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Article

Involvement of Epithelial to Mesenchymal Transition in Canine Mammary Tumor Progression

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Simple Summary: The effect of Doxorubicin presents efficiency on canine mammary tumor therapy and induces the alteration of expression of the key genes involved in the epithelial to mesenchymal transition processes.

Abstract: Canine mammary tumors represent one of the leading malignant pathologies in female dogs, displaying the importance of efficient therapeutic findings, beside the golden standard surgery, able to limit the development of the disease. Studies in human cancers demonstrated that Doxorubicin presents a good effect in different biological processes like apoptosis, autophagy, cell cycle, cell invasion and epithelial to mesenchymal transition. Our study followed the effects of Doxorubicin on two canine mammary cancer cell lines P114 and CMT-U27, showing an inhibitory effect in cell proliferation and an expression alteration of the EMT related genes

Keywords: cancer; canine; mammary tumors; epithelial to mesenchymal transition; doxorubicin

1. Introduction

Mammary tumors represent the most frequent mammary gland neoplasia in female dogs. In epidemiological studies, the annual incidence of mammary tumors is 47.7% for benign and 47.5% for malignant tumors [1]. In the USA, mammary tumors in canines have decreased because of early neutering, but in European countries still are the most frequent mammary cancer forms in female dogs [1,2]. The golden standard treatment procedure worldwide is represented by the surgical excision of the mammary chain but with unsatisfactory results. The high number of dogs with mammary cancer show lymphatic or vascular invasion and high rates of recurrence and metastasis [3,4]. There is limited information about chemotherapy and radiotherapy efficacy in treating canine mammary tumors CMT, probably because the procedures are expensive. Despite some case reports regarding the responses to chemotherapeutics such as doxorubicin [5], mitoxantrone [6], paclitaxel [7,8] and carboplatin [9], it is not clear if the chemotherapy improves overall survival of the treated dogs [2,10].

One of the most used therapeutic agents in human cancer is the anthracycline Doxorubicin (DOXO) [11–13]. In the last years, a few studies have shown the efficiency of this drug in canine patients [4,14]. Doxorubicin induces the production of free radicals, intercalation into DNA strands and inhibition of topoisomerases I and II, causing DNA damage and activating caspases, ultimately leading to apoptosis [12,15].

Epithelial-to-mesenchymal transition (EMT) is essential in embryonic development and tissue regeneration. An aberrant reactivation of EMT is associated with malignant properties of tumor cells during cancer progression and metastasis, including migration and invasiveness, increased tumor stemness, and enhanced resistance to therapy [16–18]. Studies performed in humans identified EMT

as involved in DOX-resistance to therapy, stimulating migration of tumor cells and metastasis; furthermore, EMT mediates resistance of cancer cells to DOX-mediated apoptosis [18,19].

The present study aimed to analyze the inhibitory effect of Doxorubicin in *vitro* models of canine mammary cancer to evaluate the efficiency of this therapeutic agent and the expression levels of key genes involved in the EMT process.

2. Materials and Methods

Cell lines and cell culture

Our experiment was conducted in two canine mammary carcinoma cell lines (P114 and CMT-U27) kindly donated by Prof. Gerard Rutteman from the Netherlands and Prof. Eva Hellmen from Sweden. The two canine cell lines were cultured in RPMI supplemented with 10% Fetal Bovine Serum (FBS) and 1% Glutamine for P114, respectively DMEM low glucose supplemented with 10% Fetal Bovine Serum (FBS) and 1% Glutamine for CMT-U27. The culture medium and supplements were obtained from Sigma-Aldrich.

The P114 cell line is a canine anaplastic mammary carcinoma (AMC) cell line shown in dogs and felines. In dogs, AMC is considered the most aggressive type of mammary tumor, showing a low frequency, and is associated with a short survival time. This histologic presentation is characterized by a diffuse infiltrating neoplasm composed of large, pleomorphic, multinucleated giant cells, frequently showing acidophilic cytoplasm, and dispersed nuclear chromatin. The CMT-U27 cell line is a canine mammary carcinoma cell line [20–22]

MTT cell viability assay

1x10⁴ cells/well were cultured in 96-well culture plates for 24h at 37°C in 5% CO₂ atmosphere incubators. After 24h incubation, cell cultures were treated with Doxorubicin – Sigma Aldrich (DOXO) doses. At 48h after treatment, the medium was discarded, and 100 µl MTT solution was added to every well. After 2h incubation at 37°C, the MTT solution was removed, and the formazan crystal was solubilized with 100 µl DMSO (dimethyl sulfoxide) (Sigma-Aldrich). The absorbance was measured at 570/690 nm for the cell viability assay in a microplate reader (Synergy H1 Hybrid Reader Biotech).

Apoptosis assay by fluorescence microscopy

The Multi-Parameter Apoptosis Kit (Cayman) was used for the fluorescence microscopy evaluation of apoptosis. Cell staining was performed according to the manufacturer's protocol. Stained cells were analyzed at UV wavelength for Hoechst and 560/595 nm for TMRE staining on an Olympus IX71 inverted microscope. Hoechst is a specific stain for the nucleus, while TMRE indicates the mitochondrial membrane activity potential.

Apoptosis evaluation was also performed using a triple fluorescence marking by Actin-stain 488 Fluorescent Phalloidin (Cytoskeleton, Inc.), Mitotracker (Invitrogen) for mitochondria labelling and DAPI (4',6-diamidino-2-phenylindole) (Invitrogen) for nucleus staining.

Confluency assay

Cell lines were cultured in a 96-well culture plate for 48h and then treated with 2.5 µM DOXO. After 48 h treatment, cell confluency was analyzed using a Celigo Image Cytometer (Nexcelom).

