Vitamin D modulates the basal secretion of inflammatory cytokines in breast cancer cells: An *in-vitro* study

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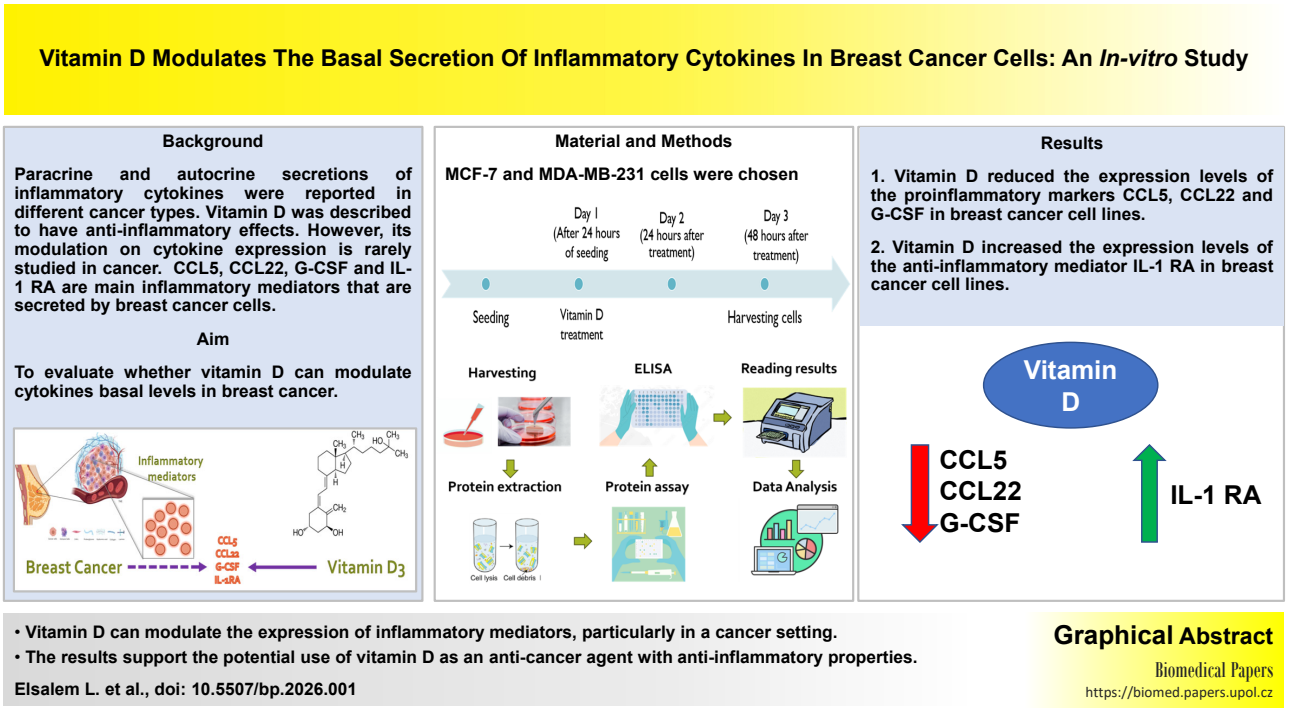
Background. Proinflammatory cytokines are a pivotal factor in cancer initiation and progression. The paracrine and autocrine secretions of such inflammatory cytokines are reported in different cancer types. Recently, C-C motif ligand (CCL) 5 (CCL5), CCL22, granulocyte-colony stimulating factor (G-CSF), and interleukin-1 receptor antagonist (IL-1 RA) have been detected in basal secretions of breast cancer (BC) cells and linked to BC pathogenesis. While Vitamin D has been found to have anti-inflammatory effects, its modulation on cytokine expression is rarely studied in relation to cancer.

Aims. In this study, we evaluated whether vitamin D could modulate cytokine basal levels in BC.

Materials and Methods. This *in vitro* study was conducted on BC cell lines, MCF-7 and MDA-MB-231. Cells were treated with vitamin D (10 and 100 nM), and cytokine levels were measured using the Enzyme-Linked Immunosorbent Assay (ELISA).

Results. There was a significant reduction in CCL5 total levels in MCF-7 cells at both concentrations ($P \leq 0.01$) and in MDA-MB-231 at 100 nM ($P \leq 0.05$). Both concentrations reduced CCL22 levels significantly in MCF-7 ($P \leq 0.05$) and MDA-MB-231 ($P \leq 0.01$). Regarding G-CSF, a significant reduction was found in MCF-7 at both concentrations ($P \leq 0.05$) and at 100 nM in MDA-MB-231 ($P \leq 0.01$). In comparison, IL-1 RA levels were significantly elevated in MCF-7 (100 nM ($P \leq 0.01$)) and MDA-MB-231 (10 nM ($P \leq 0.05$) and 100 nM ($P \leq 0.01$)).

Conclusion. Vitamin D down-regulated the pro-inflammatory and up-regulated the anti-inflammatory cytokines. This indicates that vitamin D can modulate the expression of inflammatory mediators, particularly in a cancer setting. The results support the potential use of vitamin D as an anti-cancer agent with anti-inflammatory properties.



Key words: chronic inflammation, inflammatory cytokines, vitamin D, breast cancer, CCL5, CCL22, G-CSF, IL-1 RA

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INTRODUCTION

Chronic inflammation has been linked to various diseases, including inflammatory bowel disease (IBD), chronic gastritis and endometriosis[1-3]. Recently, mounting evidence indicates its role in cancer pathogenesis[4]. Findings from epidemiological and animal model studies support chronic inflammation's role as a trigger in carcinogenesis[4-6]. The worldwide records of cancer deaths revealed that 15–20% of these deaths are due to infectious and inflammatory responses[4]. Of note, inflammation has been recognized an enabling hallmark of cancer[5].

Intricate analysis of the milieu of the tumor microenvironment (TME) that involves cancer and inflammatory cells has revealed a rich environment full of interactions mediated by inflammatory infiltrates (cytokines, chemokines, and growth factors (GFs)) (ref.[7]). Such interactions drive cancer processes into activation[7]. Notably, these infiltrates are due to the extensive population of different inflammatory cells[7].

Accumulating research on inflammation-related cancer is leading to new preventive and therapeutic strategies to target inflammatory markers[8]. Accordingly, experiments have been conducted to explore the effect of modulating the expression of different inflammatory mediators on the progression of different cancer types[9-11].

Breast cancer (BC) is the most common neoplasia among women worldwide and a leading cause of death due to its heterogeneity in relation to the molecular, morphological, and phenotypic characteristics[12,13]. Although different new cancer detection techniques in the early phases have been improved, the mortality rate is still increasing due to inflammatory and microenvironmental factors involved in BC progression[14].

The inflammatory process mediating BC results from the different interactive pathways among inflammatory cells, stromal cells, and cancer cells[15]. These ultimately keep the inflammatory cycle on due to high infiltration rates of inflammatory cells and high production rates of inflammatory mediators[15]. The effects of these mediators are conducted in a paracrine loop as they are secreted by inflammatory cells and target cancer cells[16].

