THE ROLE OF VITAMIN D IN THE CARCINOGENESIS AND PROGRESSION OF BREAST CANCER

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Nair Susana da Costa Florim Ribeiro Lopes

Porto, 2012

Programa Doutoral em Biomedicina

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IPATIMUP
Instituto de Patologia e Imunologia Molecular da Universidade do Porto

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Ao abrigo do Artigo 8.º do Decreto-Lei N.º 388/70, a secção **Original Articles** contém todos os trabalhos que fazem parte desta dissertação, publicados ou em publicação, e que são os seguintes:

1. Sousa B, Paredes J, Milanezi F, **Lopes N**, Martins D, Dufloth R, Vieira D, Albergaria A, Veronese L, Carneiro V, Carvalho S, Costa JL, Zeferino L, Schmitt F. P-cadherin, Vimentin and CK14 for identification of basal-like phenotype in breast carcinomas: an immunohistochemical study. *Histology and Histopathology*. 25:963-974, 2010.

2. **Lopes N**, Sousa B, Martins D, Gomes M, Vieira D, Veronese LA, Milanezi F, Paredes J, Costa JL, Schmitt F. Alterations in Vitamin D signalling and metabolic pathways in breast cancer progression: a study of VDR, CYP27B1 and CYP24A1 expression in benign and malignant breast lesions. *BMC Cancer*. 10:483-492, 2010.

3. **Lopes N**, Carvalho J, Durães C, Sousa B, Gomes M, Costa JL, Oliveira C, Paredes J, Schmitt F. 1alpha,25-dihydroxyvitamin D_3 induces *de novo* E-cadherin expression in triple-negative breast cancer cells by *CDH1* promoter demethylation. *Anticancer Research*. 32:249-257, 2012.

A seguinte publicação não faz parte integrante desta dissertação. No entanto, foi utilizada para a introdução:

4. **Lopes N**, Paredes J, Costa JL, Ylstra B, Schmitt F. Vitamin D and the mammary gland: a review on its role in normal development and breast cancer. Review 2012 (Submitted for publication)

Em cumprimento do disposto no referido Decreto-Lei, a candidata declara que participou activamente na recolha e análise de dados em 1, 2 e 3 e que escreveu os artigos 2, 3 e 4 com a colaboração dos co-autores. Colaborou ainda activamente na escrita do artigo 1.

Nota Explicativa

A presente dissertação encontra-se escrita em Inglês na sua quase totalidade, uma vez que será discutida por arguentes estrangeiros.

Para aqueles que todos os dias lutam contra esta terrível doença!

To those who struggle with this terrible disease everyday!

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Recomeça…
Se puderes,
Sem angústia e sem pressa.
E os passos que deres,
Nesse caminho duro do futuro,
Dá-os em liberdade.
Enquanto não alcances
Não descanses.
De nenhum fruto queiras só metade.

E, nunca saciado,
Vai colhendo
Ilusões sucessivas no pomar.
Sempre a sonhar
E vendo,
Acordado,
O logro da aventura.
És homem, não te esqueças!
Só é tua a loucura
Onde, com lucidez, te reconheças.