Cell Cycle Assay by Celigo

Cell lines were cultured in a 96-well culture plate for 48 h and then treated with 2.5 µM DOXO. After 48 h treatment, cells were fixed in ice-cold methanol at 4°C 30 minutes. After fixation, the cells were marked with RNase A and Propidium Iodide (PI) solution for 45 minutes in dark 37°C in 5% CO₂ atmosphere incubators. The cell cycle analysis was performed using Celigo Image Cytometer (Nexcelom).

Colony formation assay

P114 and CMT-U27 cells were incubated with 2.5 μ M doxorubicin for 48h to induce DNA damage. The cells were detached after 48 hours, counted, and reseeded in the standard medium in triplicate for each condition and cellular type (500 cells/6 well plate). Cells returned to the incubator for 7 days to form colonies and then were fixed with ice-cold methanol for 15 minutes, dried, and stained with 0.5% crystal violet in 25% methanol for 20 minutes. After washing with water and drying, colonies were counted manually using the Celigo Image Cytometer (Nexcelom).

Scratch assay

Canine mammary cancer cells were pre-treated individually with DOXO for 48 hours in 24-well plates. After treatment, cell proliferation was blocked by a 30-minute pretreatment with mitomycin C (40 μ g/ml). A scratch was then made in each well using a 20- μ l pipette tip, and cells were maintained in a medium with 1% serum. The distances between the edges were scaled at 0, 8, and 24h.

Cell invasion assay

Canine cells (15X10³ cells/well) were cultured in the upper 8 μ m Falcon cell culture inserts pre-coated with 100 μ l Matrigel in 500 μ l media without serum. In the lower chambers (24 well plates), 750 μ l complete medium containing 10% FBS was added. After 48 h of incubation, the cells that had invaded through the pores and attached to the bottom surface of the membrane were stained with Calcein-AM (Invitrogen) according to the manufacturer's protocol. Images of the stained invaded cells were captured under an Olympus IX71 inverted fluorescent microscope.

Gene expression evaluation by PCR Array

For the extraction of total RNA, the Trizol (TriReagent Sigma-Aldrich) protocol was used, and the quantitative and qualitative control was performed using the Nanodrop-1000 spectrophotometer (Thermo Scientific), and the Agilent Bioanalyzer 2100. CDNA synthesis was performed using the RT² First Strand Kit (Qiagen), according to the manufacturer's recommendations. For the RT² Profiler PCR Array (PAFD-090ZE-1 Dog Epithelial to Mesenchymal Transition – EMT- Qiagen), we used the SYBR Green Master Mix and the ViiA7 instrument from Applied Biosystems according to the manufacturer protocol. Alterations of gene expression levels for the studied genes were evaluated using the Qiagen RT² Profiler PCR Array and Assays Data Analysis software.

3. Results

3.1. Doxorubicin inhibits cell viability in both *in vitro* canine mammary cancer models

Treatment of P114, CMT-U27 mammary cancer cell lines with different concentrations of DOXO for 48 hours shows inhibition of viability in a dose-dependent manner for both canine cell lines. Moreover, the two cell lines have similar values for the half maximal inhibitory concentration with an approximate dose of 13 μ M Doxorubicin (Figure 1A,B). Because of the high toxicity of the DOXO we choose to apply the smallest concentration which shows an inhibitory effect in the cell proliferation in the canine mammary tumor cell lines. Due to the relatively equal concentration of DOXO corresponding to the IC₅₀ of each cell line, we decided to continue the experiments with 2.5 μ M of therapeutic agent to compare the response of each cell line under standard conditions.