The autocrine secretion of inflammatory mediators by cancer cells is a central concept in which cancer cells are producing and responding to their secretions[17]. Recent MILLIPLIX assay data on BC has detected the autocrine (basal) secretion by BC cells[7]. Upon comparing the expression levels of inflammatory mediators between different BC cell lines; the estrogen-dependent cell line; MCF-7 and the invasive, triple negative cell line; MDA-MB-231, the (C-C motif) ligand (CCL) 5 (CCL5), CCL22, the granulocyte-colony stimulating factor (G-CSF), and the anti-inflammatory cytokine; interleukin-1 receptor antagonist (IL-1 RA) were found to be detectable in both cell lines compared to other markers reported in the study.

CCL5 belongs to the CC-chemokine family[18]. In BC tissues, CCL5 has been observed in different specimens, being highly elevated in tissues of advanced disease stages[19]. Also, its receptor CCR5 is highly expressed among BC cells[20]. The enrichment of CCL5/CCR5 is consistent with increased activity of signaling pathways and lymphocyte activation[21]. Thus, CCL5 is incorporated in BC progression, invasion, and metastasis[9,21].

CCL22 belongs to the CC family and mediates its action through binding to its receptor; CCR4 (ref.[22]). In BC, CCL22 is secreted by macrophages and BC cells[23]. The secretion of CCL22 in BC is reported in patients' serum samples. It is elevated in BC patients compared to healthy individuals, and directly correlated with the tumor stage[24].

G-CSF belongs to the hematopoietic GF family called colony-stimulating factors (CSFs) (ref.[25]). It is a potent regulator of neutrophil lineage activity and production[25]. Recent studies proved that G-CSF is a potent pro-tumor GF participating in tumor progression, angiogenesis, metastasis, and suppression of apoptosis in different cancer types, including BC (ref.[25-27]). In BC, elevated serum levels of G-CSF were linked with disease stage and poor prognosis[25,28].

IL-1 RA belongs to the IL-1 family[29]. It shares homology with other members of the family (IL-1α and IL-1β) and binds to IL-1 receptor (IL-1 R) competitively by antagonizing the binding of IL-1α and IL-1β (ref.[29]). In BC, IL-1 RA was detected in tissues of invasive and ductal carcinoma in situ, and to a lesser extent in the surrounding stromal cells[30]. It was found that estrogen receptor (ER) positive subtypes of BC have high levels of IL-1 RA that dominate over IL-1 levels[30]. This domination causes decreased tumor activation and proliferation[30].

Based on the aforementioned findings about the role of the inflammatory mediators, CCL5, CCL22, G-CSF, and IL-1RA in cancer, and the recent evidence from the MILLIPLIX assay data on BC (ref.[7]) indicating the detection of these markers as basal secretions, the potential modulation of the expression levels of these markers appears as an attractive area for investigations.

Vitamin D plays key roles in mediating physiological functions, including bone remodeling, calcium homeostasis, and immune promotion[31-33]. Also, other beneficial effects on inflammatory diseases were described[34].

In cancer, numerous studies have explored the potential role of vitamin D in various cancer types. In colorectal carcinoma (CRC), vitamin D has demonstrated the ability to inhibit proliferation[35] and angiogenesis[36] processes, while also promoting apoptosis[37] and differentiation[38]. In prostate cancer, vitamin D has likewise been shown to suppress proliferation[39] and angiogenesis[40], and enhance apoptosis[41]. Studies in ovarian[42] and gastric[43] cancers have also revealed an anti-proliferative effect of vitamin D.

In BC, vitamin D is reported to affect different cancerous processes and was revealed to have anti-proliferative, pro-apoptotic, anti-migratory, and anti-invasive effects[44-47]. However, limited research exists regarding its anti-inflammatory roles or its modulatory effect on the expression of inflammatory mediators in the cancer setting, particularly in BC.

This *in vitro* study aimed to investigate the impact of vitamin D treatment on the basal secretion and expression level of CCL5, CCL22, G-CSF, and IL-1RA in BC cell lines MCF-7 and MDA-MB-231.

MATERIALS AND METHODS

Cell culture

Human BC cell lines (MCF-7 and MDA-MB-231) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in complete RPMI 1640 medium (EuroClone SPA, Cat. NO ECB2000L, Italy) supplemented with 10% fetal bovine serum (FBS) (CAPRICORN Scientific GmbH, Cat. NO FBS-HI-11A, Germany), 1% L/Glutamine (EuroClone, SPA, Cat NO ECB3000D, Italy), and 1% sodium pyruvate (EuroClone SPA, Cat NO ECM0542D, Italy). Cells were incubated at 37 °C in a humidified chamber (Model: S@ FEGROW 188 PRO, Serial Nr. S2524, Euro clone) supplemented with 5% CO_2. Cells were used for subsequent experiments with an early passage number (less than 10).

Vitamin D stock solution

Vitamin D (1α,25-Dihydroxyvitamin D3 (1,25-$(OH)_2$ D3)) was purchased from Sigma-Aldrich (SIGMA-ALDRICH, Catalog Number D1530, CAS RN 32222-06-3), and it was maintained in air and light-tight conditions (−20°C). A 10 µM vitamin D stock solution was prepared in 95% ethanol.

Cell seeding and vitamin D treatment

Each mammary cell line (MDA-MB-231 and MCF-7) was seeded into each well of the 6-well plates (SPL Life Sciences Cat. NO 30006) with a cell density equal to $25*10^4$ cells/mL in 2 mL of fresh media ($5*10^5$ cells/well, considering that each well contains 2 mL). Cells were then incubated at 5% CO_2, 37 °C, and 100% humidity for 24 h. Cells were then treated with vitamin D at concentrations of 100 nM and 10 nM prepared in complete RPMI media, and incubated for a further 48 h.

Regarding control untreated cells, vitamin D was replaced with 95% ethanol. This was calculated depending on this equation: C1*V1 = C2*V2, considering that C1 is the vitamin D stock concentration (10 µM) and C2 is the first concentration prepared in each trial (100 nM).

According to this formula, a volume of 20 µL (V1) of 95% ethanol is added to fresh media to prepare a volume of 2 mL (V2) media for control samples.

Treatment concentrations and incubation times were selected based on previous studies using vitamin D (ref.[48-51]). After incubation, cells and their supernatants were harvested in phosphate-buffered saline (PBS). Cell pellets and supernatants were kept at −80 °C for later use.

Cell lysis and protein extraction

The stored samples of cell pellets were lysed for protein extraction using 4 mL of Radioimmunoprecipitation assay (RIPA) lysis buffer (Sigma-Aldrich, Catalogue No. 632424), 20 µL of phosphatase inhibitor (Sigma-Aldrich P5726), and 20 µL of protease inhibitor (Sigma-Aldrich P8849).

