

**Vitamin D Receptor (VDR) polymorphism and antiproliferative activity of  
cholecalciferol in cancer cells**

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colecalciferol em células cancerígenas**

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**Abstract**

Vitamin D (VD) is a steroid hormone with multiple biological functions in the body and its activity requires the binding to the receptor named VDR. VDR polymorphisms seems to be involved in the development of several types of cancer. Herein we performed the genotyping of two VDR polymorphisms (Fok I and Taq I) in MCF-7 breast cancer and U87-MG glioblastoma (GBM) cell lines and investigated the antiproliferative effect of the VD analog cholecalciferol. Polymorphisms were identified by PCR-RFLP and the effect of VD was determined by viability and clonogenic assays. VD inhibited the growth of both tumor cells in

vitro. MCF-7 cells were more sensitive than U87-MG cells at concentrations ranging from 0.1nM to 1000nM. The same primer pairs used for PCR amplification of VDR gene in MCF-7 failed to amplify a fragment of expected size in the U87-MG cell line. VDR Fok I and Taq I polymorphisms in breast cancer MCF-7 cells were characterized as FF (CC) and TT respectively. The absence of amplification of VDR gene fragment in U87-MG suggests a possible chromosomal rearrangement and/or impairment of gene expression of VDR which could interfere in the sensitivity of this cell line to vitamin D.

**Keywords:** Breast cancer; Glioblastoma; Vitamin D receptor; PCR-RFLP; SNP; Brain cancer.

### **Resumo**

A vitamina D (VD) é um hormônio esteróide com múltiplas funções biológicas no corpo e sua atividade requer a ligação ao receptor denominado VDR. Os polimorfismos do VDR parecem estar envolvidos no desenvolvimento de vários tipos de cânceres. Aqui, realizamos a genotipagem de dois polimorfismos VDR (Fok I e Taq I) em linhagens celulares de câncer de mama MCF-7 e glioblastoma U87-MG (GBM) e investigamos o efeito antiproliferativo do colecalciferol análogo da VD. Os polimorfismos foram identificados por PCR-RFLP e o efeito da VD foi determinado por viabilidade e ensaios clonogênicos. A VD inibiu o crescimento de ambas as células tumorais in vitro. As células MCF-7 foram mais sensíveis do que as células U87-MG em concentrações que variam de 0,1 nM a 1000 nM. Os mesmos pares de primers usados para amplificação por PCR do gene VDR em MCF-7 não conseguiram amplificar um fragmento de tamanho esperado na linha de células U87-MG. Os polimorfismos VDR Fok I e Taq I em células MCF-7 de câncer de mama foram caracterizados como FF (CC) e TT, respectivamente. A ausência de amplificação do fragmento do gene VDR no U87-MG sugere um possível rearranjo cromossômico e/ou prejuízo da expressão gênica do VDR que poderia interferir na sensibilidade dessa linhagem celular à VD.

**Palavras-chave:** Câncer de mama; Glioblastoma; Receptor de vitamina D; PCR-RFLP; SNP; Tumor cerebral.

### **Resumen**

La vitamina D (VD) es una hormona esteroide con múltiples funciones biológicas en el cuerpo y su actividad requiere unirse al receptor llamado VDR. Los polimorfismos de VDR parecen estar involucrados en el desarrollo de varios tipos de cánceres. Aquí, genotipamos dos polimorfismos VDR (Fok I y Taq I) en las líneas celulares de cáncer de mama MCF-7 y

glioblastoma U87-MG (GBM) e investigamos el efecto antiproliferativo del análogo de colecalciferol de la VD. Se identificaron polimorfismos. mediante PCR-RFLP y el efecto de VD se determinó mediante ensayos de viabilidad y clonogénicos. VD inhibió el crecimiento de ambas células tumorales in vitro. Las células MCF-7 eran más sensibles que las células U87-MG a concentraciones que variaban de 0,1 nM a 1000 nM. Los mismos pares de iniciadores utilizados para la amplificación por PCR del gen VDR en MCF-7 no lograron amplificar un fragmento del tamaño esperado en la línea celular U87-MG. Los polimorfismos de VDR Fok I y Taq I en células de cáncer de mama MCF-7 se caracterizaron como FF (CC) y TT, respectivamente. La ausencia de amplificación del fragmento del gen VDR en U87-MG sugiere un posible reordenamiento cromosómico y/o expresión del gen VDR alterada que podría interferir con la sensibilidad de esta línea celular a VD.

**Palabras clave:** Cáncer de mama; Glioblastoma; Receptor de vitamina D; PCR-RFLP; SNP; Tumor cerebral.

## 1. Introduction

Vitamin D is a steroidal hormone derived from 7-dehydrocholesterol with more than 50 described metabolites, presenting two main biochemical forms: vitamin D3 or cholecalciferol, produced in the skin by ultraviolet B radiation, and vitamin D2 or ergocalciferol obtainable from diet (Zerwekh, 2008; Garland et al., 2011). The VD has a wide variety of biological functions in the organism. In addition to its classic action in bone mineralization and calcium absorption by the intestine (Boneti & Fagundes, 2013), VD also present immunomodulatory property (Evans et al., 2018), anti-inflammatory activity (Hardiman et al., 2016, Mousa et al., 2018) and participates in cell differentiation and apoptosis processes (PAN et al., 2010) angiogenesis modulation (Grundmann, et al., 2012), tumor invasion and metastasis (Bao et al., 2006,10).