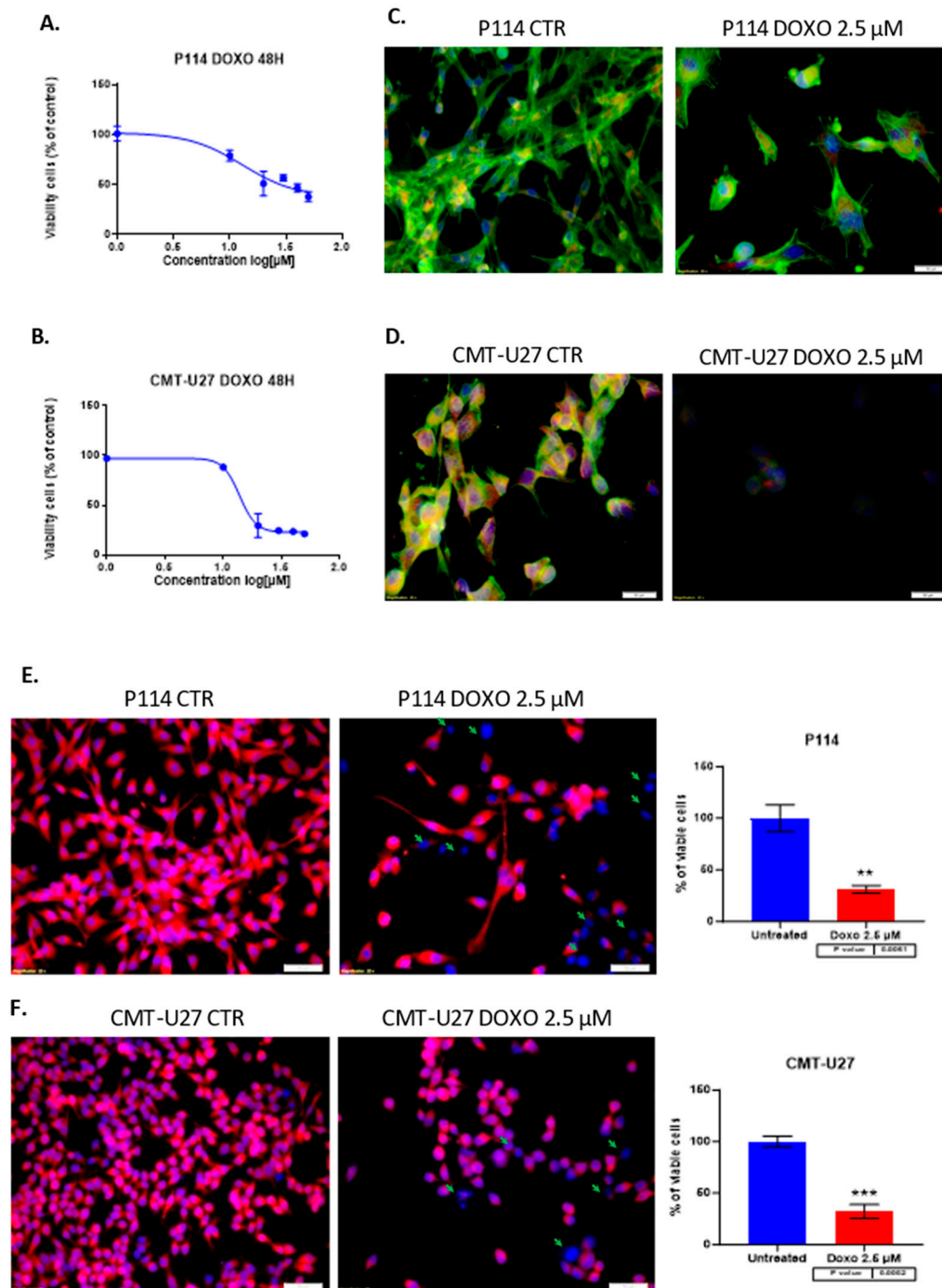


Figure 1. MTT cell viability assay and IC₅₀ value identification after treatment with gradual doses of Doxorubicin in P114 and CMT-U27 canine mammary cancer cell lines for 48h. Doxorubicin inhibits cell viability within all cell lines with an IC₅₀ of 13 μM for P114 and 13.80 μM for CMT-U27 cell line (A/B). Actin damaging and mitochondrial membrane potential activity and nuclear morphology evaluation. Observation of the Phalloidin-FITC/Mitotracker/DAPI staining by Olympus IX71 inverted fluorescence microscope after 48h of treatment with 2.5 μM of Doxorubicin; image magnification 20X (C/D) and the mitochondrial potential activity inhibition, cytoskeleton damage and nuclear morphology changes using TMRE/ Hoechst (E/F).

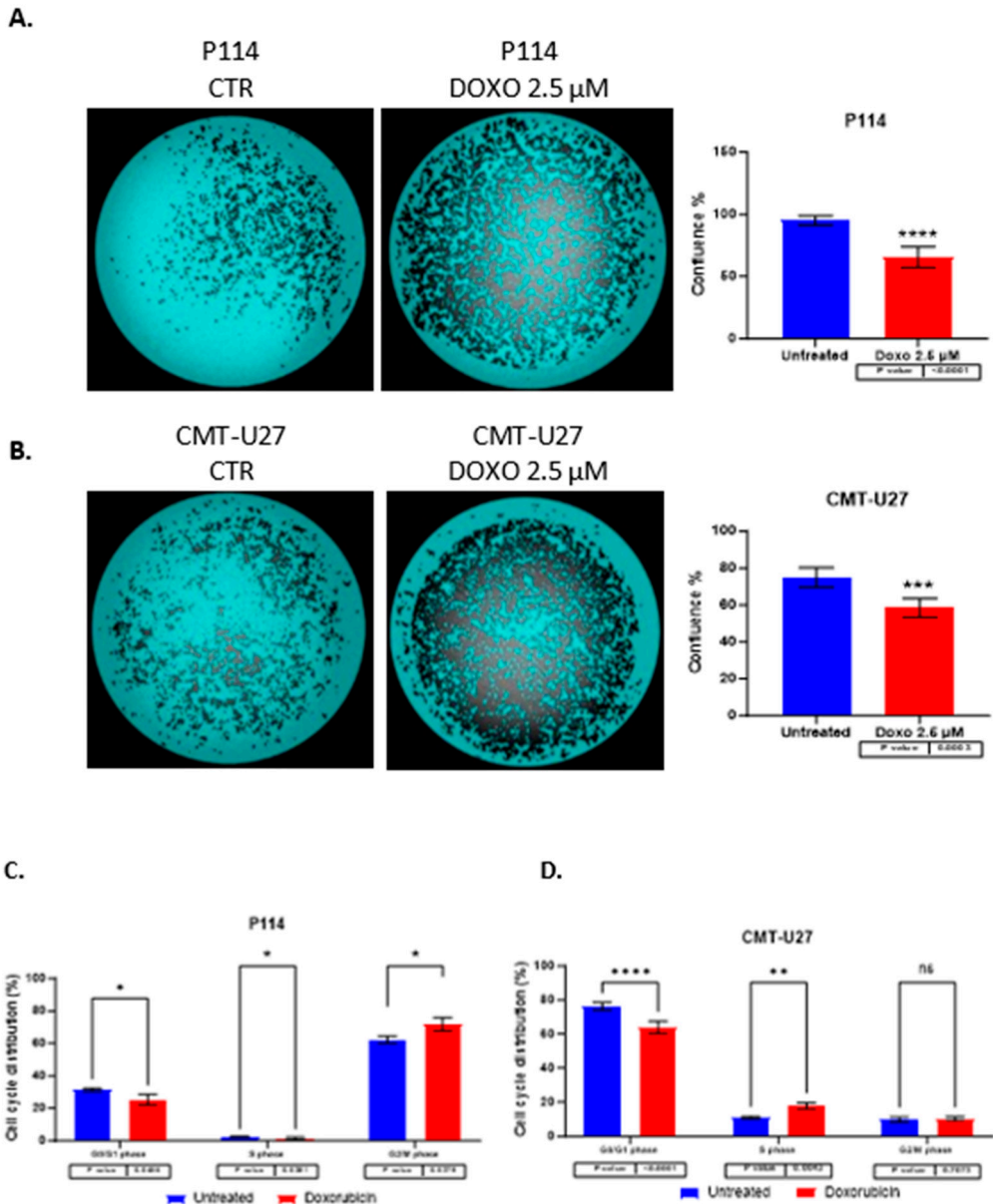
3.2. Doxorubicin treatment decreases cell viability and induces apoptosis in cell lines

The triple fluorescence staining for the cytoskeleton, mitochondria, and nucleus shows damage to the cells by the treatment (Figure 1C,D). Fluorescence microscopy assay revealed that DOXO

treatment after 48h induced in P114 and CMT-U27 cell lines a decreased mitochondrial membrane potential activity and morphological changes, including nuclear DNA condensation, nuclear shrinkage, and fragmentation (marked with green arrows). We can observe a significant reduction of the viable cell's percentage to 30% (p-value = 0.0061) in the P114 treated cells versus untreated cells and 32% for the CMT-U27 cells (p-value = 0.0002) (Figure 1E,F).