Miguel Torga

Table of contents

Abbreviations

α-SMA	**alpha-S**mooth **M**uscle **A**ctin
1α-OHase	**1alpha-H**ydroxyl**ase** or CYP27B1
1,25D$_3$-MARRS	**M**embrane-**A**ssociated **R**apid **R**esponse **S**teroid binding protein
24-OHase	25-Hydroxyvitamin D **24**-**H**ydroxyl**ase** or CYP24A1
25-OHase	**25-H**ydroxyl**ase** or CYP27A1
5-aza-dC	**5-aza**-2-**d**eoxy**c**ytidine
ADH	**A**typical **D**uctal **H**yperplasia
ALDH1	**Al**dehyde **Deh**ydrogenase **1**
Bcl-2	**B c**ell **l**ymphoma **2**
BMP	**B**one **M**orphogenetic **P**rotein
BRCA1	**Br**east **Ca**ncer Susceptibility Gene **1**
BSA	**B**ovine **S**erum **A**lbumin
CAM	**C**horio**a**llantoic **M**embrane Assay
CCL	**C**olumnar **C**ell **L**esion
CD	**C**luster of **D**ifferentiation
CDH1	**C**a**dh**erin **1** or E-cadherin gene
CDK	**C**yclin **D**ependent **K**inase
cDNA	**c**omplementary **DNA**
C/EBPα	**C**CAAT **E**nhancer **B**inding **P**rotein **alpha**
C/EBPβ	**C**CAAT **E**nhancer **B**inding **P**rotein **beta**
CK	**C**yto**k**eratin
CKI	**C**yclin Dependent **K**inase **I**nhibitor
CpG island	DNA sequence with high frequency of **CG** nucleotides (the "**p**" stands for the phosphate group that connects one base to another)

DAPI	4,6-**Dia**mino-2-**p**heny**l**indole
DBP	Vitamin **D** **B**inding **P**rotein
DCIS	**D**uctal **C**arcinomas *In Situ*
DMBA	7,12-**dim**ethyl**b**enz**a**nthracene
DMSO	**Dim**ethyl **S**ulf**o**xide
DN101	Vitamin D Analogue
DNA	**D**esoxyribo**n**ucleic **A**cid
E-cadherin	Human **E**pithelial **Cadherin** (or Cadherin 1)
EB1089	Vitamin D Analogue
EDTA	**E**thylene**d**iamine**t**etra**a**cetic acid
EGF	**E**pidermal **G**rowth **F**actor
EGFR	**E**pidermal **G**rowth **F**actor **R**eceptor (or HER1)
EMT	**E**pithelial to **M**esechymal **T**ransition
EpCAM	**Ep**ithelial **C**ell Adhesion **M**olecule
ER	O**e**strogen **R**eceptor
ESA	**E**pithelial **S**pecific **A**ntigen
FGF	**F**ibroblast **G**rowth **F**actor
GAPDH	**G**lycer**a**ldehyde**p**hosphate **Deh**ydrogenase
HDAC	**H**istone **Deac**etylase
HER2	**H**uman **E**pidermal Growth Factor **R**eceptor **2**
HIF	**H**ypoxia **I**nducible **F**actor
HRP	**H**orse**r**adish **P**eroxidase
H&E	**H**ematoxylin and **E**osin stain
IGF	**I**nsulin **G**rowth **F**actor
LBD	**L**igand **B**inding **D**omain
MET	**M**esenchymal to **E**pithelial **T**ransition
miRNA	**mi**cro**RNA**

MNU	N-**m**ethyl-N-**n**itroso**u**rea
mRNA	**m**essenger **RNA**
MMP	**M**atrix **M**etallo**p**roteinase
MMTV	**M**ouse **M**ammary **T**umour **V**irus
MUC1	**Muc**in **1**
N-cadherin	Human **N**eural **Cadherin** (or Cadherin 2)
p21	**p**rotein **21** (or cyclin-dependent kinase inhibitor 1A)
p27	**p**rotein **27** (or cyclin-dependent kinase inhibitor 1B)
p63	**p**rotein **63**
P-cadherin	Human **P**lacental **Cadherin** (or Cadherin 3)
PBS	**P**hosphate **B**uffered **S**aline
PCR	**P**olymerase **C**hain **R**eaction
PgR	**P**ro**g**esterone **R**eceptor
PKC	**P**rotein **K**inase **C**
pRb	**p**hosphorylated **R**etino**b**lastoma
PSA	**P**rostate **S**pecific **A**ntigen
Ras	**Ra**t **S**arcoma
RNA	**R**ibo**n**ucleic **A**cid
RXR	**R**etinoid **X** **R**eceptor
SERM	**S**elective O**e**strogen **R**eceptor **M**odulator
siRNA	**s**mall **i**nterfering **RNA**
Slug	Zinc Finger transcription factor (E-cadherin transcription repressor)
Snail	Zinc Finger transcription factor (E-cadherin transcription repressor)
TCF-4	**T**-**c**ell **F**actor **4**
TDLU	**T**erminal **D**uctal **L**obular **U**nit
TEB	**T**erminal **E**nd **B**ud
TGF-β	**T**ransforming **G**rowth **F**actor **beta**