The biological actions of VD are mediated by the VD steroid nuclear receptor (VDR) which is found in different cell types and tissues of the organism (Wang, Zhu & De luca, 2012). The gene encoding the VDR receptor is located on the long arm of chromosome 12 (12q12-14) and encompasses a region of 100 kb. About 200 polymorphisms have been identified in different regions of VDR gene and some of them is considered risk variant for some diseases. One of these polymorphisms is the Fok I (T / C) (rs2228570) located in exon-II and results in the use of one of two alternative translation initiation start site leading to an altered VDR receptor protein. Restriction Fragment Length Polymorphism - RFLP assay can

identify the base exchange in Fok I polymorphism where “f” denotes the presence of Fok I restriction site and “F” denotes its absence. Thus, the f allele contains both ATGs start sites whereas the F allele has only the second ATG and predicts a shorter VDR protein (Gross et al., 1998). Additionally, the Taq I (T/C) (rs731236) polymorphism is located in exon-IX and leads to a synonymous change at codon 352 (isoleucine) (Lu, Jing & Zhang, 2016; Rai et al., 2017).

The correlation between VDR polymorphisms and carcinogenesis or cancer risk is not well established, and many contradictory results can be found in the literature (Lu, Jing & Zhang, 2016; Rai et al., 2017). Some of the reasons for the conflicting results include the differences in study design and ethnic populations investigated, the requirement for VDR to associate with retinoid X receptor (RXR) and vitamin D response elements (VDREs) in order to induce several genes, the consumption and the levels of active vitamin D circulating in the blood and the different polymorphisms in other genes involved in the complex metabolic pathway of vitamin D (Giovannucci, 2005).

Glioblastoma is the most common malignant brain tumor in adults with five-years survival rate less than 5% (Dolecek, 2012). Despite the advances in the treatment of these tumors, currently available therapies do not improve the survival significantly. On the other hand, breast cancer is the most commonly occurring cancer in women and the second most common cancer worldwide and leading cause of death following lung cancer (Bray et al., 2018). These statistics demonstrate the need for new alternatives of treatment to decrease mortality and improve the quality of the life of patients. Therefore, the present study investigated the antiproliferative activity of the VD metabolite cholecalciferol in the glioblastoma U87–MG (native protein p53) and breast cancer MCF–7 (hormone sensitive) cell lines and determined the presence of two Fok I and Taq I polymorphisms.

## **2. Methodology**

This is an experimental study that used molecular and cellular methods to address the hypothesis that vitamin D present antitumoral activity.

### **2.1 Cell culture**

The glioblastoma multiforme U87–MG and breast cancer MCF–7 cell lines (American Type Culture Collection® (ATCC)) were maintained in Dubecco's modified Eagle's medium

(DMEM), supplemented with 5% and 10% fetal bovine serum (Gibco®) respectively, 50U/ml penicillin, 0.1 µg/ml streptomycin (Cultilab®) at 37°C, 5% CO<sub>2</sub> in a humidified incubator (Sanyo InCu saFe®). For all the experiments it was used viable cells at a confluence of about 70 to 80%.

## 2.2 Genotyping of VDR gene polymorphisms

The genomic DNA of the cell lines was extracted by the phenol: chloroform: isoamyl alcohol method (25: 24: 1), according to Koh, (2013). The Fok I and Taq I polymorphisms in the VDR gene were determined by PCR-RFLP. For Taq I, it was used the primer oligonucleotides described by Yaylım-Eraltan et al., (2007), and for Fok I, the primers described by Goknar et al., 2016 (Table 1). The PCR reaction for Taq I consisted of 100ng of DNA, 2.5mM MgCl<sub>2</sub>, 0.2mM of each dNTP, 0.2µM of each primer, and 1U of AmpliTaq Gold (Thermo Fisher ®), in 25µl of final volume. The PCR conditions included an initial denaturation at 94 °C for 4 min, followed by 35 cycles at 94 °C for 1 min denaturation, 62.4 °C for 1 min annealing and 72 °C for 45 sec extension and then a final extension at 72°C for 4 min. The amplified products (745 bp) were digested with the restriction enzyme Taq I at 65 °C (Jena Bioscience®) for 2h, electrophoresed on a 2% agarose gel and the bands visualized after staining with ethidium bromide in a UV transiluminador. For the normal homozygous genotype (TT) it was expected bands of 494 and 251 bp, for (TC) bands of 494, 293, 251 and 201 bp and for polymorphic homozygous (CC) bands of 293, 251, 201 bp.

The Fok I PCR reaction consisted of 100ng of DNA, 2mM MgCl<sub>2</sub>, 0.2mM of each dNTP and 0.25 µM of each primer and 1U of Taq Pol in a final volume of 25µl. The conditions of the PCR included an initial denaturation at 94 °C for 4 minutes followed by 35 cycles at 94°C for 1 min, annealing at 62 °C for 1 min and extension at 72 °C for 1 min and then a final extension at 72°C for 4min. The amplified products were digested at 37 °C for 2h with the restriction enzyme Fok I and the fragments were visualized after electrophoresis on a 2% agarose gel stained with ethidium bromide. The expected genotypes were as follow: polymorphic homozygous (CC) or FF, one fragment of 272 bp; heterozygous (CT) ou Ff three fragments of 272, 198, 78 bp, and normal homozygous (TT) or ff two fragments of 198 and 78 bp.