3.3. Reduced cell confluency in the treated versus untreated cells

P114 and CMT-U27 treated with 2.5 μM of DOXO for 48h presents a lower confluency rate than untreated cells. We can observe that the treated cells show between 30-40% reduced confluency versus the untreated cells. (Figure 2A,B)



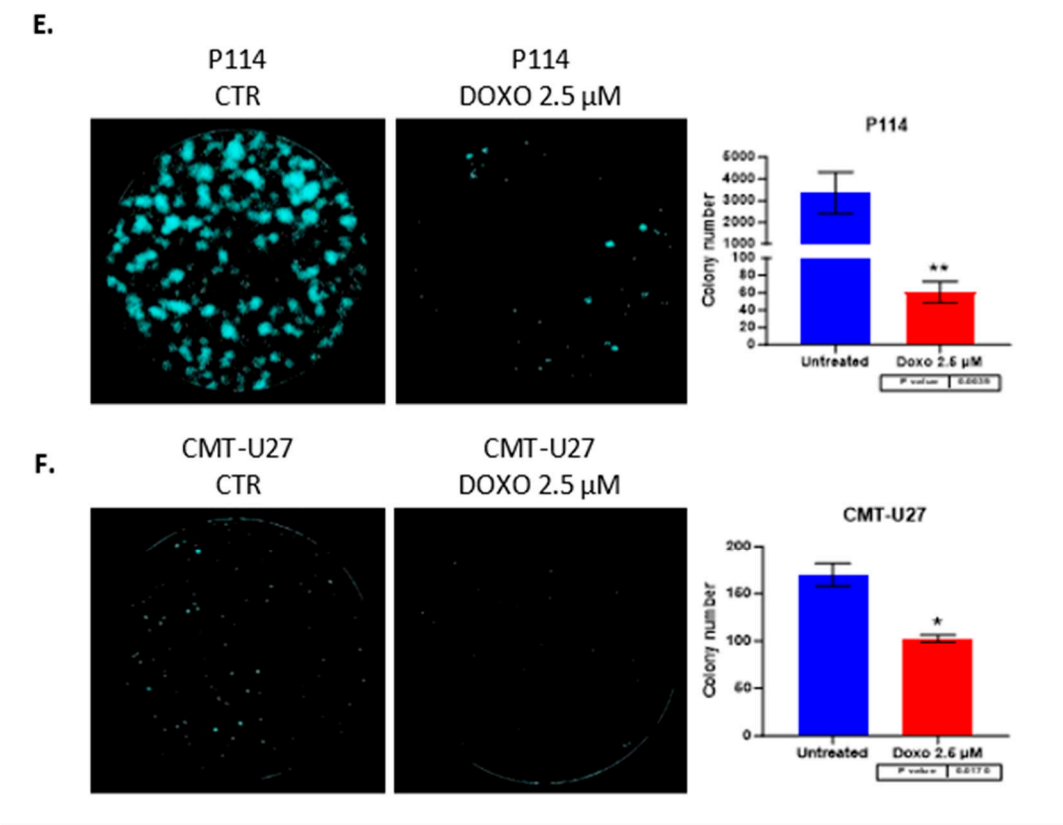


Figure 2. The confluency quantification after 2.5 μ M DOXO treatment for 48h. P114 and CMT-U27 cell lines treated with Doxorubicin show decreased confluency evaluated by Celigo Image Cytometer (A/B). Cell cycle distribution after 2.5 μ M DOXO treatment for 48h. P114 and CMT-U27 cell lines treated with Doxorubicin show a cell cycle arrest in G0/G1 phase (C/D). Colony formation after 2.5 μ M DOXO treatment for 48h. P114 and CMT-U27 cell lines treated with Doxorubicin show decreased colony formation capacity. Quantifying colonies in treated cells versus untreated cells after 7 days by Celigo Image Cytometer (E/F).

3.4. Cell cycle arrest induced by Doxorubicin

Doxorubicin induces cell cycle arrest in both cell lines in the G0/G1 phase. For the evaluation of cell growth inhibition induced by ABT-199, a cell cycle assay was performed by Celigo imaging flow cytometry. In both cell lines was observed a decreased percentage of cells in the G0/G1 phase in treated cells versus control cells. In the P114 treated cell line, the S phase distribution is slightly decreased (p-value 0.0456), the G2/M phase distribution increased from 62% to 72% (p-value 0.0379), and the G0/G1 phase distribution decreased from 31% to 25% (p-value 0.0456). CMT-U27 treated cells show an increased distribution in the S phase from 10% to 17% (p-value 0.0043), while the G0/G1 phase distribution decreased from 76% to 64% (p-value <0.0001). In the G2/M phase, no statistically significant changes were observed (Table 1) (Figure 2C,D).

Table 1. Cell cycle distribution in both canine mammary cancer cell lines.