TMA	**T**issue **M**icro**a**rray
TNFα	**T**umour **N**ecrosis **F**actor **alpha**
TSA	**T**richo**s**tatin **A**
UDH	**U**sual **D**uctal **H**yperplasia
VDR	**V**itamin **D** **R**eceptor (human gene or protein)
Vdr	**V**itamin **D** **R**eceptor (mouse gene)
VDRE	**V**itamin **D**-**R**esponsive **E**lement
VEGF	**V**ascular **E**ndothelial **G**rowth **F**actor
ZO	**Z**onula **O**ccludens

Summary / Sumário

Breast cancer is the leading cause of death by cancer in women. It is a heterogeneous disease, with different molecular subtypes that are associated with diverse biological behaviours, various responses to therapy and clinical outcome. Although some of these molecular subgroups have a targeted therapy, the most aggressive tumours, the triple-negative basal-like carcinomas, still lack a molecular target. This has led to intensive research in order to find the best immunohistochemical criterion to identify triple-negative basal-like carcinomas, as well as potential therapeutic approaches for this particular type of tumours.

In the first part of this thesis, we aimed to establish an immunohistochemical surrogate panel, easily applied on formalin-fixed paraffin-embedded samples, which could identify triple-negative basal-like carcinomas. We demonstrated that P-cadherin, Vimentin and CK14 are useful biomarkers to include in immunohistochemistry panels in order to distinguish triple-negative basal-like carcinomas, due to their consistent values of sensitivity and specificity.

In addition, Vitamin D has been shown to have anti-carcinogenic effects in various cancer models, including breast cancer, by inhibiting proliferation, invasion, angiogenesis and by preventing the formation of metastasis. Furthermore, studies have demonstrated that it is also an inducer of apoptosis and differentiation.

Based on that, we decided to evaluate the expression levels of molecules involved in the signalling and metabolic pathways of Vitamin D. A series of human mammary lesions was studied for the expression of VDR, CYP27B1 and CYP24A1. We observed that, upon malignant transformation and breast cancer progression, there is a decrease in VDR and CYP27B1 expression, whereas the expression of CYP24A1 is augmented. These results suggest that, during carcinogenesis, there is an unbalance in the Vitamin D signalling and metabolic pathways in order to favour tumour progression, since tumour cells lose their ability to synthesise and respond to Vitamin D, while increase their ability to degrade it.

Finally, we decided to explore the ability of Vitamin D as a potential therapy for triple-negative basal-like carcinomas, since VDR was expressed in a significant number of invasive breast carcinomas that were negative for ER, PgR and HER2 expression. We observed that Vitamin D induces the *de novo* expression of the epithelial and differentiation marker E-cadherin in metastatic and triple-negative basal-like breast cancer cells. We demonstrated that the induction was dependent on the duration of the treatment and on the dose of Vitamin D given to the cells; we have also shown that this effect was mediated by VDR, since its inhibition abrogated this effect. Additionally, we demonstrated that Vitamin D is more potent than the demethylating agent 5-aza-dC in inducing E-cadherin expression and that the treatment with Vitamin D seems to be important for the correct localisation of E-cadherin at the cell membrane. Finally, our results suggest that Vitamin D may function as a demethylating agent in *CDH1* / E-cadherin gene promoter.