A volume of 600 µL of lysis buffer cocktail was added to each eppendorf tube. The Ultra X Cordless Ultrasonic activator device (Cat. NO SKU: 6280001, Germany) was then used to lyse cells efficiently. After that, centrifugation at 15000 rpm for 10 min was achieved to separate cell debris from cell lysates. Cell lysates were then distributed into eppendorf tubes with known volumes and stored at −80 °C freezer for further use.

Protein quantification assay protocol

The colorimetric assay DC protein assay kit (BIO-RAD, Cat. NO 500-0111, 500-0112, 500-0116) was used for protein quantification according to the manufacturer's instructions using 96-well plate and absorbance microplate reader (750 nm) (Bio Tek Instruments Inc., SN 1402047, USA).

The enzyme-linked immunosorbent assay (ELISA)

Four cytokines were investigated in the experiment: IL-1RA, G-CSF, CCL22, and CCL5. The kits related to IL-1RA (Catalogue # SEA223Hu), CCL22 (Catalogue # SEA091Hu), and CCL5 (Catalogue # SEA116Hu) were purchased from Cloud-Clone Corp (CCC, USA). Regarding G-CSF, the kit was purchased from R&D Systems (Catalog # DY214, Bio-Techne Brand).

The levels of these mediators were determined in each treated and control untreated sample in triplicate, according to the instruction booklets supplied by the manufacturers. The color change is measured spectrophotometrically at a wavelength of 450 nm ± 10 nm using the absorbance microplate reader (BioTek Instruments Inc., SN 1402047, USA).

Each reading of the 96-well plates of these kits was presented as an absorbance value after reading on the absorbance microplate reader, then these values were plotted against standard concentration values to build a proper standard curve (according to each kit protocol). The results were expressed as optical densities, and they were converted into concentration values by extracting each standard curve equation using Microsoft Excel.

Statistical analysis

Microsoft Excel Office was used to calculate the mean concentration and the standard error of the mean (SEM) for each marker from 3 independent experiments. Using the student's *t*-test, the significance of the difference between the control untreated and treated cells was assessed. *P* values that were equal to or less than 0.05 were considered significant. The figures regarding each marker were generated using Prism 8.0.2 (GraphPad Software, San Diego, California, USA).

RESULTS

Effect of vitamin D on CCL5 level

CCL5 level in supernatant samples

Regarding the expression of CCL5 in supernatant samples, both MCF-7 and MDA-MB-231 showed that the highest levels were in control untreated cells compared to cells treated with vitamin D (Fig. 1A and 1D, respectively). Vitamin D treatment has reduced the cytokine level in a dose-dependent manner. This was statistically significant in MCF-7 cells at vitamin D concentrations of 10 nM

(*P*=0.0476) and 100 nM (*P*=0.0634), reducing the protein concentration to 23.7% and 7.7%, respectively, compared with control untreated cells. Regarding MDA-MB-231, the reduction in expression was only significant at 100 nM (*P*=0.0317), and the expression was up to 55.9% compared with control untreated cells (Table S1).

CCL5 levels in cell lysates

Referring to Fig. 1B, it is noted that the lysate samples from control untreated MCF-7 cells generally exhibit higher CCL5 expression levels than their corresponding treated samples. Vitamin D treatment has resulted in a significant reduction at 10 nM (*P*=0.0277) and 100 nM (*P*=0.0361) concentrations to a level of 56.7% and 58.5%, respectively, compared to control cells (Table S1). In comparison, no statistically significant differences were observed in MDA-MB-231 cells as illustrated in Fig. 1E and Table S1.

Total CCL5 levels

Fig. 1C shows that vitamin D has resulted in a significant reduction in total CCL5 levels in MCF-7 cells in a dose-dependent manner at 10 nM (*P*=0.0036), and at 100 nM (*P*=0.0050). The total CCL5 secretion among the 10 nM and 100 nM was 43.9% and 39.5%, respectively, compared to the control untreated cells (Table S1). In comparison, only the 100 nM vitamin D concentration has resulted in a significant reduction (*P*=0.0479) in total CCL5 level in MDA-MB-231, being 72.2% compared to control untreated cells (Fig. 1F and Table S1).

Effect of vitamin D on CCL22 levels

CCL22 levels in supernatant samples

As illustrated by Fig. 2 (A and D) and S2 Table, the supernatant results of MCF-7 cells and MDA-MB-231 revealed that vitamin D treatment decreased the level of CCL22 in a dose-dependent manner compared to control untreated cells, but this was statistically significant only in MDA-MB-231 cells (*P*=0.0398) at vitamin D concentration of 100 nM. At this concentration, the expression was reduced to 49% compared with the control untreated cells.