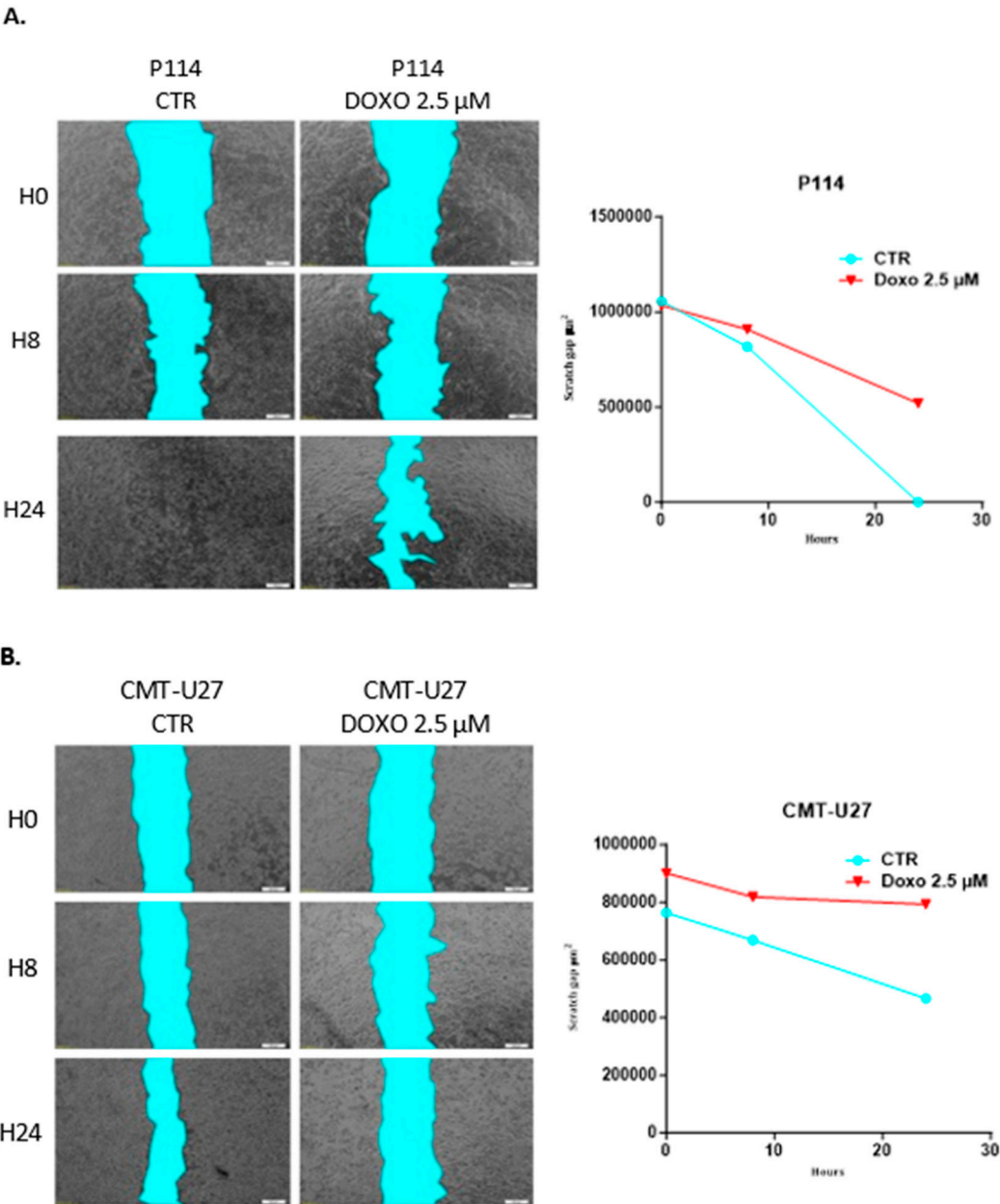
Cell line	Mean number of cells (%)					
	G0/G1	S	G2/M	G0/G1	S	G2/M
	CTR			DOXO		
P114	31.34	2.47	62.17	25.28	1.37	71.84
CMT-U27	76.35	10.86	9.69	63.95	17.79	10.20

3.5. Doxorubicin reduces colony formation in both canine mammary cancer cell lines

Both canine cell lines treated with 2.5 μ M of DOXO for 48h show a significantly reduced colony formation capacity demonstrated through the ability of untreated cells to form more colonies compared to the treated (P114 p-value 0.0039; CMT-U27 p-value 0.017) ones where we can observe a significant low number of colonies after 7 days from the treatment. (Figure 2E,F)

3.6. Doxorubicin impairs cell migration in both canine in vitro mammary cancer models

CMT-U27 and P114 cell lines treated with 2.5 μ M of DOXO for 48h show a significantly reduced migratory capacity demonstrated through the ability of control cells to close the gap from the scratch assay compared to the treated ones where the gap is still significantly visible after 48h (Figure 3A,B).