**Table 1.** Set of primers used for the genotyping of Fok I and Taq I polymorphisms and expected PCR product size.

Polymorphisms	Sequence of primers	Product size (pb)
Taq I*	F 5' – CAGAGCATGGACAGGGAGCAAG–3' R 5'– GCAACTCCTCATGGCTGAGGTCTCA–3'	745
Fok I**	F 5'– GATGCCAGCTGGCCC TGGCACTG–3' R 5'– ATGGAAACACCTTGCTTCTTCTCCCTC–3'	272

\*Yaylım-Eraltan et al., (2007), \*\* Goknar et al., (2016). Source: Authors.

### 2.3 Cell viability

The U87–MG and MCF–7 cells were seeded in 96-well sterile plates, at a density of 1500 cells/ well and incubated overnight at 37 °C for attachment. After this period, the medium was removed, and cells were treated with cholecalciferol in 0.1 % ethanol/DMEM at concentrations of 0.1 nM, 1 nM, 10 nM, 100 nM and 1000 nM in quadruplicates. Controls consisted of DMEM medium with 0.1 % ethanol. Cells were incubated for 24 h, 48 h, 72 h and 96 h and then the antiproliferative effect of VD was determined by counting the number of viable cells in a Neubauer chamber under an optical microscope (Leica®), after staining with 4% trypan blue in phosphate buffer. Non-viable cells incorporate trypan blue dye while viable cells exclude the dye and remain unstained (Strober, 2011).

### 2.4 Clonogenic assay

The clonogenic assay allows to evaluate the capacity of individual cells to divide and form colonies in a culture plate, and it can be used to determine the effectiveness of cytotoxic agents (Franken, 2006). The assay was performed in triplicates and repeated four times. U87-MG cells were seeded in sterile 35 x 10mm Petri dishes, at a density of 150 cells per plate in a final volume of 3 ml and after attachment overnight, medium was removed and exchanged to fresh medium supplemented with cholecalciferol at concentrations of 100nM, 1000nM and 10000nM in 0.1% ethanol/DMEM. Controls consisted of cells treated with ethanol 0.1%. Cells were then incubated for 8-10 days under humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. The MCF-7 cells were plated at a density of 500 cells per 35 x 10mm Petri dishes in

medium already supplemented with cholecalciferol at concentrations of 100nM, 1000nM and 10000nM of VD in 0.1% ethanol/DMEM. After the incubation period, the colonies formed were stained with Wright's solution (0.06%). Analysis of the colony size and number was performed using Image J ® software (NIH, Bethesda, MD, USA).

## **2.5 Statistical analysis**

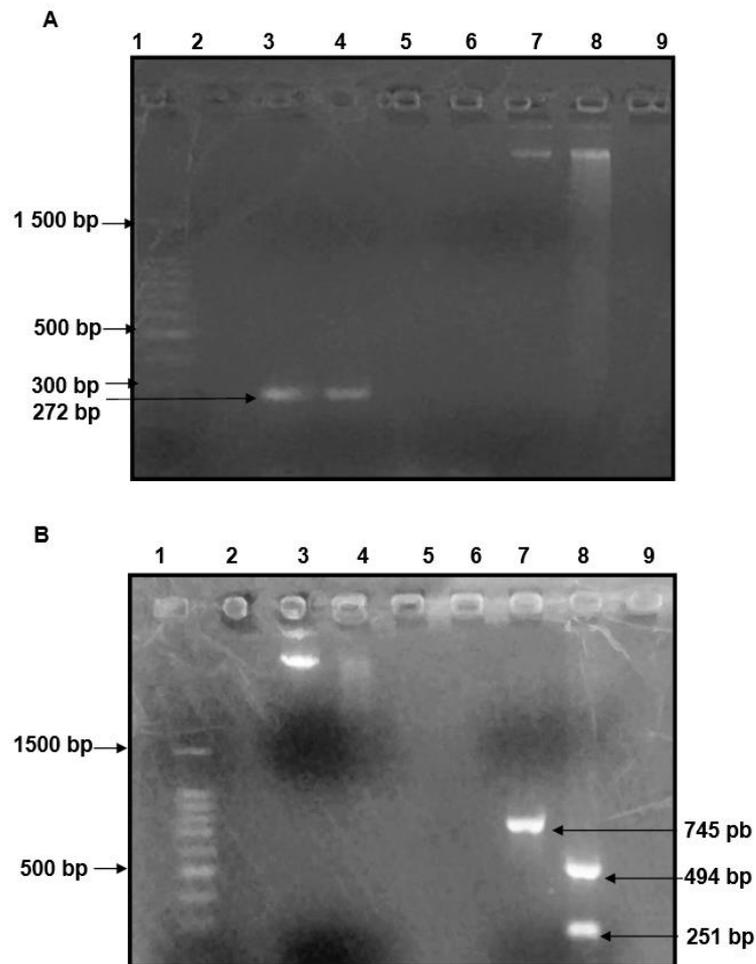
The differences between treated and untreated cells were performed using the GraphPad prism statistic software (version 5.0). The results were compared by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test, two-way analysis (ANOVA), followed by Bonferroni post hoc test with significance level  $p < 0.05$ .