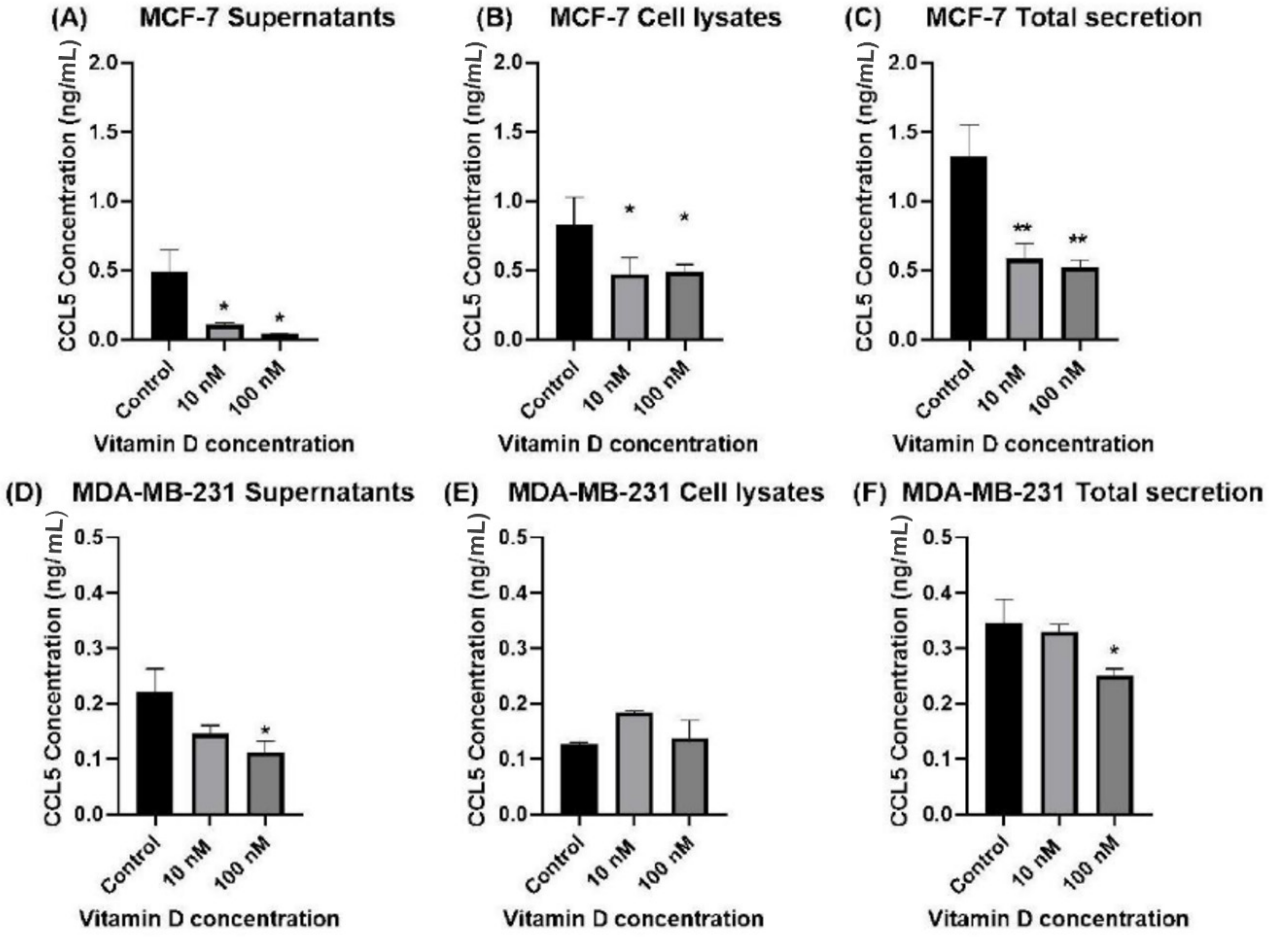

Fig. 1. Vitamin D treatment effect on CCL5 secretion (ng/mL) in both MCF-7 and MDA-MB-231 cells.
The effect of the treatment was studied in each sample type (supernatants, cell lysates, and total) of each breast cancer cell line (MCF-7 (A–C), MDA-MB-231 (D–F)). Data represent the mean concentration of 3 independent experiments and error bars are the standard error of the mean (SEM). Stars represent significance levels as * refers to a significant difference ≤0.05, ** refers to a significant difference ≤ 0.01, and *** refers to a significant difference ≤0.001.

CCL22 levels in cell lysates

Vitamin D treatment has resulted in a significant reduction of CCL22 expression levels in cell lysate samples of MCF-7 cells at 10 nM (*P*=0.0224) and at 100 nM (*P*=0.0174) concentrations (Fig. 2B). The expression levels were reduced at 10 nM to 62.1% and at 100 nM to 56.9% compared with control untreated cells (Table S2**)**. Regarding MDA-MB-231 cells, vitamin D treatment has also resulted in a significant reduction at 10 nM concentration (*P*=0.0031), and at 100 nM (*P*=0.0004) compared with control, untreated cells. The expression levels at 10 nM concentration were reduced to 42.5% and at 100 nM concentration to 46.2% compared with control untreated cells (Fig. 2E and Table S2).

Total CCL22 levels

Both Fig. 2 (C and F) and Table S2 show that vitamin D treatment resulted in a dose-dependent reduction in CCL22 levels in both MCF-7 and MDA-MB-231 cells. This was found to be statistically significant (*P*=0.0316) at 10 nM and (*P*=0.0138) at 100 nM concentrations in MCF-7 cells, reducing the levels to 74.8% and 69.4%, respectively, compared to control untreated cells. Regarding MDA-MB-231, the reduction was also statistically significant upon treatment with 10 nM (*P*=0.0031) and 100 nM (*P*=0.0004), and the level of expression was reduced to 52.8% and 47.2%, respectively.

Effect of vitamin D on G-CSF level

G-CSF levels in supernatant samples

Regarding the expression of G-CSF in supernatant samples of control untreated cells, as shown in Fig. 3 (A and D) and Table S3, MDA-MB-231 cells expressed a very high level (856.556 pg/mL) in comparison to MCF-7 cells (1.826 pg/mL). Studying the changes in G-CSF expression upon vitamin D treatment showed a decrease in its expression at the highest concentration (100 nM), and this was only significant (*P*=0.0364) in MDA-MB-231 cells.

G-CSF levels in cell lysates

Both Fig.3B and Table S3 show that treatment of MCF-7 cells with vitamin D resulted in a significant reduction in G-CSF level in cell lysate samples at both investigated concentrations to a level of 69% and 67.6%

