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Posted Date: 22 November 2023

doi: 10.20944/preprints202311.1423.v1

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Antitumor Effect of Novel Berberine Analogues in a Canine Mammary Tumor Cell Line and In Zebrafish Reporters via Wnt/β-Catenin and Hippo Pathways

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Abstract: The heterogeneous nature of human breast cancer (HBC) can still lead to therapy inefficacy and high lethality and new therapeutics are needed as well as new spontaneous animal models to benefit translational HBC research. Dogs are primarily investigated since they spontaneously develop tumors which share many features with human cancers. In recent years, different natural phytochemicals including berberine, a plant alkaloid, have been reported to have antiproliferative activity *in vitro* in human cancers and in rodent animal models. In this study, we report the antiproliferative activity and mechanism of action of berberine, of its active metabolite berberrubine, and of eight analogues, on a canine mammary carcinoma cell line and in transgenic zebrafish models. We demonstrate both *in vitro* and *in vivo* the significant effects of specific analogues on cell viability, via induction of apoptosis, also identifying their role in inhibiting the Wnt/β-catenin pathway and activating the Hippo signals with a downstream reduction of *CTGF* expression. Particularly the berberine analogues NAX035 and NAX057 show the highest therapeutic efficacy, deserving further analyses to elucidate their mechanism of action more in detail, and *in vivo* studies on spontaneous neoplastic diseases aiming at improving veterinary treatments of cancer as well as translational cancer research.

Keywords: berberine derivates; dog; mammary tumors; zebrafish; Hippo; Wnt; β-catenin

1. Introduction

Human breast cancer (HBC) is the most common type of tumor in women accounting for 30% of all diagnosed cancers [1]. Despite recent advancements in detection, diagnosis and treatment of HBC, the heterogeneous nature of this disease leads, in some cases, to therapy inefficacy [2]. For this reason, spontaneous animal models of HBC are highly important to study the molecular mechanisms underlying the onset and progression of these tumors. They can serve as a useful intermediary between traditional preclinical models and human clinical trials and therefore for the identification of new therapies [3,4].

Canine mammary tumors (CMTs) are a highly heterogeneous group of tumors representing the most common type of tumors in female dogs. Approximately 50% of diagnosed CMTs are malignant [5] but recently, an increase in malignant *vs*. benign tumors was observed with a similar trend that

 doi:10.20944/preprints202311.1423.v1

has been also described in human oncologic patients [6]. CMTs are spontaneous in dogs as well as in women, and many anatomic, clinical, and histological features of CMTs are similar to those reported in HBC [7–9]. As companion animals, dogs share environment and lifestyle with humans and become consequently exposed to many of the same carcinogens. Better than genetically modified mice, dogs have higher physiological similarities with humans, such as comparable telomerase activities [10] and a high incidence of spontaneous cancers [11], making them a valuable cancer model for HBC research [12]. This spontaneous animal model could be highly beneficial to translational HBC research, particularly for the identification of new therapeutic targets and the collection of highly predictive data that may accelerate human cancer clinical research.

In the last decade, the Wnt/β-catenin pathway, involving β-catenin and the adenomatous polyposis coli (APC) proteins, has emerged as an important player in many human tumor types, including HBC [13–15]. The reduced membrane expression of E-cadherin and β-catenin has been associated with a poor prognosis in feline and canine MTs as well as in HBC [16,17]. E-cadherin in association with other adhesion molecules of the β-catenin class binds to the actin cytoskeleton. This association is important for the epithelial cell function and for tissue integrity [18]. Moreover, β-catenin has a double function as an adhesion molecule and as an activator of the Wnt/β-catenin pathway. Specifically, in normal cells, free cytoplasmic β-catenin is entrapped by a complex containing axin, glycogen synthase kinase 3 (GSK3), and APC that facilitates the phosphorylation of β-catenin which consequently can be degraded. In tumor cells, this complex is not able to phosphorylate β-catenin that, as a result, cannot be degraded [19,20]. To the best of our knowledge, only little information is known about the role played by the Wnt/β-catenin signaling pathway in CMTs [21–23].

Additionally, the Hippo pathway has been identified as highly important in HBC onset and progression. Cordenonsi and co-authors demonstrated that the activity of the transducer of the Hippo pathway TAZ is required to sustain tumor-initiation capacities and self-renewal of cancer stem cells (CSCs) in the breast. TAZ protein level is elevated in CSCs and in poorly differentiated HBC with a poor prognosis [24,25]. To date, the role of Hippo pathway in CMTs has not been deeply investigated and not fully clarified [23,26–28].

Berberine (BBR) is a bitter-tasting isoquinoline quaternary alkaloid isolated from many kinds of plants such as *Hydrastis canadensis*, *Berberis vulgaris* and other plant species. A mainstay of Traditional Chinese Medicine, BBR is also in use in Ayurvedic and Native American herbal medicines for its anti-microbial and anti-inflammatory properties [29,30]. BBR has multiple pharmacological properties including antibacterial, anti-inflammatory, antidiabetic, hepatoprotective, and neuroprotective. It also controls the expansion of blood vessels and the inhibition of platelet aggregation and, therefore, has a wide spectrum of medical applications against, *inter alia*, gastroenteritis, hyperlipidemia, non-alcoholic fatty liver disease, coronary artery disease, hypertension, diabetes, and Alzheimer's disease [31–34]. There is an increasing interest in the clinical efficacy of BBR as manifested by almost 90 completed and ongoing clinical studies, including five for cancer indications.

In vitro studies using HBC cell lines demonstrated that BBR inhibits cancer cell migration and proliferation and affects cell viability, inducing apoptosis [15,35–37]. More recently, it has been shown that BBR could be a promising drug able to suppress cell growth and cell invasiveness of triple-negative HBC (TNBC) cells through IL-8-related mechanisms [38,39]**.** BBR was also demonstrated to be a promising drug in prevention and treatment of colon cancer through the inhibition of Wnt/β-catenin signaling [40] and in exerting anticancer activity delaying the development of MTs in transgenic mice for the HER-2/neu oncogene [41–43].

Berberrubine (BRR) is the first-pass metabolism product of BBR [44], and little is known about its antitumor properties [45,46]. BRR has been reported to induce topoisomerase II-mediated DNA cleavage [47], and to bind G-quadruplex nucleic acid structures [48].