## **3. Results**

### **3.1 VDR polymorphism genotyping**

After PCR with the set of primers designed to amplify a region encompassing the Fok I and Taq I polymorphisms it was expected to obtain products with band sizes of 272 bp and 745 bp respectively. As shown in Figure 1, DNA extracted from MCF-7 cell lines worked as template for both set of primers allowing the amplification of VDR gene with the expected sizes. After digestion with the restriction enzymes Taq I and Fok I the presence of polymorphism could be determined. Based on the size of the bands produced after digestion, MCF-7 demonstrated the absence of Fok I restriction site, producing a band the same size as the undigested PCR product (272 pb). Therefore, it was genotyped as CC also known as (FF) in the first start codon of VDR gene, which changes the nucleotide sequence from ATG to ACG. For Taq I polymorphism MCF-7 was genotyped as TT (normal homozygous), showing bands of 494 and 251 pb after restriction digestion (Figure 1 A and B). Interesting, the same set of primers under the same conditions failed to generate PCR products with the expected size even with different DNA extractions obtained from the U87-MG cell line. Then we tried different PCR conditions and still could not obtain a PCR product as we got with DNA from MCF-7 cells.

**Figure 1.** MCF-7 VDR polymorphism genotyping by PCR-RFLP.



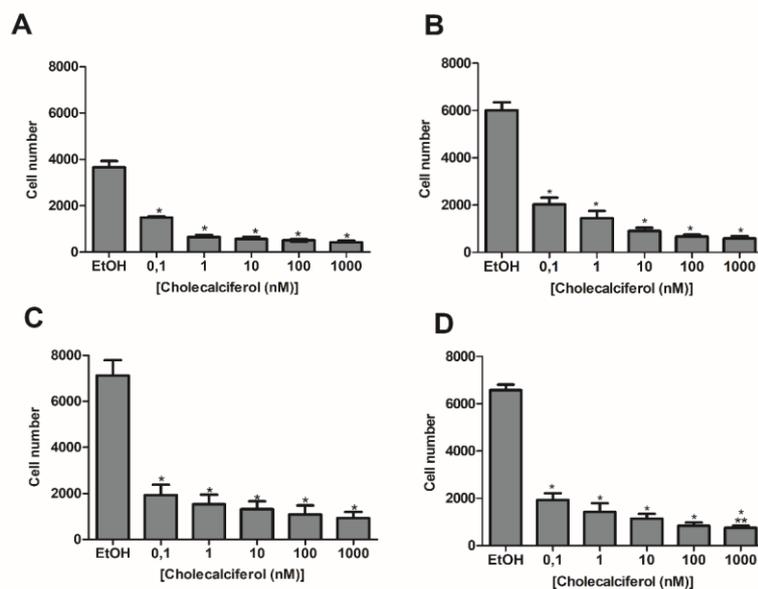
Source: own study.

Figure shows DNA bands profile as seen in agarose gel after PCR amplification and digestion with restriction enzymes. A) VDR Fok I genotyping. PCR products were analyzed on 2% agarose gel stained with ethidium bromide and visualized on a UV transilluminator. Lane1: 100bp molecular weight marker, lane3: undigested PCR product, 4: Fok I digested PCR product – FF homozygosity (272bp), lane 5: negative control, lane 7: undigested MCF-7 genomic DNA, lane 8: Fok I digested MCF-7 genomic DNA used as digestion control. B) VDR Taq I genotyping. PCR products were analyzed on 2% agarose gel stained with ethidium bromide and visualized on a UV transilluminator. Lane 1: 100bp molecular weight marker, lane 3: undigested MCF-7 genomic DNA, lane 4: Taq I digested MCF-7 genomic DNA (digestion control), lane 6: negative control, lane 7: undigested PCR product (745 bp), lane 8: Taq I digested PCR product duct - homozygous TT genotype (494) and (251) bp band sizes.

### 3.2 Effect of cholecalciferol on cell growth

Cholecalciferol inhibited the growth of MCF-7 breast cancer cell line at all concentrations tested ranging from 0.1 to 1000 nM. This effect was observed as early as 24 hours after exposure and continued until 96 hours. Although a significant decrease in cell proliferation could be observed in cells treated with different concentrations compared to the control, this effect seems not to be concentration dependent. Except for the time 96 hours exposure, the concentration of 1000 nM induced a significant decrease in the number of cells compared to the concentration 0.1 nM (Figure 2). Based on the values obtained for the inhibition of growth, an estimated IC<sub>50</sub> (half maximum inhibitory concentration) was calculated as (3.0 x 10<sup>-3</sup> nM) R<sup>2</sup>= 0.9153 in 24 h, (1.6 x 10<sup>-3</sup> nM) R<sup>2</sup> = 0.8862 in 48 h, (2.5 x 10<sup>-4</sup> nM) R<sup>2</sup>= 0.7813, (7 x 10<sup>-4</sup> nM) R<sup>2</sup>= 0.9039.

**Figure 2.** Antiproliferative effect of different concentrations of cholecalciferol in MCF-7 cells.

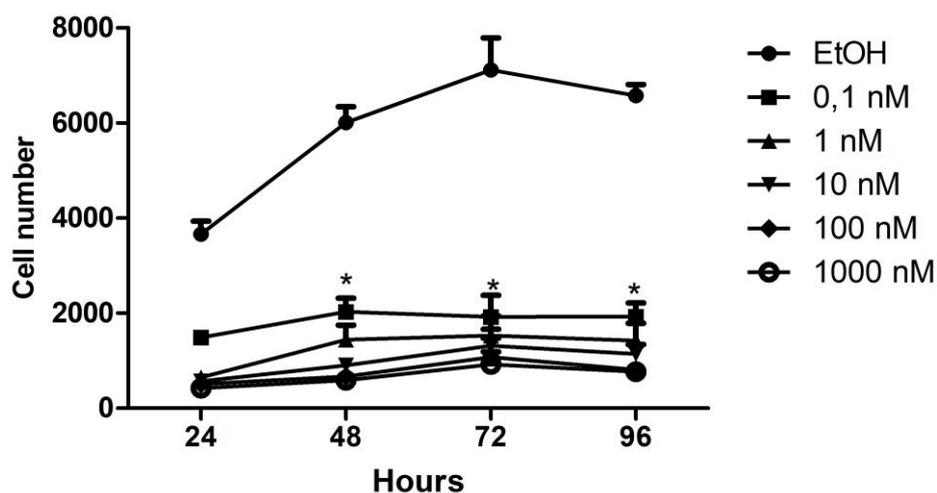


Source: own study.