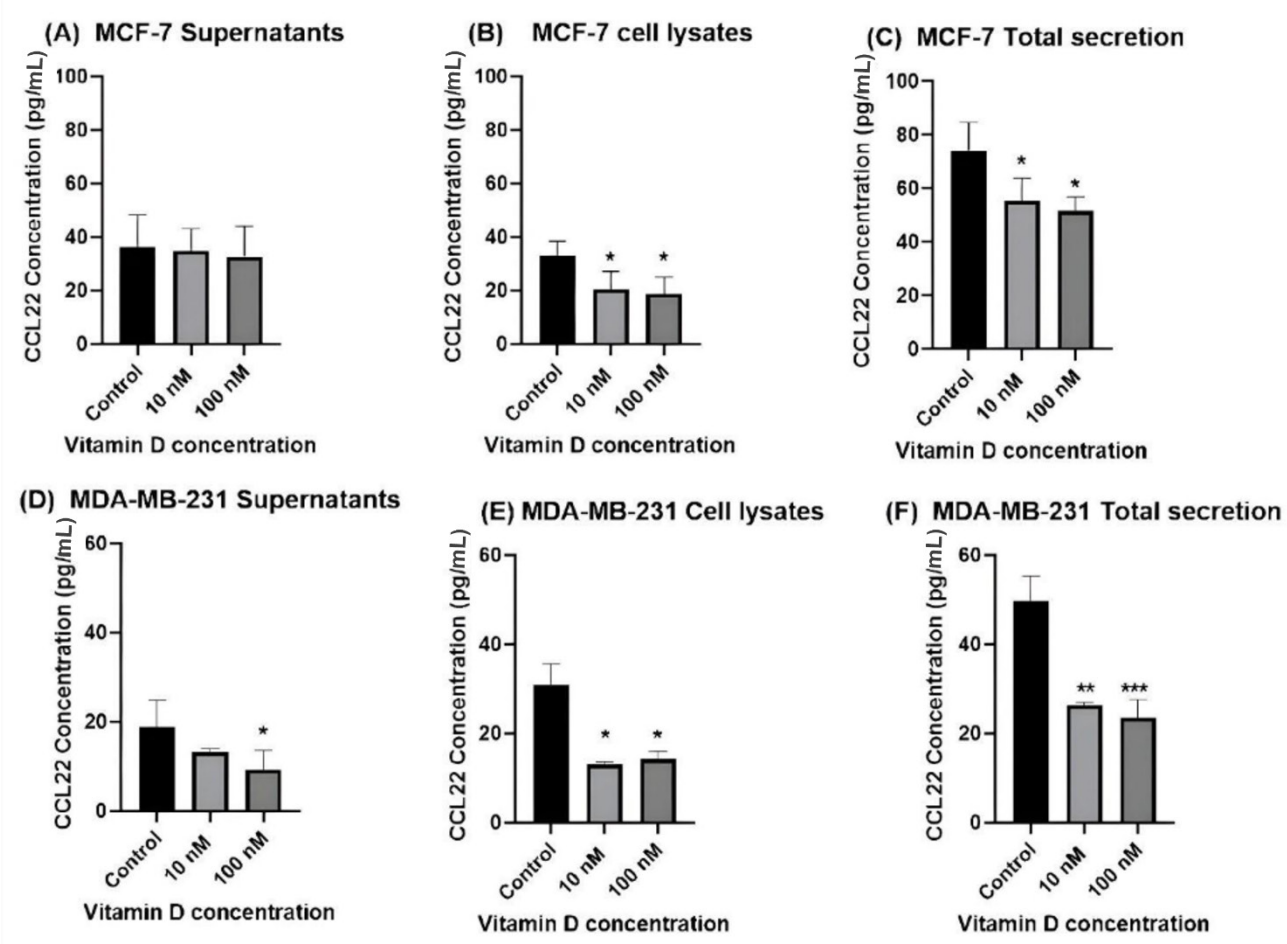

Fig. 2. Vitamin D treatment effect on CCL22 secretion (pg/mL) in both MCF-7 and MDA-MB-231 cells.
The effect of the treatment was studied in each sample type (supernatants, cell lysates, and total) of each breast cancer cell line (MCF-7 (A-C), MDA-MB-231 (D-F)). Data represent the mean concentration of 3 independent experiments and error bars are the standard error of the mean (SEM). Stars represent significance levels as * refers to a significant difference ≤0.05, ** refers to a significant difference ≤ 0.01, and *** refers to a significant difference ≤0.001.

upon treatment with 10 nM (P=0.0042), and 100 nM (P=0.0268) compared with control untreated cells. Regarding MDA-MB-231, G-CSF levels were decreased in a dose-dependent manner, and this was statistically significant at both vitamin D concentrations of 10 nM (P=0.0456) and 100 nM (P=0.0085) where the levels were reduced to 75.4% and 58.8%, respectively, compared with control untreated cells (Fig. 3E).

Total G-CSF levels

Fig. 3C and Table S3 show that vitamin D treatment resulted in a dose-dependent significant reduction in total G-CSF level in MCF-7 treated cells to a level of 77.9% and 75.5% compared with control untreated cells upon treatment with 10 nM (P=0.0149) and 100 nM (P=0.0095), respectively. However, upon vitamin D treatment of MDA-MB-231, the total expression was only decreased at the highest concentration (100 nM) by 2.9% (P=0.0094) (Fig. 3F and Table S3).

Effect of vitamin D on IL-1 RA level

IL-1 RA levels in supernatant samples

Fig. 4A shows no significant differences in the expression level of IL-1 RA in supernatant samples of MCF-7 treated cells compared with control untreated cells. In comparison, MDA-MB-231 cells showed that upon vitamin D treatment, the levels of IL-1 RA were elevated in a dose-dependent manner, being statistically significant at 10 nM (P=0.0217) and 100 nM (P=0.0036) concentrations, and increased by 3.8% and 5.9% compared to control untreated cells, respectively (Fig. 4D and Table S4).

IL-1 RA levels in cell lysate samples

In MCF-7 cells, the IL-1 RA levels in cell lysate samples were increased upon vitamin D treatment, but this was found to be statistically significant (P=0.0033) only at the concentration of 100 nM (Fig. 4B). The levels increased by 23.2% compared to the control, untreated cells. Regarding the MDA-MB-231 cells, although vitamin

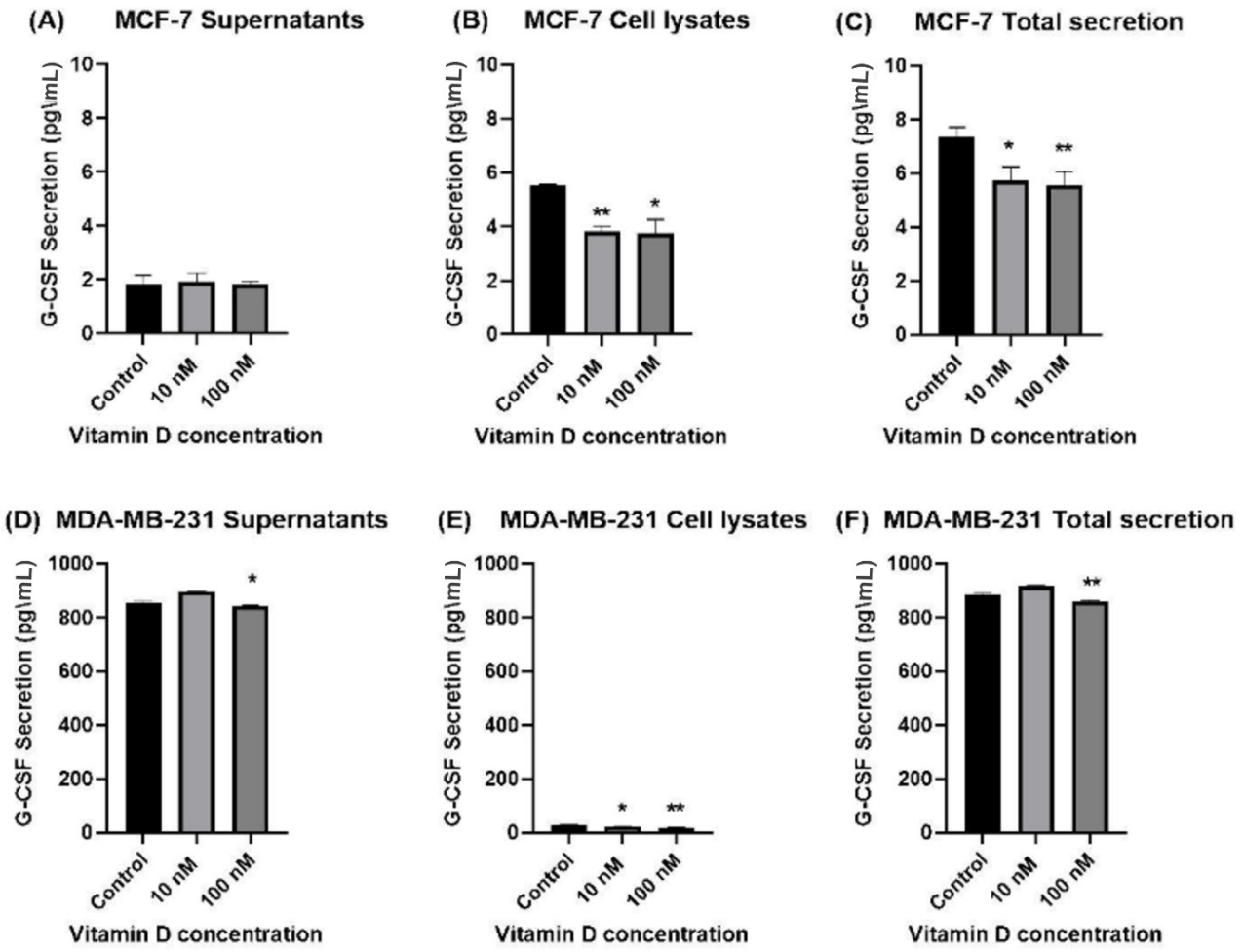

Fig. 3. Vitamin D treatment effect on G-CSF secretion (pg/mL) in both MCF-7 and MDA-MB-231 cells.
The effect of the treatment was studied in each sample type (supernatants, cell lysates, and total) of each breast cancer cell line (MCF-7 (A–C), MDA-MB-231 (D–F)). Data represent the mean concentration of 3 independent experiments and error bars are the standard error of the mean (SEM). Stars represent significance levels as * refers to a significant difference ≤0.05, ** refers to a significant difference ≤0.01, and *** refers to a significant difference ≤0.001.