The aim of our study was to evaluate the potential of BBR, BRR, and eight structurally related analogues as anticancer agents. We conducted *in vitro* experiments to examine the biological effects of these compounds on a malignant CMT cell line in order to identify candidates that might become potential anticancer agents. The analogues under investigation possess aromatic groups attached to

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the 13-position of the parent alkaloid skeleton through a hydrocarbon linker [49]. Their effects on Wnt/β-catenin signaling [50] and on some relevant human tumor types have been reported, both *in vitro* [36,51–55] and *in vivo* [41,56].

To elucidate the mechanism involved in the antitumor effect of BBR, of BRR, and of selected analogues, we investigated the association with the Wnt/β-catenin signaling and Hippo pathways. More specifically, we used Wnt/β-catenin and Hippo signaling pathway reporter zebrafish lines to confirm the *in vivo* effect of these BBR and BRR analogues on these pathways. We expected to identify at least one analogue able to kill cancer cells mainly by apoptosis and better clarify how the Wnt/β-catenin and Hippo signaling pathways are involved.

2. Materials and Methods

2.1. Chemicals

Berberine (BBR - chloride form, Trust & We, Shanghai, China), berberrubine (BRR), and analogues NAX012, NAX014, NAX035, NAX053, NAX057, NAX060, NAX085, NAX118 were provided by Naxospharma (Figure 1).

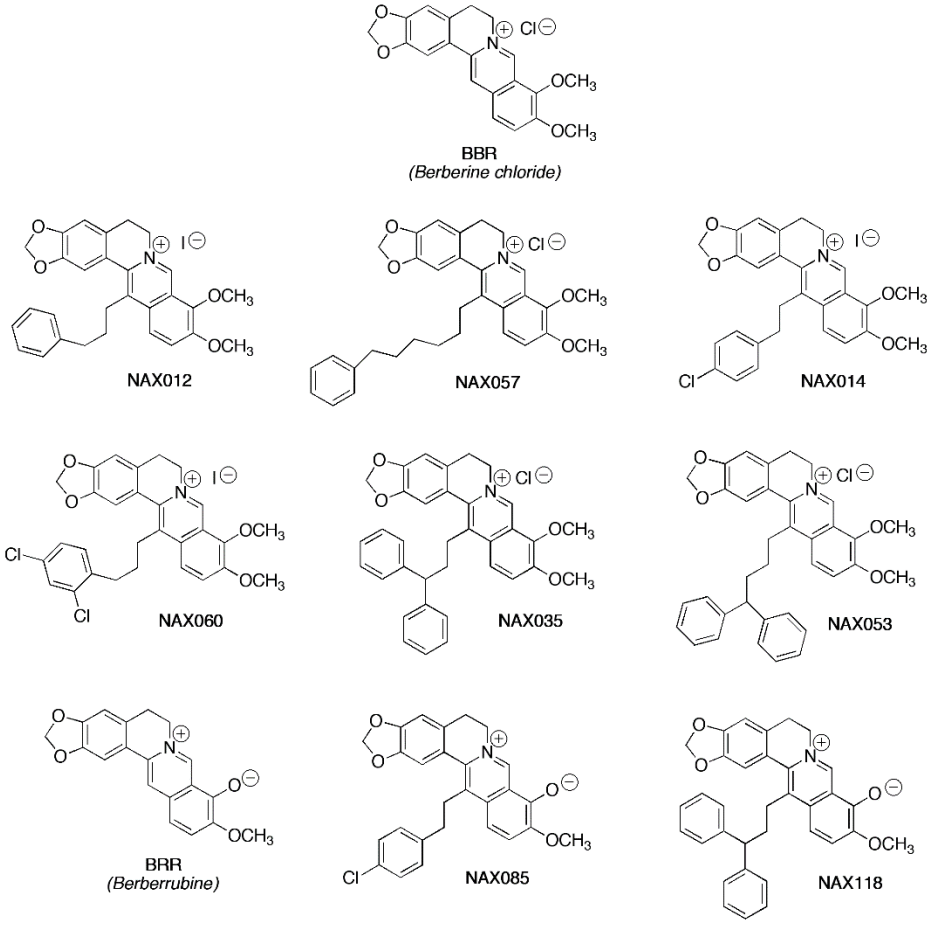

Figure 1. Biochemical structure of berberine (BBR), berberrubine (BRR), and analogues.

2.2. Cell Culture and Drug Treatment

The canine mammary carcinoma cell line CF33 (kindly provided by Dr. R. De Maria, University of Turin, Italy) was maintained in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 units/ml penicillin, and 100µg/ml streptomycin under a humidified atmosphere of 5% CO_2 at 37°C. BBR, BRR, and analogues were dissolved in dimethyl sulfoxide (DMSO), aliquoted, and stored at −20 °C with a stock concentration of 40mM. The final concentration of DMSO did not exceed 0.1%.

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For the *in vitro* experiments, CF33 cells were plated and treated separately with BBR, BRR, NAX012, NAX014, NAX035, NAX053, NAX057, NAX060, NAX085 and NAX118. Two different experiments were performed. In the first experiment, we used different drug concentrations, from 2 μM to 40 μM, for a steady incubation time of 24 hours. In the second experiment, we used the same drug concentration, 10 μM, for different incubation times (from 3 to 24 hours). Control cells were incubated with a respective maximal percentage of DMSO alone (<0.1%). The two experiments were performed in triplicate.

2.3. Cell Viability Assay

CF33 cells were plated in a 96-well plate at a density of $1x10^4$ cells/well in 100 μl of complete medium. After 24 hours, a medium containing the specific drug was added. To measure cell viability, cells were incubated with the different drugs and the *in vitro* toxicology assay kit TOX8 (Sigma-Aldrich) was used, following manufacturer's instructions. The experiment was performed in triplicate. Experimental results were expressed as percentage of cell viability in comparison with control DMSO-treated cells.