Cells were exposed to different concentrations of cholecalciferol for A) 24 h, B) 48 h, C) 72 h and D) 96 h and the number of cells were determined. The results represent the mean  $\pm$  standard deviation of three independent experiments (four replicates for each experimental set). \*Statistically significant difference in relation to the control,  $p < 0.05$ , One-way ANOVA and Tukey post hoc test.

The effect of cholecalciferol along the time of exposure is better visualized in Figure 3 where although it is clear the effect on the cell proliferation, this decrease is not changed over the time. The inhibitory effect of cholecalciferol was not evident for the glioblastoma cell line U87-MG as demonstrated for MCF-7 (Figures 4 and 5). Cells incubated for 24 and 48 hours in the presence of different concentrations of cholecalciferol did not show a significant decrease in the number of cells. In contrast, after 72 hours exposure a significant decrease in cell proliferation could be observed for all concentrations tested, but this inhibition was not sustained, since at 96 hours cells were able to reassume growth. If the relative inhibition of cell growth promoted by cholecalciferol is compared between U87-MG and MCF-7 the last one demonstrated more susceptibility to vitamin D (Figure 6).

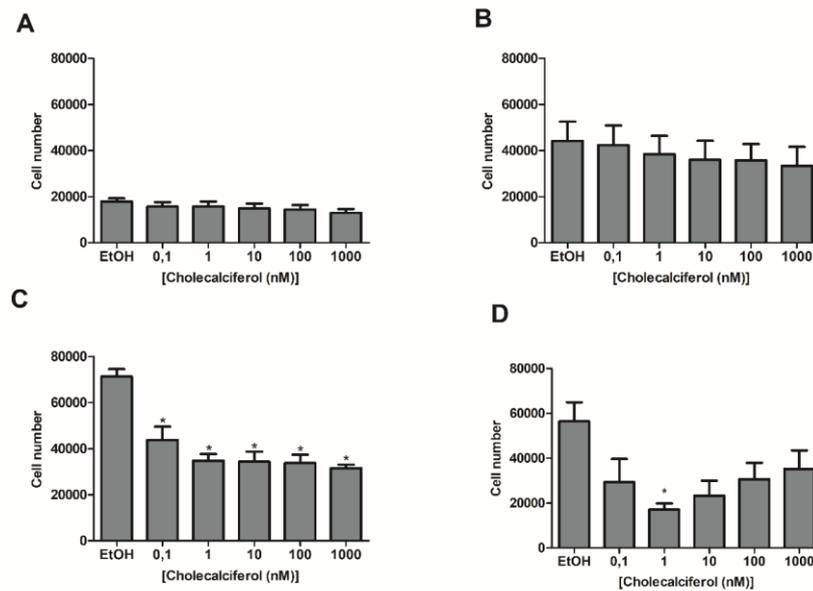
**Figure 3.** Effect of the time of exposure to cholecalciferol on the proliferation of MCF-7 cells.



Source: own study.

The figure shows the rate of growth of MCF-7 cells exposed to different concentrations of cholecalciferol compared to the vehicle (ethanol-EtOH) alone used to solubilize the compound. Results represent the mean  $\pm$  standard deviation of three independent experiments (four replicates for each experimental set). \* Statistically significant difference,  $p < 0.05$ . Two-way ANOVA and Bonferroni post hoc test.

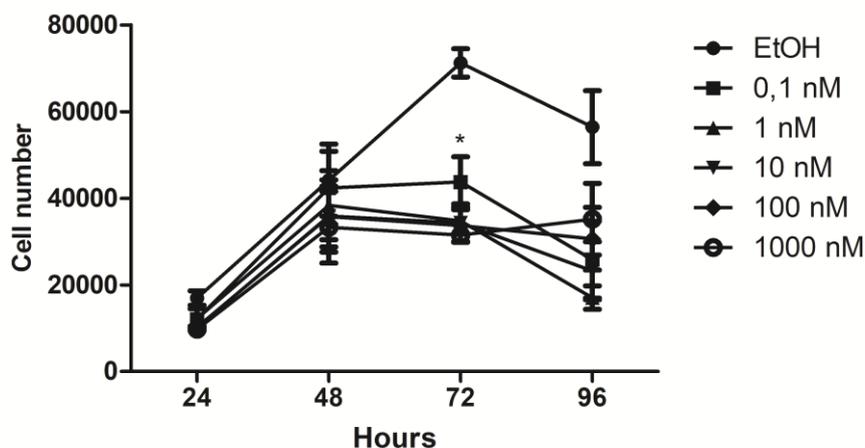
**Figure 4.** Antiproliferative effect of different concentrations of cholecalciferol in U87–MG.



Source: own study.

Cells were exposed to different concentrations of cholecalciferol for A) 24 h, B) 48 h, C) 72 h and D) 96 h and the number of cells were determined. The results represent the mean  $\pm$  standard deviation of three independent experiments (four replicates for each experimental set). \*Statistically significant difference in relation to the control,  $p < 0.05$ , One-way ANOVA and Tukey post hoc test.