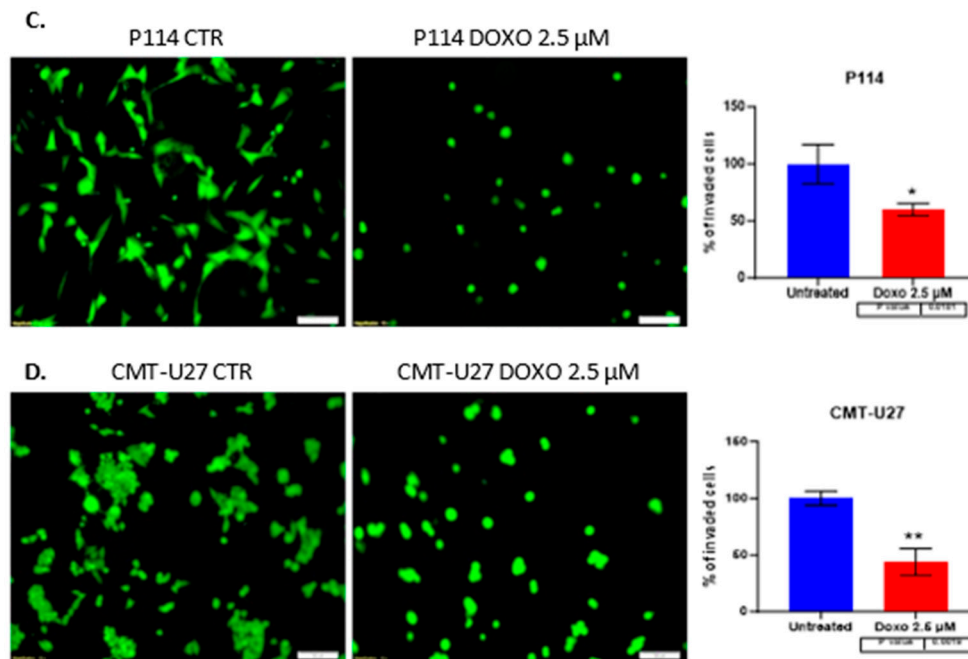


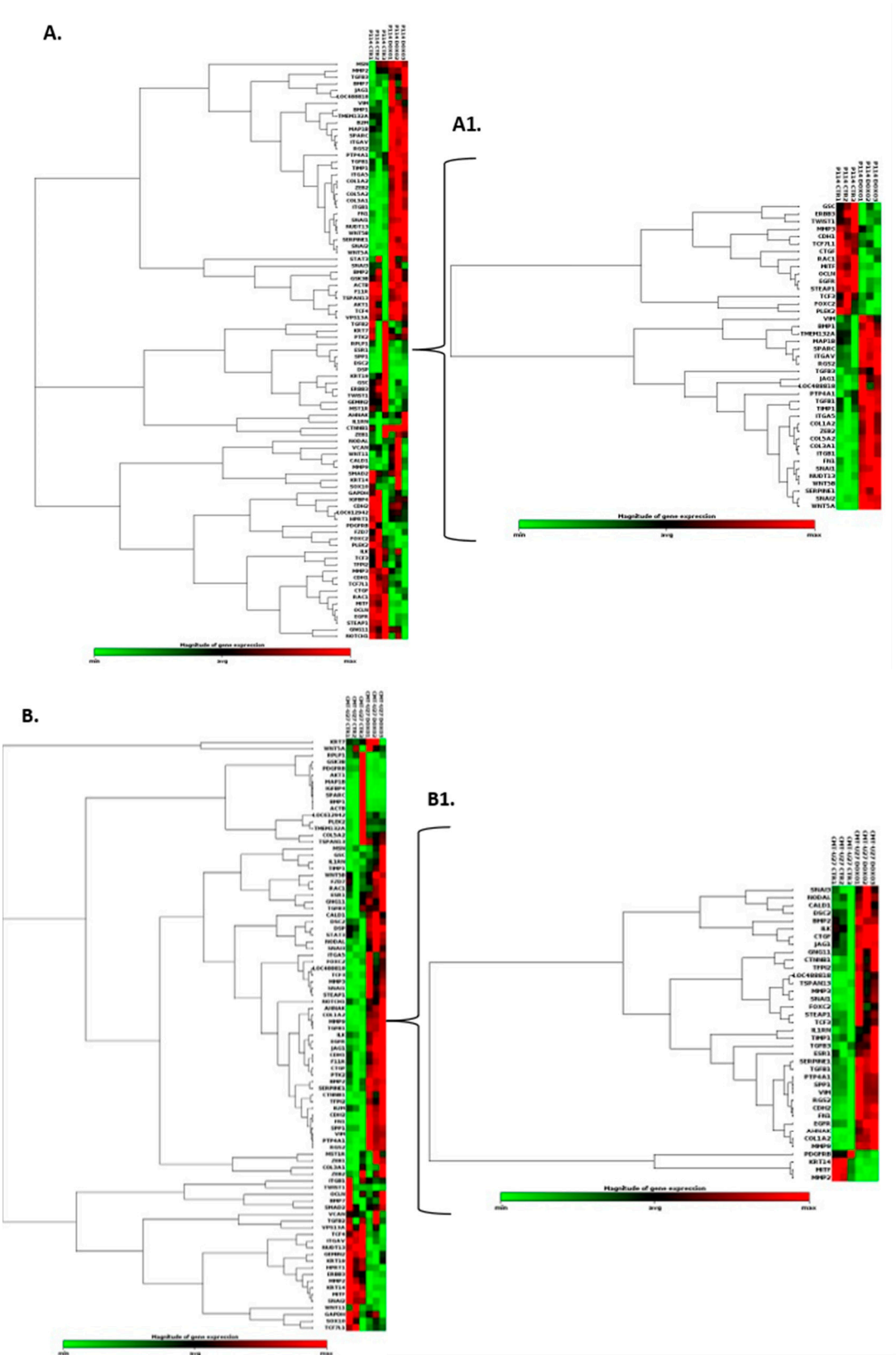
Figure 3. Scratch assay and quantification of cell migration after 2.5 μ M DOXO treatment for 48h. P114 and CMT-U27 cell lines treated with Doxorubicin show decreased migratory capacity after 48 hours. Quantifying wound area in control cells versus treated ones was evaluated after 0h, 8h and 48h by an Olympus IX71 microscope, objective 4X and CellSens Imaging software (A/B). Invasion capacity evaluation using Matrigel (Invitrogen) and fluorescence microscopy. In both canine cell lines treated with DOXO, we can observe a reduced percentage of invaded cells versus the untreated cells. The fluorescence microscopy evaluation was done by marking the cells with Calcein-AM and observed by Olympus IX71 inverted fluorescence microscope; image magnification 20X (C/D).