2.4. Quantification of Necrosis/Apoptosis by Flow Cytometry

In order to assess the rate of necrosis and apoptosis induced by BBR, BRR, and analogues at the same concentration (10 μM) and at the different incubation times (6, 12, 18, and 24 hours of incubation), two biological replicates (both performed in technical triplicates) of CF33 were analyzed by flow cytometry using the Annexin V-Fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (eBioscience, Thermo Scientific) following the manufacturer's instruction. Briefly, cells were trypsinized and centrifuged at 1100 rpm for 10 min at 4°C. Cells were resuspended in 200 μL of binding buffer with 5 μL of annexin V–FITC and incubated for 10 min in the dark at room temperature. After the incubation, 200 μL of binding buffer were added to the cells that were subsequently centrifuged at 1100 rpm for 10 min at 4°C, and the supernatant was discarded. Then, 10 μL of Propidium Iodide (PI) and 900 μL of binding buffer were added. After 5 minutes of incubation in the dark at room temperature, the cells were acquired using the flow cytometer CyFlow Space (Partec-System, Sysmex Europe GmbH, Norderstedt-Amburgo, Germany) and the data were analyzed with the open-source software FCSalyzer (version 0.9.16-alpha). For each tube, 20,000 events were analyzed. Necrotic cells were positive for both Annexin-V FITC and PI while apoptotic cells were positive for Annexin-V FITC only.

2.5. Protein Extraction and Western Blot Analysis on CF33 Cells

At the end of incubation time (24 hours), CF33 cells treated with BBR, BRR or analogues at a final concentration of 2 μM, were washed twice with ice-cold phosphate buffered saline (PBS) and total lysate was obtained by solubilization in 5% sodium deoxycholate supplemented with protease inhibitors (Sigma-Aldrich). Protein concentration was determined by the bicinchoninic acid- protein assay (Pierce), using bovine serum albumin as standard. Protein fractions were stored at −80 °C. Proteins were resolved by SDS-PAGE using 7.5% polyacrylamide gels [57] and the same amount of protein (20μg) was loaded for all the samples. After electrophoresis, proteins were transferred onto nitrocellulose which was stained with Ponceau S (Sigma-Aldrich). The proteins were transferred to the membrane and blocked in 5% skim milk before being probed overnight at 4°C with the primary antibodies. The blots were probed with the following antibodies: rabbit polyclonal antibodies specific for WW domain-containing transcription regulator protein 1 (WWTR1) (1:1000, Sigma-Aldrich #HPA007415), recognizing both YAP and TAZ human proteins; rabbit monoclonal antibodies to non-phosphorylated (active) β-Catenin (1:1000, Cell Signaling #8814); mouse monoclonal antibodies to β-catenin (1:1000, BD Biosciences #610154). After washing the membrane, an HRP-conjugate secondary antibody was added and incubated with the membrane for 1 hour at room temperature. The membrane was washed and analyzed by a chemiluminescence analyzer using the chemiluminescence ECL reagent.

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2.6. RT-PCR and Semi-Quantitative PCR

Total RNA was isolated from control CF33 cells and CF33 cells 24 hours after treatment with 2 μM of drug using RNeasy Micro Kit (Qiagen) following the supplier's protocol. The obtained RNA was then quantified using Qubit Fluorometric Quantitation (LifeTechnologies) and 500 ng were reverse transcribed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific), according to manufacturer's instructions. Five μl of cDNA products were amplified with 1 unit of GoTaq (Promega) in the buffer provided by the manufacturer that contains MgCl2, with dNTPs and in the presence of the specific primers for β-catenin, YAP, TAZ, CTGF. Actin-beta was used as a housekeeping gene. Primers and PCR Conditions are summarized in Table 1. A first cycle of 2 minutes at 95°C was followed by 30 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72 °C for 27 cycles. The used number of cycles was chosen so that none of the samples reached a plateau at the end of the amplification protocol; therefore, all the samples were in the exponential phase of amplification. Each set of reactions always included a negative control, where the sample was replaced by nuclease-free water and was performed in triplicate.

Table 1. Primers and condition used for the semi-quantitative PCR analysis.

	Primer F (5′-3′)	**Primer R (5′-3′)**	**Amplicon length (pb)**	**Number of cycles**
ACTB	TGGCACCACACCTTC TACAA	CCAGAGGCGTACAGG GATAG	182	25
β-catenin	ACACGTGCAATCCCT GAACT	CACCATCTGAGGAGA ACGCA	138	26
TAZ	TCCAATCACCAGTCC TGCAT	AGCTCCTTGGTGAAGC AGAT	125	28
YAP	CCCAGACTACCTTGA AGCCA	CTTCCTGCAGACTTGG CATC	107	28
CTGF	CGACTGGAAGACACG TTTGG	AGGAGGCGTTGTCATT GGTA	136	27

2.7. Gel Electrophoresis, Acquisition of Gel Images and Quantitative Analysis

The PCR products were loaded onto a 2% agarose gel stained with gel-red (Promega, Italy). A 100 bp DNA ladder molecular weight marker (Life Technologies, Rockville, MD) was run on each gel to confirm the expected molecular weight of the amplification product. Images of the RT-PCR agarose gels were acquired with BioRad gel imaging systems and band quantification was performed using ImageJ software. The ratio between the gene of interest and actin-beta was calculated to normalize for initial variations in sample concentration and as a control for reaction efficiency. Mean and standard deviation of all experiments performed were calculated after normalization to actin-beta.

2.8. Ethics Statements

Zebrafish embryos and adults were raised, staged, and maintained at the Zebrafish Facility of the University of Padua, under standard conditions [58]. All husbandry and experimental procedures complied with European Legislation for the Protection of Animals used for Scientific Purposes (Directive 2010/63/EU) and with Italian law on animal experimentation (D.L. 4 March 2014, n.26). All the procedures were carried out under authorization n. 407/2015-PR from the Italian Ministry of Health. The project was also examined and approved by the Ethics Committee of the University of Padua with protocol number 18746.