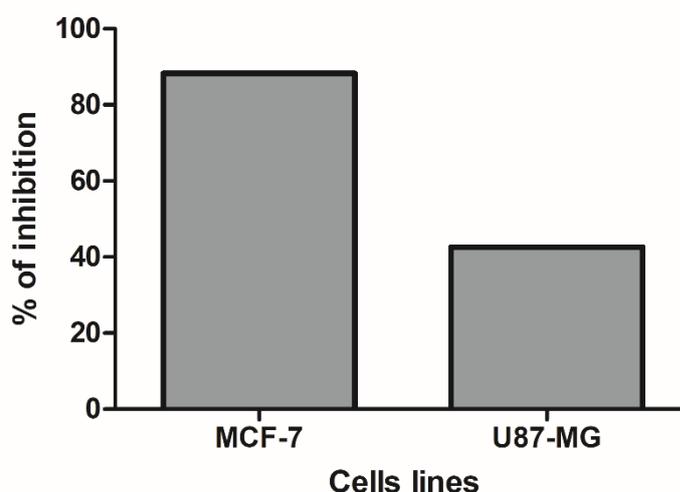
**Figure 5.** Effect of the time of exposure to cholecalciferol on the proliferation of U87–MG cells.



Source: own study.

Rate of growth of U87-MG cells exposed to different concentrations of cholecalciferol compared to the vehicle (ethanol-EtOH) alone used to solubilize the compound. The results represent the mean  $\pm$  standard deviation of three independent experiments (four replicates for each experimental set). \*Statistically significant difference,  $p < 0.05$ . Two-way. ANOVA and Bonferroni post hoc test.

**Figure 6.** Comparison of the cholecalciferol growth inhibition activity between MCF-7 and U87-MG tumor cell lines.



Source: own study.

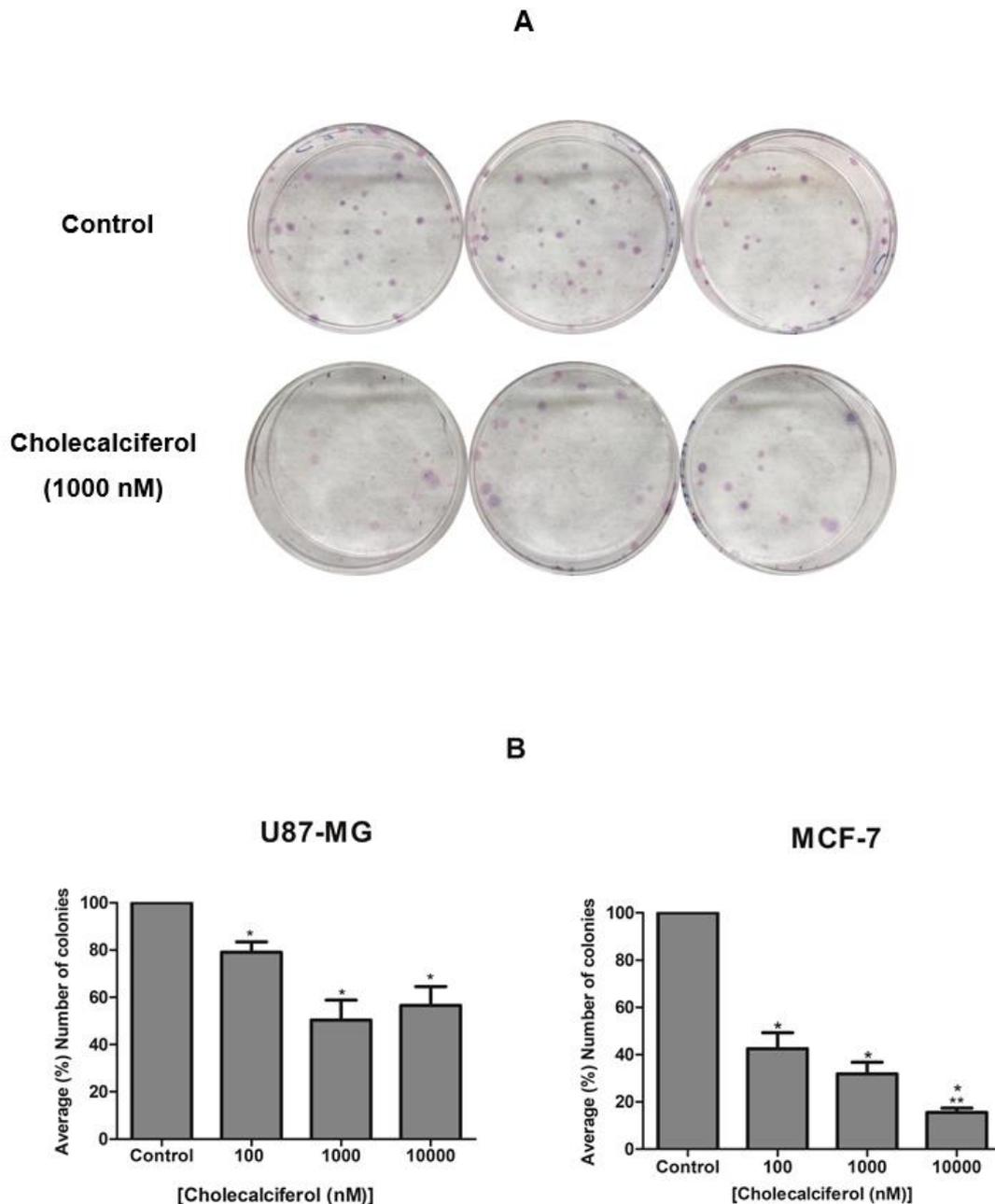
Cholecalciferol induces different rates of inhibition in MCF-7 and U-87-MG cell lines. The percentage of inhibition related to untreated control was determined for each cell line after normalizing for the rate of growth. The results represent an average of inhibition determined for all concentrations 0,1 to 1000 nM time 96 h exposure. \* Statistically significant difference,  $p < 0.05$ .

