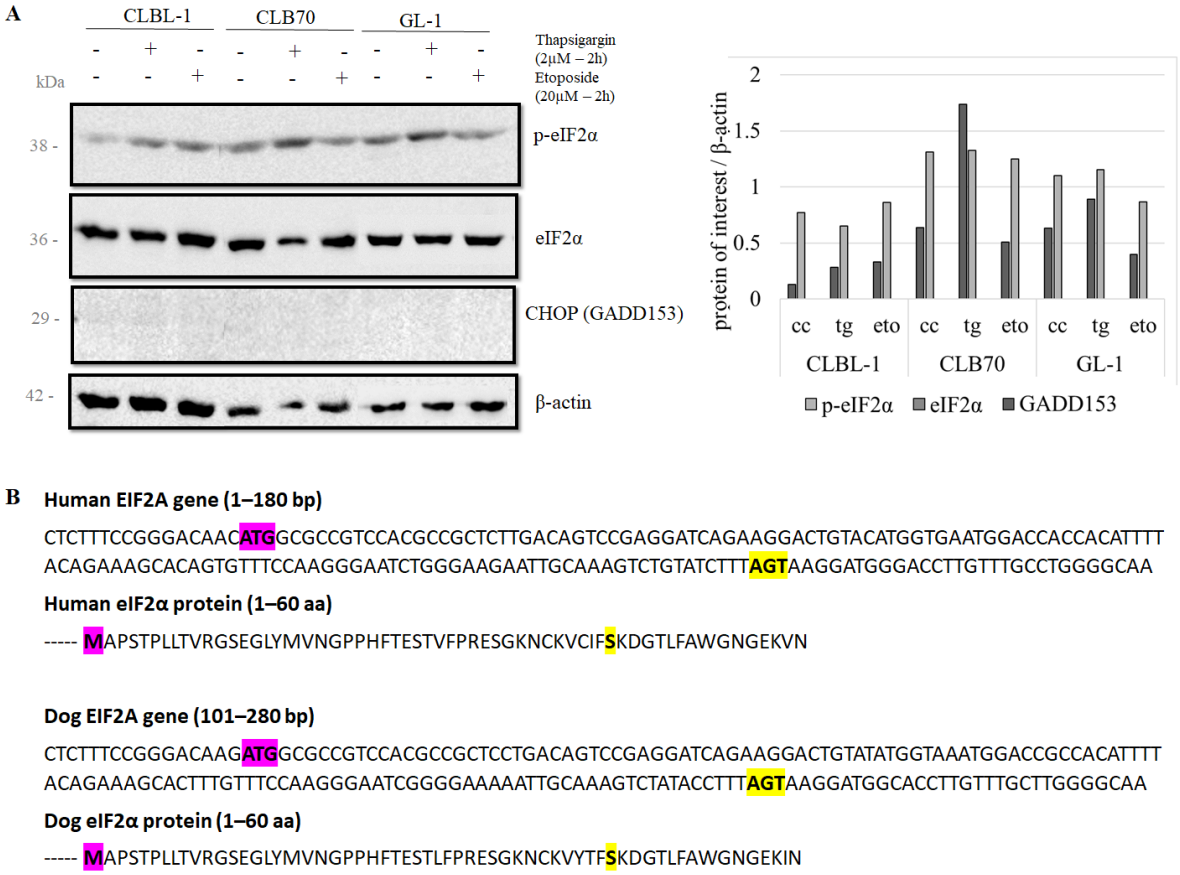

Fig. 3. Expression of unfolded protein response (UPR) proteins by canine lymphoma (CLBL-1 – B-cell lymphoma) and leukaemia (CLB70 – B-cell chronic lymphocytic leukaemia and GL-1 – B-cell leukaemia) cell lines treated with thapsigargin in order to induce endoplasmic reticulum stress (A). Expression levels 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.