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2.9. Zebrafish Housing and Maintenance

Zebrafish used for all the experiments were taken from the breeding stocks of the Zebrafish Facility of the University of Padua and fed four times a day on a variable diet of dried food and live Artemia. Fish were kept in a 14:10 light:dark cycle in a 10L multi tank constant flow system. Water temperature was held at a constant 28.5℃ and replaced at a rate of 10% per day. Eggs were collected between 9:00–11:00 am and transferred in petri dishes. The following day, dead eggs were removed, and dishes were cleaned. Zebrafish embryos were incubated at 28.5℃ in system water mixed with methylene blue (2 ml 0.1% methylene blue per 1 L). Humane endpoints were not used considering that all the experimental procedures were performed in wild type and transgenic larvae under 5 days post fertilization. For anesthesia or euthanasia of zebrafish embryos and larvae, Tricaine was added to the fish water at 0.16 or 0.3 mg/mL, respectively. The study was carried out in compliance with the ARRIVE guidelines as reported by Percie du Sert and colleagues [59]. All the experimental procedures have been performed by specifically trained personnel by the veterinary staff working in the Zebrafish Facility of the University of Padua.

2.10. Zebrafish Tg(7xTCF-Xla.-Siam:mCherry) and Tg(Hsa.CTGF:mCherry) Transgenic Lines

In order to confirm the *in vivo* effect of BBR and NAX035, NAX053 and NAX057 on Wnt/β-catenin and Hippo pathways, we used two zebrafish transgenic lines named Tg(7xTCF-Xla.-Siam:mCherry) [60] and Tg(Hsa.CTGF:mCherry), respectively [61]. Fish of all strains, maintained in Padua Zebrafish facility, were monitored daily for the presence of signs of sickness, pain, distress, suffering, or moribund conditions; all treated zebrafish larvae were euthanized before the phenotypic analysis.

2.11. LC50

The median lethal concentration (LC50) of BBR and analogues identified as the drug dosage able to kill 50% of 24 hours post fertilization (hpf) zebrafish larvae incubated with the drugs for 24 hours, was calculated with the Quest Graph™ LD_{50} Calculator, (AAT Bioquest, Inc., Sunnyvale, CA, United States). Different dosages of drugs were directly added to the fish water in 96-well plates. For each treatment we used at least 96 embryos, and the experiment was performed in triplicate.

2.12. In Vivo Drug Treatments

Zebrafish embryos were incubated with 100 μM BBR (BBR LC50) and with 10 μM of NAX035, NAX053 and NAX057 (LC50) at 24 hpf for 24 or 48 hours. Drugs were added directly to the fish water in 6-well plates. For each treatment performed in triplicates, at least 10 embryos were used.

2.13. Microscopy and Image Acquisition

The mCherry-expressing embryos belonging to the transgenic lines named Tg(7xTCF-Xla.-Siam:mCherry) and Tg(Hsa.CTGF:mCherry) were analyzed using a Leica M165FC epifluorescent microscope. All pictures were acquired with a Leica DC 500 digital camera and contrast and brightness elaborated with Adobe Photoshop 6.0 software. In each transgenic embryo, to quantify the level of fluorescence (that is strictly associated with the activity of Wnt/β-catenin of Hippo pathways) the integrity density was calculated using ImageJ software. With ImageJ we were able to analyze the embryos' fluorescence in the head, cardiac region, and spinal cord. The yolk sac was not included since it usually shows autofluorescence after drug treatment.

2.14. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9 software. To verify mean differences among groups, the one-way ANOVA with Tukey's multiple comparison test was used when data were normally distributed. The Kruskal-Wallis test with Dunn's multiple comparison test was used

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when data were not normally distributed. Shapiro-Wilk test was used to check normality. Level of significance was set at $p < 0.05$.

3. Results

3.1. BBR, BRR, and Analogues Induce a Dose-Dependent Inhibition of Tumor Cell Viability

To evaluate the *in vitro* anti-proliferative effect of BBR, BRR, and analogues (Figure 1) on CF33 cells, we measured cell viability after treatment. In the first experiment, cells were treated with BBR and analogues for 24 hours using different dosages (2 μM, 5 μM, 10 μM, 20 μM, and 40μM) (Figure 2A). The half maximal inhibitory concentration (IC50) was analyzed for each compound and reported in Table S1. Consequently, we performed a second cell viability assay at different time points, using a concentration of 10 μM for all the compounds (Figure 2B). In this experiment, cells were treated with each drug at the dosage of 10 μM and cell viability was measured at different times (3, 6, 12, 15, 18, and 24 hours) (Figure 2B). Treatments with BBR, BRR, and analogues exerted a dose- and time-dependent inhibition of cell viability (Figure 2).

NAX012, NAX035, NAX053, NAX057, and NAX060 were identified as promising drug candidates able to kill more than 50% of tumor cells after 3h. NAX035, NAX053, NAX057, and NAX060 were able to kill more than 85% of tumor cells after 24h (Figure 2B) and were selected for further analyses. BRR was also included as a comparison being an apparently less efficient compound.

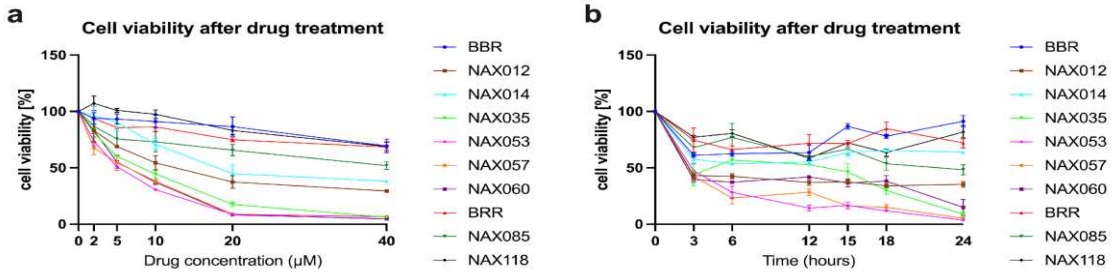

Figure 2. Effect of berberine (BBR), berberrubine (BRR) and other analogues on CF33 cell viability. A) Cells were treated with 2 μM, 5 μM, 10 μM, 20 μM, and 40 μM of the different compounds for 24 hours. B) Cells were treated with 10 μM of the different compounds and cell viability was measured at different time points (3, 6, 12, 15, 18, and 24 hours). BBR, BRR and analogues exerted a dose- and time-dependent inhibition of cell viability.