D treatment resulted in an elevation in IL-1 RA expression, this was not statistically significant (Fig. 4E and Table S4).

Total IL-1 RA levels

Upon treatment with vitamin D, the the total levels of IL-1 RA were higher compared with control untreated cells in both cell lines at both investigated concentrations. However, in MCF-7 cells, this was statistically significant only at 100 nM (P=0.0088) with a 15% elevation in expression compared with control untreated cells, as illustrated in Fig. 4C. In comparison, the elevation in total expression in MDA-MB-231 cells was dose-dependent and statistically significant at both concentrations. The increase in the level was by 6.5%, and 6.4% compared with control untreated cells at concentrations of 10 nM (P=0.0333) and 100 nM (P=0.0027), respectively (Fig. 4F and Table S4).

DISCUSSION

Considering that inflammatory mediators are the main components and drivers of different cancer processes, their modulation appears an attractive approach to be investigated. In this study, we focused on the basal release of three pro-inflammatory mediators (CCL5, CCL22, and G-CSF) and one anti-inflammatory mediator (IL-1RA) by BC cells using a cell culture-based model. This basal expression was compared with samples treated by vitamin D (1α, 25-dihydroxyvitamin D3) at different concentrations (10nM and 100nM) to detect if this treatment has an impact on the expression of these mediators.

It is critical to mention that inflammation is considered a new hallmark of cancer progression when it works chronically[5]. Many studies have investigated the anticancer effects of vitamin D, in addition to its anti-inflammatory properities[52]. Vitamin D exerts actions on the different cellular events of growth, differentiation, and apoptosis of immune cells such as dendritic cells (DCs), monocytes, T cells and natural killer (NK) cells. This is reflected on the inflammatory engine in TMEs as these cells, along with stromal cells are main components[53-55]. Investigations have proved that vitamin D ex-

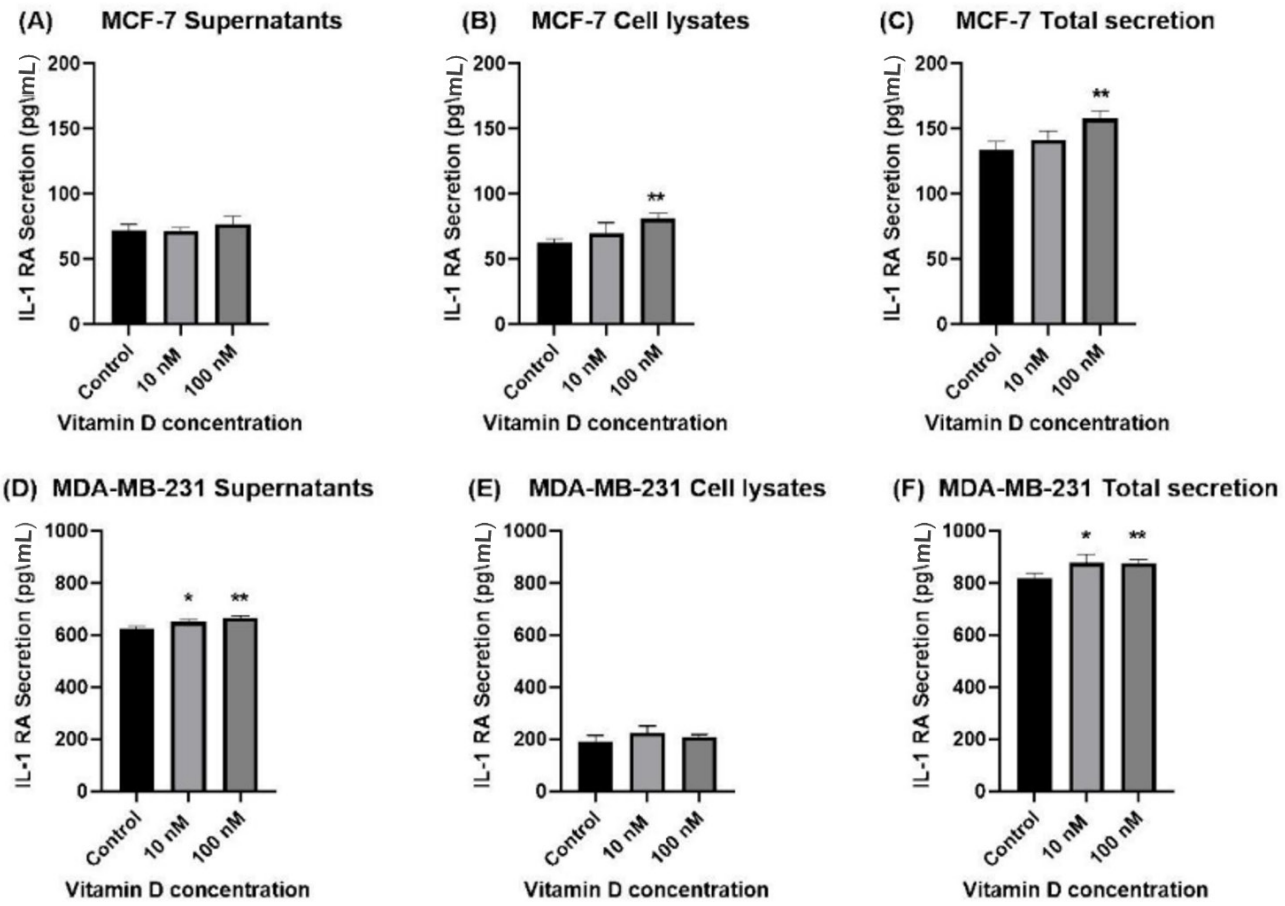

Fig. 4. Vitamin D treatment effect on IL-1 RA secretion (pg/mL) in both MCF-7 and MDA-MB-231 cells.
The effect of the treatment was studied each sample type (supernatants, cell lysates, and total) of each breast cancer cell line (MCF-7 (A–C), MDA-MB-231 (D–F)). Data represent the mean concentration of 3 independent experiments and error bars are the standard error of the mean (SEM). Stars represent significance levels as * refers to a significant difference ≤0.05, ** refers to a significant difference ≤0.01, and *** refers to a significant difference ≤0.001.

erts anti-tumor effects generated by targeting cells such as cancer-associated fibroblasts (CAFs) and tumor-derived endothelial cells (TDECs) (ref.[54,56]). In pancreatic cancer, the suppressive effect of vitamin D on CAFs exhibited anti-tumor and anti-metastatic effects[54,57]. Also, the use of vitamin D in prostatic and breast tumor models reflected an angiogenic suppression as TDECs are inhibited in their proliferation[56,58,59].