### 3.3 Anticlonogenic effects of cholecalciferol

The clonogenic assay is able to demonstrate the ability of individual cells proliferate and form colonies after a specific period of time. The initial appropriate number of cells as well as the plate efficiency for each cell line was previously determined as 100 cells per plate and 500 cells per plate for U87-MG and MCF-7 respectively. A representative set of plates are shown in Figure 7A. Cholecalciferol was able to decrease the clonogenicity of MCF-7

and U87–MG as demonstrated by the number of colonies formed after 8-10 days exposure to different concentrations of the vitamin D metabolite. MCF–7 was more susceptible to cholecalciferol than U87–MG and at 10.000 nM these cells showed a tendency to resume growth (Figure 7B).

**Figure 7.** Effect of cholecalciferol on the clonogenicity of U87–MG and MCF–7.



Source: own study.

Demonstration of inhibition of cell proliferation by clonogenic assay. A) representative petri dishes of an experimental set showing reduced number of colonies after seeding 100 cells per plate in the absence (control) and presence of 1000 nM cholecalciferol for 8–10 days followed by fixation and staining with Wright 0.06% inPBS. B) Effect of 100, 1000 and 10000 nM cholecalciferol on the number of colonies formed after 8–10 days incubation. The results represent the mean  $\pm$  standard deviation of the number of colonies counted of three independent experiments (three replicates for each experiment). \*Statistically significant difference in relation to the control group \* $p < 0.05$ , \*\*Statistical difference between concentrations 10000 and 100 nM for MCF–7. One-way ANOVA and Tukey post hoc test.

#### 4. Discussion

The hypothesis that vitamin D may have anticancer benefits was suggested several decades ago based on the fact that higher rates of total cancer mortality is seen in regions with less UV-B radiation, associated with lower levels of circulating vitamin D in the body (Giovannucci, 2005). Although some data on the effect of vitamin D and cancer are promising others show contradictory effects, adding a challenge to support a definite conclusion.

Herein we demonstrated that cholecalciferol inhibits proliferation of MCF–7 breast cancer cells and glioblastoma U87–MG brain tumor cells at different degrees. MCF–7 cells showed more sensitivity to cholecalciferol than U87–MG. Polymorphisms in the VDR gene have been considered one of the factors that could help understand the putative anticancer activity of vitamin D and cancer risk prevention, however the relationship between the polymorphism and the function of the VDR is still not clear. Genotyping of the Fok I (exon II) and Taq I (exon IX) polymorphisms, showed homozygosity of the wild alleles (TT) for Taq I and polymorphic CC (FF) alleles in the Fok I analysis in MCF–7 cell line.

According to Alimirah et al., (2011), Fok I polymorphisms can alter the structure and consequently the function of VDR in human breast cancer cells, where the VDR FF variant is more efficient than VDR ff in modulating Vitamin D action. MCF–7 as shown here has FF genotype and demonstrated sensitivity to treatment with cholecalciferol. The ff variant of VDR gene has both translation initiation codon intact and the protein expressed is longer than its FF genotype. The alternate start codon (SCP) used in the FF genotype is located three codons downstream the original start site, leading to a shorter protein. It is not clear how the

short version of VDR could lead to a sensitivity of MCF-7 cells to cholecalciferol, but it is known that VDR FF protein is more stable and VD increases its half-life. Also, once associated with its ligand, VDR ff upregulates antiapoptotic and pro-inflammatory genes in contrast with VDR FF cells (Alimirah et al., 2011). Then, the inhibitory growth effect observed in MCF-7 may be associated in part to the Fok I FF alleles.

Nevertheless, other factors must be better understood to translate these findings into a definite conclusion. Vitamin D is involved in different biochemical pathways and polymorphisms in other genes associated to VDR may also influence the activity of the vitamin D. Fok I polymorphism alone or in combination with other VDR polymorphisms has been investigated in breast cancer with contradictory results. In one study it was shown that Fok I FF allele with other polymorphisms increased breast cancer risk in a Caucasian population in United Kingdom (Guy et al., 2004). However, two other studies showed that women with ff genotype were more susceptible to breast cancer (Sinotte, et al., 2008; Chen, et al., 2005). Yet, another study showed no correlation between Fok I polymorphism and increased breast cancer risk in postmenopausal women (McCullough et al., 2007). In a study by Raza et al., 2019, evaluating VDR polymorphism in 125 breast cancer patients in India, the Fok I ff genotype was significantly associated with an increased risk of breast cancer. Contradictory, Ahmed et al., 2019 carried out a study in Ethiopia evaluating 392 patients with breast cancer and found no association between homozygous ff or FF with the risk of breast cancer. These contradictory results are probably due to the different ethnic groups assessed in each study and the genetic profile of each individual and tumor type.