3.2. Induction of Necrosis/Apoptosis in Canine Mammary Tumor Cells by BBR Analogues

Next, we investigated whether the inhibitory effects of BBR and analogues were due to apoptotic or necrotic cell death. CF33 cells were exposed to 10 μM of each drug and early apoptosis and late apoptosis/necrosis were measured after 6, 12, 18, and 24 hours of treatment by flow cytometry (Figure 3). Over time, an increased rate of apoptosis versus necrosis was observed for most drugs, from 6 to 18 hours (Figure 3A–C).

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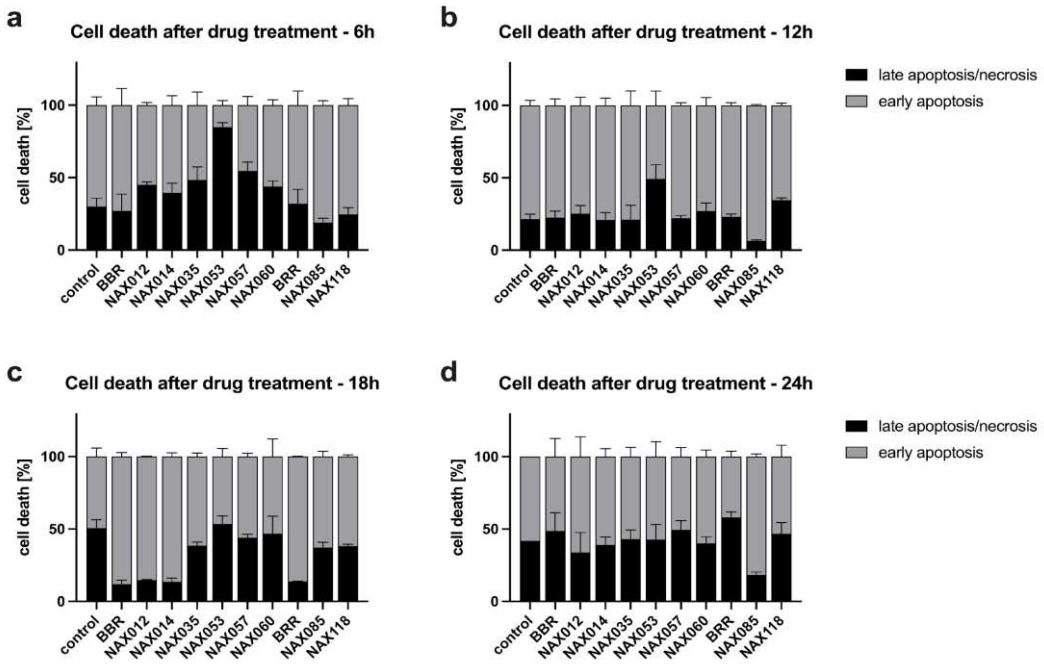

Figure 3. Cell death after drug treatment. Early apoptosis and late apoptosis/necrosis rates of CF33 cells treated with 10 μM of the different compounds at A) 6h, B) 12h, C) 18h, and D) 24h, measured using flow cytometry with double staining for annexin V and propidium iodide.

3.3. NAX035, NAX053, NAX057, and NAX060 Induce a Downregulation of Wnt/β-Catenin and an Activation of the Hippo Signaling Pathways in CF33 Cells

To evaluate the effect of BBR and the most efficacious analogues on Wnt/β-catenin and Hippo pathways, we analyzed the protein expression of total β-catenin, active β-catenin, YAP, and TAZ after treatment.

Western blot analysis revealed a decreased amount of total β-catenin in CF33 cells treated with NAX035, NAX053, NAX057, and NAX060. Interestingly, cells treated with the same compounds showed a notable lower expression of active β-catenin when compared to the controls and to other analogues, suggesting a down-regulation of the Wnt/β-catenin pathway (Figure 4).

Considering the Hippo pathway, western blot analysis also revealed a down-regulation of YAP and TAZ proteins in cells treated with NAX035, NAX053, NAX057, and NAX060 when compared to the control, which was more evident for TAZ (Figure 4). These results suggest a down-regulation of the Wnt/β-catenin and an activation of the Hippo pathway, respectively, when cells are treated with NAX035, NAX053, NAX057, and NAX060, when compared to the control cells.

We focused on genes specifically involved in the Wnt/β-catenin and Hippo pathways. *β-catenin* mRNA expression was not significantly altered after treatment with NAX035, NAX053, NAX057 and BRR in comparison with cells treated with BBR and control. *β-catenin* mRNA expression was significantly higher ($p < 0.01$) in cells treated with NAX060 when compared to the control (Figure 5A).

Interestingly, *YAP* mRNA expression was significantly higher in cells treated with BBR, NAX053, and NAX060, when compared to the control ($p < 0.05$) (Figure 5B), whereas *TAZ* mRNA expression was not altered after treatment (Figure 5C). We also looked at the mRNA expression of *CTGF*, which is regulated by the Hippo pathway. Notably, *CTGF* mRNA expression was significantly lower in cells treated with NAX035 ($p < 0.01$) and NAX057 ($p < 0.001$) when compared to the control (Figure 5D), indicating an activation of the tumor-suppressor Hippo pathway after treatment with these BBR analogues.

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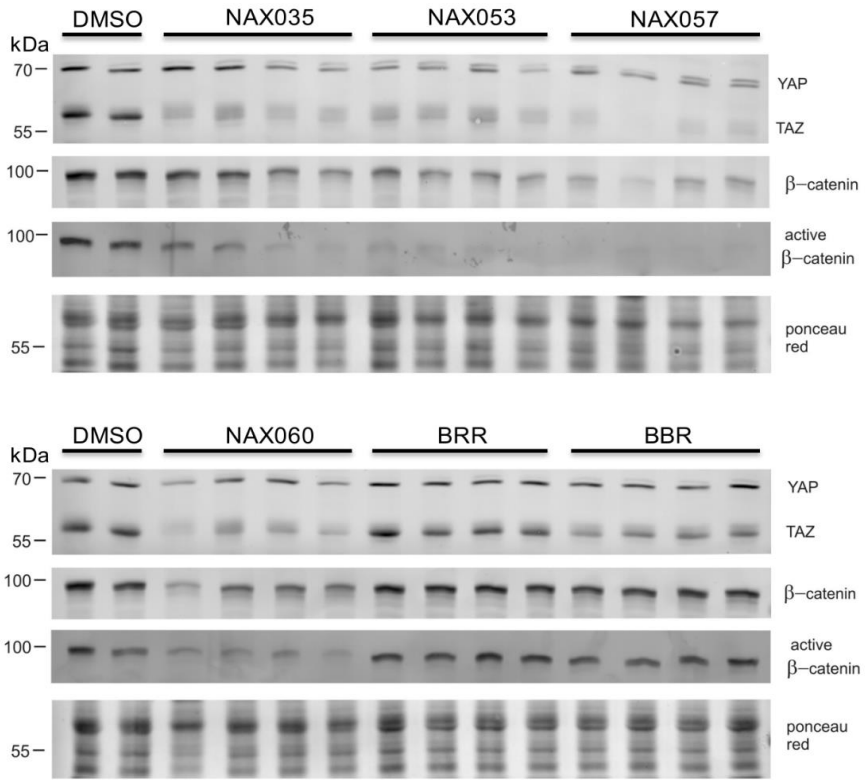