Regarding the inflammatory mediator contents within the TME, limited studies have been conducted in the literature regarding the effect of vitamin D on their expression levels and consequently their effects on cancer processes[55].

CCL5 is a pro-inflammatory mediator that belongs to the chemokine family. It has been detected in different cancer types, as it can be provided in TMEs by inflammatory and cancer cells[21]. In our study, the expression of BC cells of CCL5 was monitored and results showed that its expression in MCF-7 cells is more than in MDA-MB-231 cells, which was apparent and consistent with findings from a previous study[7]. The general readings of CCL5 expression in MCF-7 cells revealed that vitamin D treatment has resulted in a significant reduction in its level in supernatants and cell lysates compared with control untreated cells. On the other hand, MDA-MB-231 readings revealed that only the highest concentration (100 nM) reduced the expression of CCL5 in supernatants and the total expression compared to control untreated cells. Our results revealed that vitamin D generally diminishes CCL5 expression in both cell lines. This is a proof of concept that vitamin D can modulate the expression of inflammatory cytokines, and the observed reduction might be reflected in inflammation, which subsequently compromises the nourishing inflammatory fuel of cancer supplied by this mediator.

By inference to previous studies that incorporate CCL5 levels with different treatment approaches, the decreased levels of CCL5 were linked to subsequent inhibitory effects on different cancerous processes such as growth, immunosuppression, invasion, and metastasis[9,20,21,60,61]. Accordingly, this suggests that the observed reduction of CCL5 levels upon vitamin D treatment in this study might be reflected in the inhibition of the aforementioned cancerous processes.

CCL22 is another marker that belongs to chemokines[22]. In cancer, CCL22 is highly expressed as reported in different cancer types, and it is critical in progressing immunosuppression within cancers as it interacts mainly with regulatory T cells (Tregs) and Th2 cells[24]. Cancer cells have been studied, and results showed that they are also considered expressors of CCL22, like other immune cells[62]. In MDA-MB-231 cells, our investigations showed that vitamin D has resulted in a significant reduction of CCL22 in samples from cell lysates (at both concentrations) and supernatants (at 100 nM) compared with control untreated cells. Regarding MCF-7 cells, vitamin D also caused a significant reduction of CCL22 in cell lysate samples at both concentrations compared with control untreated cells. Regarding CCL22's roles in cancer, it was investigated by changing its expression and studying the affected cancerous processes upon this change. The two main cancerous processes that have been affected are immunosuppression and migration[62-65]. Overexpression of CCL22 levels has been induced in the prostate and liver, and stimulatory effects on immunosuppression and migration have resulted[63,65]. On the contrary, antagonizing CCL22 effect by CCL22 monoclonal antibodies in HNSCC, liver, and colon cancers or CCR4 antagonism in HNSCC and prostate cancer have resulted in reduced activities of immunosuppression and migration[62-65]. A study on BC revealed that micro-vessel density and metastasis are the main cancerous processes consistent with CCL22/CCR4 expression levels[66]. Regarding our results, the general role of vitamin D on CCL22 expression is an inhibitory effect in both cell lines. According to this, proposed inhibitory effects on migration, immunosuppression, in addition to angiogenesis, are expected by referring to the previous effects of CCL22 antagonism[62,64]. Also, suggestions to incorporate the previous CCL22/CCR4 targeted strategies with vitamin D can be highlighted as possible significant inhibitory effects can be expected.

The third marker that has been studied is a member of the CSF, which is the G-CSF. This mediator is critical in immune functions as it is responsible for neutrophil lineage production and activity, thus enhancing the inflammatory responses[25]. In cancer, it has been reported to promote cancer progression, angiogenesis, metastasis, and anti-apoptotic effects in different cancer types[26,27]. Our results showed that MDA-MB-231 cells express very high levels of G-CSF in comparison to MCF-7 cells that express very low levels, and this finding is consistent with other findings[7,25]. The differential expression suggests the possible association of this pro-inflammatory mediator with the aggressiveness of MDA-MB-231 cells (triple negative) compared with the less invasive MCF-7 cells. Regarding the MCF-7 cells, vitamin D has resulted in a significant reduction in G-CSF expression in cell lysates at both concentrations of vitamin D compared to control untreated cells. Regarding MDA-MB-231 supernatants, vitamin D treatment suppressed the expression of this cytokine only at 100 nM, while cell lysates showed dose-dependent reduction upon vitamin D treatment at 10 nM and 100 nM concentrations. Studies on G-CSF are extensive among different types of cancer, including BC. The general effects on the modulation of G-CSF levels by the different methods influence different cancer processes. Investigations related to G-CSF supplementary treatments enhanced G-CSF levels and resulted in the production of pro-inflammatory cytokines (IL-1β, IL-6, IL-12, and tumor necrosis factor-α (TNF-α)) in colon and pancreatic cancers, proliferation of colon and gastric cancer cells, and migration in prostate cancer[67-69]. On the other hand, antagonizing treatments of G-CSF using monoclonal antibodies in colon and gastric cancer reduced the migratory activity of cancer cells[69]. Of note, the most affected cancerous processes upon the use of G-CSF monoclonal Abs in BC ware migration and metastasis, while supplementing recombinant G-CSF enhanced both the

production of pro-tumor cytokines (IL-6 and monocyte chemoattractant protein-1 (MCP-1)) and migration[28, 70]. Our results revealed that this mediator has been decreased in the expression levels of both BC cells by vitamin D treatment. This encourages the successive use of vitamin D to modulate G-CSF expression and suggests a new combined treatment of both G-CSF antibodies and vitamin D that may achieve profound effects.

IL-1 RA was the fourth marker that was investigated. It belongs to the IL-1 family and competes with IL-1 mediators to bind to receptor IL-1 R1. The anti-inflammatory property of this mediator makes it a potent option in the treatment of different diseases, such as rheumatoid arthritis[71]. This also applies to cancer, as it works in antagonizing different cancer processes by inhibiting the pro-inflammatory mediators[72,73]. In BC, this cytokine was reported to be secreted by BC cells and highly secreted by MDA-MB-231 cells compared to MCF-7 cells, which coincides with our findings[7]. Regarding our results, vitamin D treatment has increased the expression of IL-1 RA in the total secretion of MCF-7 cells at the highest concentration (100 nM). This also applies to cell lysates. In MDA-MB-231 cells, the expression was significantly increased upon vitamin D treatment in supernatants at both concentrations; 10 nM and 100 nM. The use of the recombinant IL-1 RA in gastric cancer inhibited cancer cell proliferation, migration, and angiogenesis[29]. It also showed inhibition of liver metastasis of colon cancer[74]. The vast use of recombinant IL-1 RA in BC revealed inhibitory effects on growth, metastasis, and angiogenesis[75,76]. Regarding our results, the general stimulatory effects of vitamin D on the expression of IL-1 RA in both cell lines suggest potential activation of an anti-inflammatory response, as this is considered consistent with the recombinant cytokine use. Thus, we suggest incorporating vitamin D treatment to be considered an important approach to enhance the anti-inflammatory response of cancers by increasing the levels of the anti-inflammatory cytokine, IL-1 RA, and consequently may affect the general cancer processes as mentioned earlier.