3.7. Doxorubicin treatment suppresses cell invasion

A significantly suppressed invasion ability was observed in both canine mammary cancer cell lines treated with DOXO. Canine cells treated with 2.5 μ M DOXO show a reduced percentage of the invaded cells, a 60% p-value of 0.018 for P114 and a 43% p-value of 0.001 for the CMT-U27 cell line. (Figure 3C,D)

3.8. Doxorubicin treatment induces EMT gene expression level alteration

Based on the RT² Profiler PCR Array analysis of Dog Epithelial to Mesenchymal Transition genes, a total of 41 for the P114 cell line and 38 for the CMT-U27 cell line significantly (p-value < 0.05) differentially expressed EMT-related genes were identified (Figure 4A/A1, B/B1). Nineteen genes were identified to be common in the two cell lines treated with DOXO. P114 cell line treated with DOXO shows an altered expression level for five genes (LOC488818/FGF-BP1, RGS2, FOXC2, STEAP1 and MMP3) and CMT-U27 only for two genes (LOC488818 and RGS2) (Figure 4C/C1 4D/D1).



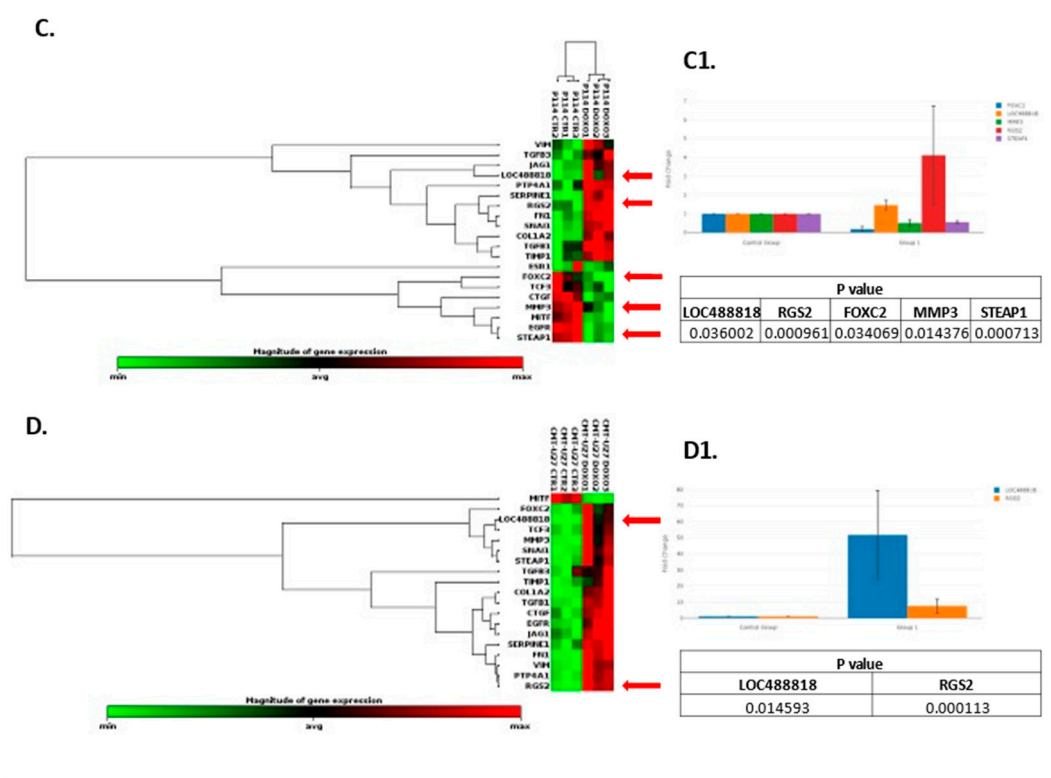
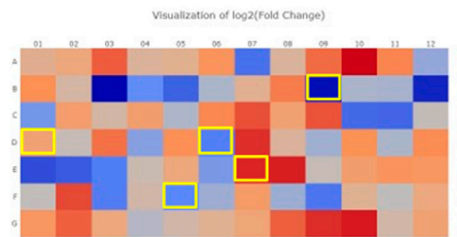
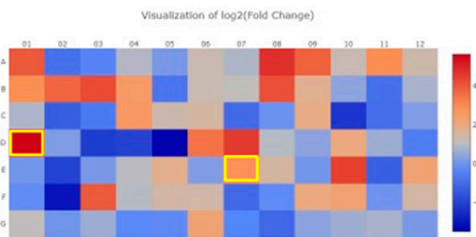


Figure 4. EMT PCR Array gene expression analysis. In both canine cell lines treated with DOXO we can observe a significantly (p -value < 0.05) EMT related gene expression alteration represented by clustergrams generated with Qiagen analysis software for P114 (A/A1) and CMT-U27 (B/B1). Clustergrams, multigroup plots and p -values for the common altered genes for the treated P114 (C/C1) and CMT-U27 cell lines (D/D1).

A. P114



B. CMT-U27



C.

	1	2	3	4	5	6	7	8	9	10	11	12
A	AHNAK	AKT1	BMP1	BMP2	BMP7	CALD1	CDH1	CDH2	COL1A2	COL3A1	COL5A2	CTGF
B	CTNBN1	DSC2	DSP	EGFR	ERBB3	ESR1	F11R	FN1	FOXC2	FZD7	GEMIN2	GNG11
C	GSC	GSK3B	IGFBP4	IL1RN	ILK	ITGA5	ITGAV	ITGB1	JAG1	KRT14	KRT19	KRT7
D	LOC488818	LOC612942	MAP1B	MITF	MMP2	MMP3	MMP9	MSN	MST1R	NODAL	NOTCH1	NUDT13
E	OCLN	PDGFRB	PLEK2	PTK2	PTP4A1	RAC1	RGS2	SERPINE1	SMAD2	SNAI1	SNAI2	SNAI3
F	SOX10	SPARC	SPP1	STAT3	STEAP1	TCF3	TCF4	TCF7L1	TFPI2	TGFBI	TGFB2	TGFB3
G	TIMP1	TMEM132A	TSPAN13	TWIST1	VCAN	VIM	VPS13A	WNT11	WNT5A	WNT5B	ZEB1	ZEB2

Figure 5. EMT PCR Array gene expression analysis. In both canine cell lines treated with DOXO, we can observe a significant (p-value < 0.05) EMT-related gene expression alteration (marked with yellow) represented by heat maps generated with Qiagen analysis software for P114 (A) and CMT-U27 (B). In the C panel is represented the list of the genes from the PCR array and the significantly (p-value < 0.05) expressed genes marked with red.