One of the hallmarks of cancer is the genetic instability that can lead to loss or rearrangement of chromosomes (Vargas-rondón, Villegas & Rondón, 2018). We tried to determine the status of Fok I and Taq I polymorphisms in the U87-MG cell line using the same set of primers able to amplify the fragment of the VDR gene in MCF-7 cells. However, we were not able to get PCR product with the expected size. Interestingly, U87-MG cells demonstrated less sensitivity to cholecalciferol than MCF-7 cells. We believe that one explanation for the lack of amplification may be due to some rearrangement, deletion or mutation in the region of annealing of the primers. We are currently investigating if this may be the case. Interestingly, Zou et al., (2000) reported different sensitivities to vitamin D in different glioma cell lines. They discovered a 220 kDa protein in glioma cells that were sensitive to VD. This protein was not a classical VDR but was recognized by anti-VDR monoclonal antibody. On the other hand, it has been shown that VDR is important in cellular

survival and migration in the T98-G glioma cell lines, which has mutated p53 tumor suppressor protein (Salomon et al., 2014).

As shown in Figure 6, cholecalciferol inhibited about 85% of cell growth of the MCF-7 strain at the end of the experiment. For the U87-MG, the inhibition was only 40%. The higher sensitivity of breast cancer lineage to VD suggests that in these cells the classic VD pathway may be active, which would not be the case in U87-MG. Since it was not possible, in our hands, to amplify regions of the VDR receptor in U-87 cells, some sequence rearrangement may be occurring and deserve to be better investigated. On the other hand, the effects of cholecalciferol on these tumor cells could not be dependent on VDR, which could explain the low inhibition observed in this cell line.

Considering the VDR polymorphism status related to brain tumor patients, VDR Fok I- ff genotype has been shown significantly higher in Turkish patients with meningioma, however, there was no association of this polymorphism in gliomas patients (TOPTAŞ et al., 2013). Another study has been shown that VDR expression is increased in GBM brain tumor tissues compared to non-malignant tissue and VDR expression is associated with improved outcome in patients with GBM (TOPTAŞ et al., 2013). In contrast, a study performed by Anic et al., (2012) demonstrated no association between the Taq I or Fok I polymorphism with lower survival in patients with Glioblastoma.

It is known that although different tumor cells can be classified as the same type of tumor, the genetic background can be very heterogeneous, which could explain differences in the sensitivity and response to therapy. In fact, it has already been demonstrated that the anti-cancer effects of VD may be dependent on VDR but tumors from different patients can show different sensitivities (Salomon et al., 2014, Ferronato et al., 2018).

Although cholecalciferol induced a decrease in the clonogenicity of U87-MG cells, we found that high concentrations of cholecalciferol was ineffective in inhibiting the growth at 24 and 48 hours. Similar results have been observed (Deberardinis Lemieux & Hadden, 2013), where VD synthetic analogues had no antiproliferative effect in U87-MG at concentrations of 1000, 5000 and 10000nM. On the other hand, the GBM T98-G strain showed sensitivity to concentrations of 1000nM only after 96h exposure (Salomon et al., 2014). This may indicate that the effect of VD on glioblastoma cells is time, cell line and VD metabolite dependent. The antiproliferative activity of VD in Glioblastoma strains is attributed to the activation of p21 and p27 protein expression and a decrease in Cyclin D1, thereby interrupting the cell cycle (Salomon et al., 2014, Ferronato et al., 2018).

The high sensitivity of MCF-7 cells to cholecalciferol reported in this work corroborates similar studies that have shown that VD modulates the growth of breast cancer cells. Saracligil et al., 2017 demonstrated inhibition of MCF-7 cell proliferation in response to calcitriol, the active form of VD, in a dose and time dependent manner, with an IC<sub>50</sub> of 145nM and apoptosis levels of 28% in 48 hours (Saracligil, et al., 2017). Antiproliferative activity of VD has also been demonstrated in the MCF-10 strain of breast cancer (Zheng et al., 2013). It has been also suggested that in response to calcitriol cancer cells may present a less aggressive or normal phenotype, indicating the influence of VD on cell differentiation and proliferation (Gocek & Studzinski, 2009). Another work demonstrated that treatment with calcitriol inhibited the growth of MCF-7 in a dose and time dependent manner and the cells were arrested in the G<sub>0</sub>/G<sub>1</sub> phase and this effect was associated with inflammatory COX<sub>2</sub>/PGE<sub>2</sub> pathway (Yuan, Jiang, Yang, Ding & Deng, 2012).

Vitamin D has a pleiotropic effect and participates in a myriad of pathways related to cell multiplication and differentiation. Several proteins interact with VDR and once the complex is formed different set of genes may be induced. However, the complexity of these interactions is still not well understood. The conflicting results observed in the literature may be explained at least in part by the type of VDR expressed in the tumor cells which may be different from the genotype performed in DNA extracted from blood cells, considering the genetic instability of tumors. Also, the metabolism of vitamin D in the organism involves the expression of many genes that in turn may present functional polymorphisms as well. This demonstrate the need for further studies to better understand the key players in the activity of vitamin D and how this activity can be driven to kill tumor cells.

## **5- Final Considerations**

We demonstrated that VDR gene in MCF-7 presents FF genotype for Fok I polymorphism and TT genotype for Taq I. These cells are sensitivity to cholecalciferol corroborating the results found by other authors. Also, our data suggest that the effect of VD is dependent on tumor cell type and the genetic background may influence in the activity of vitamin D. Considering the impossibility to amplify the VDR gene in U-87-MG cell line by the methodology used here our next step would be to sequence the region encompassing this gene in the genome of these cells to identify possible deletion or rearrangement of this gene. We also will investigate the expression of VDR at protein level in MCF-7 and U87-MG to elucidate the mechanism of action of cholecalciferol in these cells.

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