LIMITATIONS

This study has some limitations that should not underestimate the novel findings. The experiments were conducted in a 2D cell culture model. In addition, the study focused solely on breast cancer cell lines (MCF-7 and MDA-MB-231) without co-culturing with inflammatory or immune cells, which play a significant role in tumor inflammation and progression. Therefore, the effect of Vitamin D on the broader inflammatory network within the tumor microenvironment remains unclear. Furthermore, changes in the expression levels of inflammatory mediators were measured, however, no functional assays were performed to assess the downstream biological consequences of these changes. Future studies involving functional analyses are needed to provide a more comprehensive understanding of the role of vitamin D in modulating cancer-related inflammation.

CONCLUSION

Our findings revealed that vitamin D treatment has resulted in general inhibitory effects on the expression of the pro-inflammatory markers, CCL5, CCL22, and G-CSF, and upregulation effects on the anti-inflammatory marker, IL-1RA. This opens the horizons on possible roles of vitamin D in cancer setting by affecting cytokine expression levels. This suggests that vitamin D might be a potential pharmacological agent with anti-inflammatory properties that might have profound effects on cancer progression. Accordingly, future *in-vitro* and *in-vivo* studies should be conducted to evaluate the functional consequences of modulating the expression of the investigated mediators. This can also be considered in combination with currently available inhibitors or monoclonal antibodies against these mediators. Since vitamin D is considered a safe supplement, further studies are needed to assess if adding this vitamin to the treatment protocols of cancer treatment might have a valuable impact on the patient's survival.

ABBREVIATION

$1,25\text{-}(OH)_2D3$, 1α,25-Dihyroxy vitamin D3; BC, Breast cancer; CAFs, Cancer-associated fibroblasts; CCL, C-C motif ligand; CCR, CC chemokine receptor; CSFs, Colony stimulating factors; CRC, Colorectal carcinoma; DCs, Dendritic cells; ELISA, Enzyme-linked immunosorbent assay; ER, Estrogen receptor; G-CSF, Granulocyte-colony stimulating factor; GFs, Growth factors; HNSCC, Head and neck squamous cell carcinoma; IBD, Inflammatory bowel disease; IL-1 R, Interleukin 1 receptor; IL-1 RA, Interleuking-1 receptor antagonist; MCP-1, Monocyte chemoattractant protein-1; NK cells, Natural killer cells; PBS, Phosphate buffered saline; RIPA, Radioimmunoprecipitation assay; SEM, Standard error of the mean; TDECs, Tumor-derived endothelial cells; Th2 cells, T helper 2 cells; TME, Tumor microenvironment; TNF-α, Tumor necrosis factor-α; Tregs, Regulatory T cells.

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SUPPLEMENTUM

Table S1. The average readings CCL5 secretion (ng/mL) in all samples (supernatants, cell lysates, and total) of both cell types MDA-MB-231 and MCF-7.

CCL5 Concentration	MCF-7 Supernatant	Cell lysate	Total	MDA-MB-231 Supernatant	Cell lysate	Total
Control	0.493	0.828	1.321	0.22	0.126	0.346
10 nM	0.117 (*P*=0.047612)	0.470 (*P*=0.027754)	0.580 (*P*=0.003642)	0.145	0.184	0.330
100 nM	0.038 (*P*=0.634875)	0.485 (*P*=0.036171)	0.522 (*P*=0.005092)	0.112 (*P*=0.031793)	0.137	0.250 (*P*=0.047945)

CCL5 levels were measured in supernatants and cell lysates of each breast cancer cell line; total secretion was calculated as their sum.

Table S2. The average readings of CCL22 secretion (pg/mL) in all samples (supernatant, cell lysates, and the total) at both cell types MDA-MB-231 and MCF-7.

CCL22 Concentration	MCF-7 Supernatant	Cell lysate	Total	MDA-MB-231 Supernatant	Cell lysate	Total
Control	36.14675	33.155	74.139	18.969	30.82528	49.794
10 nM	34.892	20.603 * (*P*=0.02732)	55.495 * (*P*=0.03163)	13.242	13.09388 * (*P*=0.022376)	26.336 ** (*P*=0.003063)
100 nM	32.6255	18.878 * (*P*=0.014008)	51.504 * (*P*=0.013758)	9.298 * (*P*=0.039846)	14.2572 * (*P*=0.017405)	23.555 *** (*P*=0.000387)

CCL22 levels were measured in supernatants and cell lysates of each breast cancer cell line; total secretion was calculated as their sum.

Table S3. The average readings of G-CSF secretion (pg/mL) in all samples (supernatant, cell lysates, and the total) at both cell types MDA-MB-231 and MCF-7.

G-CSF Concentration	MCF-7 Supernatant	Cell lysate	Total	MDA-MB-231 Supernatant	Cell lysate	Total
Control	1.826	5.539	7.365	856.556	27.764	884.320
10 nM	1.920	3.823 ** (*P*=0.004215)	5.743 * (*P*=0.014898)	897.376	20.955 * (*P*=0.045575)	918.332
100 nM	1.816	3.748 * (*P*=0.026777)	5.565 ** (*P*=0.009543)	842.898 * (*P*=0.036399)	16.330 ** (*P*=0.008502)	859.228 ** (*P*=0.009382)

G-CSF levels were measured in supernatants and cell lysates of each breast cancer cell line; total secretion was calculated as their sum.

Table S4. The average readings of IL-1 RA secretion (pg/mL) in all samples (supernatant, cell lysates, and the total) at both cell types MDA-MB-231 and MCF-7.

IL-1 RA Concentration	MCF-7 Supernatant	Cell lysate	Total	MDA-MB-231 Supernatant	Cell lysate	Total
Control	71.719	62.358	134.078	626.926	192.926	819.853
10 nM	71.523	69.902	141.426	652.094 * (*P*=0.021727)	225.421	877.515 * (*P*=0.03327)
100 nM	76.687	81.253 ** (*P*=0.003257)	157.941 ** (*P*=0.008837)	666.507 ** (*P*=0.003575)	209.963	876.411 ** (*P*=0.002669)

IL-1 RA levels were measured in supernatants and cell lysates of each breast cancer cell line; total secretion was calculated as their sum.