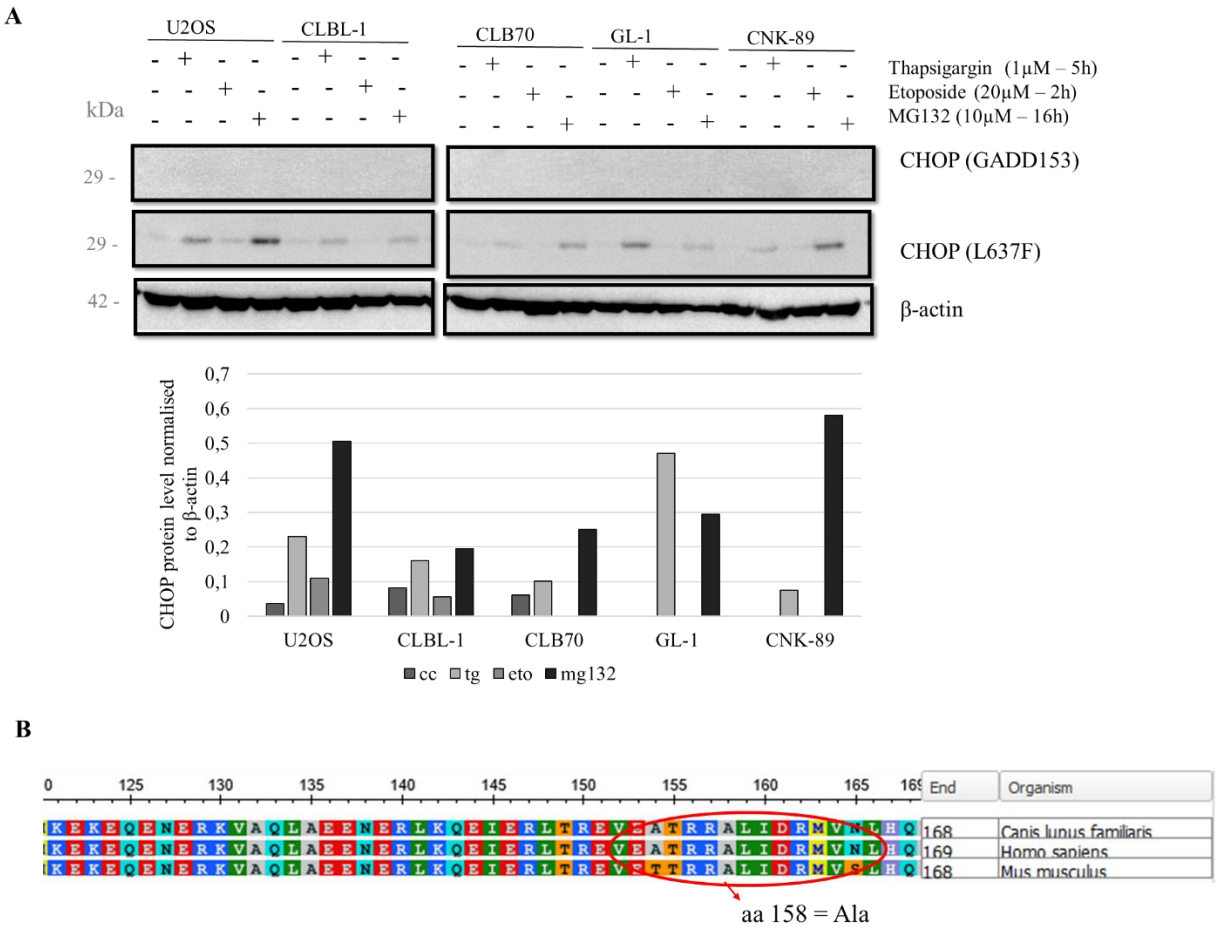

Fig. 4. Expression of CCAAT enhancer-binding protein homologous protein (CHOP) by canine lymphoma (CLBL-1 – B-cell lymphoma and CNK-89 – natural-killer-cell lymphoma), canine leukaemia (CLB70 – B-cell chronic lymphocytic leukaemia and GL-1 – B-cell leukaemia) and human osteosarcoma (U2OS) cell lines 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%.