Figure 4. Protein expression after drug treatment. Representative cropped western blots of YAP/TAZ, β-catenin and active β-catenin in protein lysates obtained from CF33 cells treated with berberine (BBR), NAX035, NAX053, NAX057, NAX060 and berberrubine (BRR) for 24 hours. Bands of the expected sizes for YAP (~70 kDa), TAZ (~55 kDa), β-catenin (~92 kDa) and active β-catenin (~92 kDa) are present in all samples. The experiment was performed in quadruplicate. Each lane corresponds to a replicate. Ponceau red was used as a loading control.

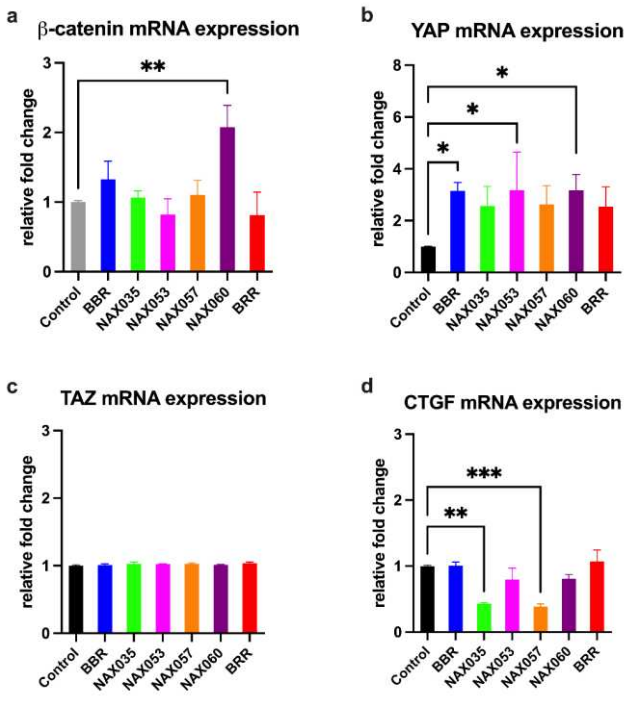

Figure 5. mRNA expression after drug treatment. Gene expression analysis by semi-quantitative PCR of genes directly or indirectly involved in Wnt/β-catenin and Hippo pathways in CF33 cells

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treated with berberine (BBR), NAX035, NAX053, NAX057, NAX060 and berberrubine (BRR). A) β-catenin, B) YAP, C) TAZ, and D) CTGF mRNA expression levels in CF33 cells. Relative gene expression levels are shown following normalization with beta-actin. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3.4. BBR and NAX057 Reduce the Activity of Wnt/β-Catenin and Activate Hippo Signaling Pathways in Zebrafish Embryos

Next, we used zebrafish embryos to study the toxicity response to BBR, BRR and analogues by assessing Median Lethal Concentration (LC50) at 24 and 48 hours of drug treatment. The LC50 of BBR, BRR, NAX085, and NAX118 was defined at 200 μM. NAX012 and NAX014 had a LC50 of 100 μM. NAX035 and NAX060 had a LC50 of 20 μM. Finally, NAX053 and NAX057 had a LC50 of 10 μM (Table S2). For all tested compounds, zebrafish embryo LC50 values were not dependent on the duration of exposure, such that longer exposures (48h) were not associated with lower LC50 values (data not shown).

We tested BBR, NAX035, NAX053, and NAX057 on Zebrafish Tg(7xTCF.Xlasiamois:nlsmCherry) and Tg(Hsa.CTGF:mCherry) transgenic lines, in which the expression of the reporter gene mCherry is regulated by specific cell signaling pathways-responsive elements (52,53). By fluorescent microscopy imaging, we analyzed the amount of fluorescence indicating the expression of specific target genes in treated fish compared with controls. The analysis of these responsive transgenic lines, in which the expression of the reporter protein mCherry is directly associated with the activity of canonical Wnt/β-catenin and Hippo signaling pathways, is simple and relatively immediate. In these *in vivo* experiments, we decided to use the drug concentrations of 100 μM for BBR, 10 μM for NAX035, and 5 μM for NAX053 and NAX057 because these concentrations were tested on zebrafish and did not induce alteration in fish morphology and development. Indeed, fluorescence analysis and quantification need to be performed on animals that are correctly formed and developed. Tg(7xTCF.Xlasiamois:nlsmCherry) reporter animals treated with BBR, NAX035, NAX053, and NAX057 showed a significant lower fluorescence when compared to the control ($p < 0.0001$) (Figure 6A), suggesting that these compounds down-regulated the canonical Wnt/β-catenin pathway *in vivo*.

Interestingly, Hippo pathway reporter animals showed a lower fluorescence when treated with BBR ($p < 0.001$), NAX035, and NAX057 ($p < 0.05$), when compared to the control (Figure 6B), while transgenic embryos treated with NAX053 showed an increased fluorescence compared to untreated siblings ($p < 0.0001$) (Figure 6B).