4. Discussion

Our *in vitro* study aimed to investigate doxorubicin's efficacy in treating CMT. Our findings suggest that therapy with Doxorubicin has apoptotic effects on canine *in vitro* models of mammary cancer and can significantly reduce cell migration, invasion, confluency, and cell colony formation and can induce cell cycle arrest in treated versus untreated cells. Moreover, Doxorubicin treatment can alter EMT gene expression levels. Consistent with our data, DOXO treatment inhibited the cell proliferation at 48h in two CMT cell lines: one from a primary tumor and one from a lymph node metastasis; furthermore, a substantial effect on the cell cycle has been observed and an increase in the gene expression of P-glycoprotein (P-gp) and Breast Cancer Resistance Protein (BCRP) compared to the controls [14].

Epithelial-to-mesenchymal transition (EMT) is a biological process that occurs in various types of tumors, including canine mammary tumors [1]. In this complex cellular phenomenon, epithelial cells are organized tightly and present specific adhesion properties. They suffer molecular and structural characteristics to become more mesenchymal cells, losing their connectivity and acquiring migratory properties [2]. This phenomenon involves cancer progression, invasion, and metastasis [3]. EMT plays a key role in cancer progression [23,24]. Our data suggests that canine mammary tumors can benefit from the activity of Doxorubicin through inhibition of EMT and different cancer-related biological processes inducing tumor suppressor mechanisms. Regulator of G protein signaling 2 (RGS2) belongs to a family of proteins that serve as a GTPase-activating protein (GAP) for $G\alpha$ subunits [25]. It has been described as a potential target in different types of human cancer, including breast cancer [25,26]. Thus, RGS2 was upregulated in MCF7 breast cancer cells inhibiting cell proliferation [25]. In breast-invasive carcinoma of no special type (BIC-NST), the downregulation of RGS2 was associated with a significantly poorer overall survival rate [25]. LOC488818 is also named FGF-BP1 (Fibroblast Growth Factor Binding Protein 1) and plays an important role in cell proliferation, differentiation, and migration. FGF-BP1 is a protein that acts as a chaperone molecule and is found to be up-regulated in breast cancers, squamous cell cancer and colon cancer [27]. Overexpression of FGF-BP1 was correlated with better prognosis in human breast cancer patients [28]. A transcription factor known as FOXC2, which plays an important role in determining the fate of mesenchymal cells during embryonic development, has also been implicated in cancer cells' ability to metastasize [29]. FOXC2 is a transcription factor that induces mesenchymal differentiation during EMT [29,30] and has the potential to be a therapeutic target in inhibition of metastasis [31]. FOXC2 overexpression was significantly associated with aggressive basal-like human breast cancers [29]. The matrix metalloproteinases (MMPs) family of zinc-containing and calcium-dependent proteinases play a crucial role in various biological processes, including tissue remodeling, wound healing and angiogenesis, and diseases such as arthritis, cancer, and tissue ulceration. As they hydrolyze extracellular matrix components (ECM), they are also known as matrixins. MMP3 is a zinc-dependent proteolytic enzyme that regulates different biological processes by altering the extracellular matrix [32]. MMP3 over-expression was correlated with tumor growth and metastasis in different types of cancer, including breast cancer [32,33]. Several changes are induced by MMP-3 (increased cell proliferation, apoptosis, angiogenesis, changes in the stroma, etc.), which may result in the development of breast cancer in women. This protein may also affect Mammary tumors in canines [34]. STEAP1 is a membrane-bound channel protein found overexpressed in various cancers [35–38] and was associated with a malignant phenotype and disease prognosis [39]. STEAP1 upregulation significantly inhibited the capacity of migration of the tumor cells and invasion potential and downregulated expression of the EMT-related genes in the human breast cancer [39].

5. Conclusions

The treatment and knowledge of canine mammary tumors (CMTs) have made significant progress in veterinary oncology as a spontaneous model for breast cancer research in the last few decades. Using Doxorubicin to target specific biological processes like apoptosis, cell cycle, migration, invasion, and EMT may result in better outcomes for patients with this disease. The canine model of breast cancer represents a good opportunity for new therapeutic strategies to be developed for human breast cancer. Doxorubicin is a widely used antineoplastic agent in humans and animals. Multiple mechanisms are involved in DOXO's action, producing free radicals, intercalation into DNA strands, and inhibiting topoisomerases I and II, causing DNA damage, activating caspases, and ultimately causing apoptosis. The importance of new therapeutic strategies development in canine mammary cancer represents a major challenge worldwide. Findings from our study provide valuable information for revealing the link between doxorubicin, phenotypic changes, and proliferation dynamics in malignant cells that may contribute to chemotherapy treatment limitations in the canine model.

Author Contributions: “Conceptualization, Madalina Gherman, Oana Zanoaga, Lajos Raduly and Ioana-Berindan Neagoe.; methodology, Madalina Gherman, Lajos Raduly, Oana Zanoaga, Liviuta Budisan: data curation, Oana Zanoaga, Lajos Raduly and Ioana-Berindan Neagoe.; writing—original draft preparation, Madalina Gherman, Oana Zanoaga, Lajos Raduly.; writing—review and editing, Oana Zanoaga, Lajos Raduly and Ioana-Berindan Neagoe.; supervision, Ioana-Berindan Neagoe. All authors have read and agreed to the published version of the manuscript.”

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Conflicts of Interest: Declare conflicts of interest or state “The authors declare no conflict of interest.”

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