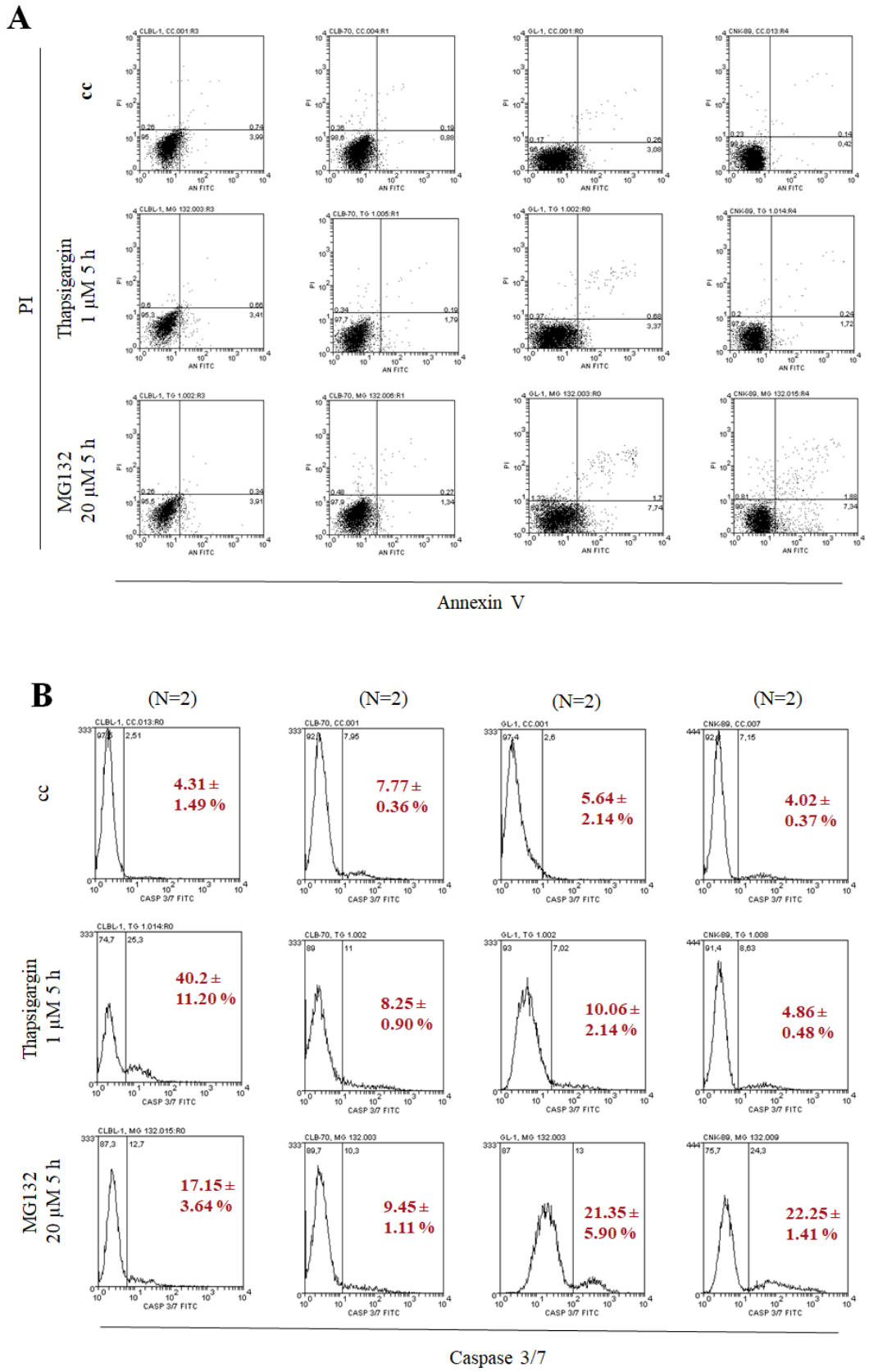

Fig. 5. Apoptosis in canine lymphoma (CLBL-1 – B-cell lymphoma and CNK-89 – natural-killer-cell lymphoma) and leukaemia (CLB70 – B-cell chronic lymphocytic leukaemia and GL-1 – B-cell leukaemia) cell lines treated with thapsigargin and MG132 to induce endoplasmic reticulum stress and stained with annexin 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.

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