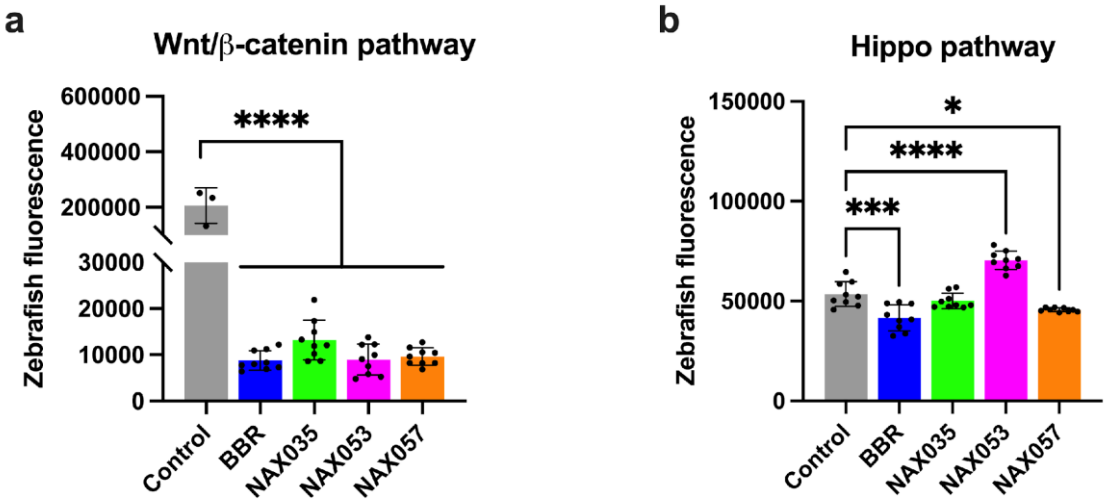

Figure 6. Wnt/β-catenin and Hippo pathways zebrafish reporters. Tissue specificity expression of mCherry under control of A) TCF/LEF and of B) CTGF genes. Quantification of mCherry fluorescence

 doi:10.20944/preprints202311.1423.v1

in control transgenic zebrafish in comparison with transgenic zebrafish treated with berberine (BBR) and other analogues (NAX035, NAX053, NAX057). *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$.

4. Discussion

HBC is the most common invasive cancer in women. Treatment of HBC depends on the subtype of breast cancer, the stage of the disease, sensitivity to hormones, patient's age, and overall health [1]. The main treatment options include radiation therapy, chemotherapy, surgery, and hormone therapy. With treatment, a woman who receives a diagnosis of HBC has a percentage chance of surviving for at least 5 years ranging from almost 100 to 22 percent depending on the stage of the disease at diagnosis and the tumor subtype [62]. For this reason, new treatment strategies are needed.

In recent years, phytochemicals, considered as bioactive ingredients present in plant products, became interesting drugs to be studied for their anti-tumor activity. This effect was demonstrated both *in vitro* and *in vivo* [63]. BBR is a phytochemical which has been reported to inhibit carcinogenesis in rats and mice [64] and in certain types of human cancers as revealed by both *in vitro* and *in vivo* studies [38,41,65,66] and in one clinical administration to human patients [40].

Regarding breast cancer, most *in vitro* studies on HBC cell lines have demonstrated BBR antitumoral effects with several involved molecules and pathways mainly regarding cell proliferation and cell cycle, apoptosis, autophagy, and metastasis [65,67]**.** Only one study investigated BBR effects *in vitro* on a canine mammary tumor cell line (CF41.Mg) showing a decrease of cell viability after 24h of BBR treatment (100 μM) [67]. *In vivo* mouse models also showed reduction of tumor volume, tumor weight or vessel density after BBR exposure [41,66,68–73]. In a recent study, Pierpaoli and collaborators explored the efficacy of the oral administration of a BBR derivative (NAX014) in a mouse model of HER2-overexpressing breast cancer. The authors showed no signs of toxicity after oral administration of a high dose of the compound, suggesting its safety [56].

A major challenge today is to design novel drugs that target tumor cells specifically, with minimal cytotoxic effects on normal cells. Marverti and collaborators showed a prominent decrease of cell viability on two ovarian cancer cell lines, but a minimal effect on normal cells [74], when treated with BBR. This might indicate that BBR specifically targets tumor cells.

In this study, we chose eight semi-synthetic BBR analogues to investigate their effects on the CMT CF33 cell line for the first time, in comparison with BBR. In order to study the *in vivo* effect of these compounds, we used a transgenic zebrafish model. Despite this preliminary study being performed with simple methodology, this type of comparison of the two models has never been performed before for CMTs. Our data demonstrated that the BBR analogues NAX035, NAX053, NAX057, and NAX060 affected cell viability, exerting a higher cytotoxic effect on cancer cells acting at a lower concentration in comparison with BBR. Generally, most of the tested drugs increased cell death by inducing cell apoptosis, which is a desirable effect for novel anticancer drugs. Apoptosis is a programmed cell death often overcome by tumors, whereas necrosis is often present in tumors and can be associated with increased metastases [75]. In line with our results, the BBR capacity to induce apoptosis is well known [65]. Wang and co-authors demonstrated that BBR inhibits proliferation and induces apoptosis of human cervical cancer (HeLa229) cells in a dose and time-dependent manner, by the up-regulation of *p53* and the downregulation of *Bcl2* and *Ptgs2* mRNA expression levels [76]. Additionally, the pro-apoptotic activity of BBR is well documented in various breast cancer cell lines and many other cell cancer types [35].

In recent years, several data have emerged regarding the involvement of Wnt/β-catenin signaling in tumors. In particular, the increase of Wnt/β-catenin signaling seems to be important for human cancer onset and progression, including tumor initiation, tumor growth, cell death, cell differentiation and metastasis onset [77]. In this study, we showed that BBR and other BBR analogues downregulated Wnt/β-catenin signaling. Our data were confirmed both at the protein level *in vitro*, by a decreased expression of active β-catenin in the tested canine cell line, and *in vivo* on zebrafish. In other studies, BBR has been found to deregulate the Wnt/β-catenin signaling, inhibiting proliferation, migration, and invasion of HBC cells *in vitro* [78], and human colorectal cancer growth both *in vitro* and *in vivo* in a mouse model [79,80]. Similarly, it was found by clinical administration

 doi:10.20944/preprints202311.1423.v1

that BBR potently attenuated intestinal polyps in familial adenomatous polyposis human patients via inhibition of Wnt signaling [40]. In our study, we showed that at the gene expression level *β-catenin* mRNA, instead, did not show a significant decrease after drug treatment, exception made for cells treated with NAX060 which curiously presented a significant increase in comparison with control cells. We can speculate that the downregulation of active β-catenin could be due to post-transcriptional or post-translational mechanisms. To date, it is well known that the mechanism whereby nuclear β-catenin drives or inhibits expression of Wnt target genes is more diverse and less characterized [81]. Additional studies will be necessary to understand better the mechanistic underpinnings of the active β-catenin-observed plasticity. A deeper and more comprehensive characterization of the protein networks which regulate β-catenin transcription using zebrafish as a model to dissect molecular mechanisms involved in tumorigenesis could possibly drive us to confirm the identification of attractive new therapeutic targets.

The Hippo pathway is a well-conserved signaling pathway able to regulate organ size and tissue homeostasis [82]. This pathway can be downregulated leading to oncogenesis through a variety of mechanisms [83]. These mechanisms include the induction of hyperproliferation, cellular invasion, metastasis, and might play a role in cancer cell maintenance and chemotherapy resistance [84]. Our *in vitro* study on zebrafish for Hippo pathway indicated an effect for BBR and for other analogues on this signaling pathway. Particularly, there was a decrease of YAP and, mainly, TAZ proteins in cells treated with NAX035, NAX053, NAX057, and NAX060. Notably, the mRNA expression of the downstream gene *CTGF* was lower in cells treated with NAX035 and NAX057, when compared to the control. The Hippo pathway has already been found to be implicated in canine mammary carcinogenesis and the described post-transcriptional/post-translational modifications of YAP and TAZ proteins justify the changes at the protein but not at the mRNA level that were found in our and other studies [23,85]. Mechanistically, YAP and TAZ accumulate within the cytoplasm to translocate into the nucleus and activate downstream tumor-promoting genes when the Hippo pathway is switched off, whereas decreased cytoplasmic YAP/TAZ levels, due to their phosphorylation and degradation, is seen when the tumor-suppressing Hippo pathway is activated [86]. More recently, a Hippo-independent regulation of YAP/TAZ has been described, that could possibly explain non-decreased YAP levels in our study [87]. Several anti-cancer mechanisms of BBR-related compounds have been extensively studied [88], but only one study detected cell cycle arrest and apoptosis induced by a novel synthetic cyclizing-berberine on human cancer cell lines by activating YAP phosphorylation [89]. No data are published on this Hippo-related anticancer effect of BBR analogues.

The analysis of the reporter gene expression in zebrafish treated for 48 hours particularly with BBR and NAX057 (NAX035 with no statistical significance) confirmed a downregulation of CTGF. These data suggest an activation of the Hippo pathway, in comparison with controls, as also supported by a significant decrease of *CTGF* mRNA expression when cells were treated with NAX035 and NAX057. Instead, treatment with NAX053 indicated an *in vivo* up-regulation of CTGF. On one side, human studies have shown that CTGF can have pro-tumorigenic or anti-tumorigenic effects in different situations [90,91]. On the other hand, CTGF regulation is not controlled exclusively by the Hippo pathway [92]. Additional analysis to better characterize the response to these drugs in the *in vivo* zebrafish model should be performed, measuring the expression of other genes and proteins.

Further studies will be necessary to clarify the interesting interaction between Wnt/β-catenin and Hippo pathways in response to BBR and other analogues. We have only touched the tip of the iceberg in understanding the intricacies and interconnections of Wnt/β-catenin and Hippo pathways responsible for the onset and progression of cancer proliferation particularly in canine mammary tumors.

5. Conclusions

In conclusion, our results demonstrated that NAX035 and NAX057 could be considered promising anti-tumoral drugs. In short, (i) they kill approximately 50% of tumor cells at low concentration, (ii) they induce cell death mainly by apoptosis, (iii) they downregulate the Wnt/β-

catenin pathway and, (iv) interestingly, they also seem to act on the activation of the well-known tumor-suppressor Hippo pathway. Our results, obtained on a canine mammary tumor cell line, are in line with similar results demonstrated only for BBR in *in vitro* and *in vivo* human cancer studies, but with less toxicity also proved in our *in vivo* zebrafish model. Considering many similarities between canine mammary tumor and human breast cancer, these BBR analogues might represent relevant candidates to be tested in further *in vivo* animal models.

Supplementary Materials: The following supporting information are available Figure S1: Protein expression of YAP, TAZ, β-catenin, and active β-catenin after treatment with berberine, berberrubine, NAX035, NAX053, NAX057, and NAX060 of a canine mammary cancer cell line. Table S1: Half maximal inhibitory concentration and standard deviation of different compounds after 24 hours of treatment of CF33 cells. Table S2: Different drugs toxicity effects and standard deviation on zebrafish larvae treated for 24 hours.

Author Contributions: Conceptualization, G.B., V.Z.; methodology, A.S., G.B., V.Z.; validation, G.B., A.V., R.S., F.B., G.S.; formal analysis, A.S., G.B.; investigation, G.B., A.S., A.V., R.S., F.B., G.S.; resources, M.E.G., L.C., S.F., C.G., P.L.; data curation, A.S.; writing—original draft preparation, G.B., A.S., V.Z.; writing—review and editing, A.S., R.S., A.V., F.B., P.L.; visualization, A.S.; supervision, V.Z.; project administration, V.Z.; funding acquisition, M.E.G., L.C., S.F., V.Z. All authors have read and agreed to the published version of the manuscript.

Funding: G.B. is supported by BIRD200003, University of Padua.

Institutional Review Board Statement: Zebrafish embryos and adults were raised, staged, and maintained at the Zebrafish Facility of the University of Padua, under standard conditions (50). All husbandry and experimental procedures complied with European Legislation for the Protection of Animals used for Scientific Purposes (Directive 2010/63/EU) and with Italian law on animal experimentation (D.L. 4 March 2014, n.26). All the procedures were carried out under authorization n. 407/2015-PR from the Italian Ministry of Health. The project was also examined and approved by the Ethics Committee of the University of Padua with protocol number 18746.

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

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