O cancro da mama é a principal causa de morte por cancro nas mulheres. É uma doença heterogénea, com diferentes subtipos moleculares que estão associados a comportamentos biológicos diversos, várias respostas à terapia e diferentes evoluções clínicas. Apesar de alguns destes subgrupos moleculares disporem de uma terapia dirigida, os tumores mais agressivos, os carcinomas triplo-negativos do tipo basal, ainda não possuem um alvo molecular. Este facto tem levado a intensiva investigação em potenciais abordagens terapêuticas para este tipo particular de tumores.

Na primeira parte desta tese, quisemos estabelecer um painel imunohistoquímico alternativo que pudesse identificar os carcinomas triplo-negativos do tipo basal e que fosse facilmente aplicável em amostras fixadas em formol e incluídas em parafina. Demonstrámos que a P-caderina, a Vimentina e a CK14 são biomarcadores úteis a incluir em painéis imunohistoquímicos para o reconhecimento dos carcinomas triplo-negativos do tipo basal, devido aos seus valores de sensibilidade e especificidade consistentes.

Adicionalmente, os efeitos anti-carcinogénicos da Vitamina D têm sido demonstrados em vários modelos de cancro, incluindo o cancro da mama. A Vitamina D inibe a proliferação, invasão, angiogénese e previne a formação de metástases. Para além disso, alguns estudos demonstram a sua capacidade de indução de apoptose e diferenciação.

Baseados nestas evidências, decidimos avaliar os níveis de expressão de moléculas envolvidas nas vias metabólica e de sinalização da Vitamina D durante a progressão do cancro da mama. Estudámos a expressão do VDR, CYP27B1 e CYP24A1 numa série de lesões mamárias humanas e observámos que, com a transformação maligna e progressão do cancro da mama, ocorre uma redução da expressão do VDR e CYP27B1, enquanto a expressão do CYP24A1 é aumentada. Estes resultados sugerem que, durante a carcinogénese, ocorre um desequilíbrio nas vias metabólicas e de sinalização da Vitamina D, de modo a favorecer a progressão tumoral, uma vez que as células

neoplásicas perdem a capacidade de síntese e de resposta à Vitamina D, ao mesmo tempo que potenciam a sua capacidade de a degradar.

Finalmente, explorámos a capacidade da Vitamina D funcionar como uma potencial terapia para os carcinomas triplo-negativos do tipo basal, uma vez que o VDR é expresso por um número considerável de carcinomas invasores da mama, negativos para a expressão de ER, PgR e HER2. Observámos que a Vitamina D induz a expressão *de novo* do marcador epitelial e de diferenciação E-caderina nas células de cancro da mama metastáticas e triplo-negativas do tipo basal. Demonstrámos que essa indução era dependente do tempo de duração do tratamento e da dose de Vitamina D administrada às células; e mostrámos ainda que esse efeito era mediado pela presença do VDR, já que a sua inibição anulava este efeito. Adicionalmente, observámos que o tratamento com Vitamina D foi mais potente que o tratamento com o agente desmetilante 5-aza-dC na indução da expressão da E-caderina e parece ainda ser importante para a correcta localização da E-caderina na membrana citoplasmática. Finalmente, estes resultados sugerem que a Vitamina D pode funcionar como agente desmetilante no promotor do gene *CDH1* / E-caderina.

Thesis outline

This thesis is organised in chapters. First, there is a general background addressing the main subjects and theoretical concepts related with the thesis theme. Afterwards, it is indicated the rationale and aims of the thesis, as well as all the material and methods used to perform the studies. Subsequently, there is a detailed description of the results obtained within this work, which were divided in 3 sub-chapters. All these sub-chapters comprise a short introduction, the results and the discussion for each proposed aim. Finally, the main conclusions from all aims are summarised.

Background

1. The mammary gland and breast cancer

1.1. Anatomy and normal development

The mammary gland is the major characteristic of the class Mammalia and consists of modified skin appendages, more specifically, sweat glands. Its function is to provide nourishment and immunologic protection for the new born individuals [1]. In humans, paired mammary glands rest on the pectoralis muscle on the upper chest wall (Figure 1) [2].
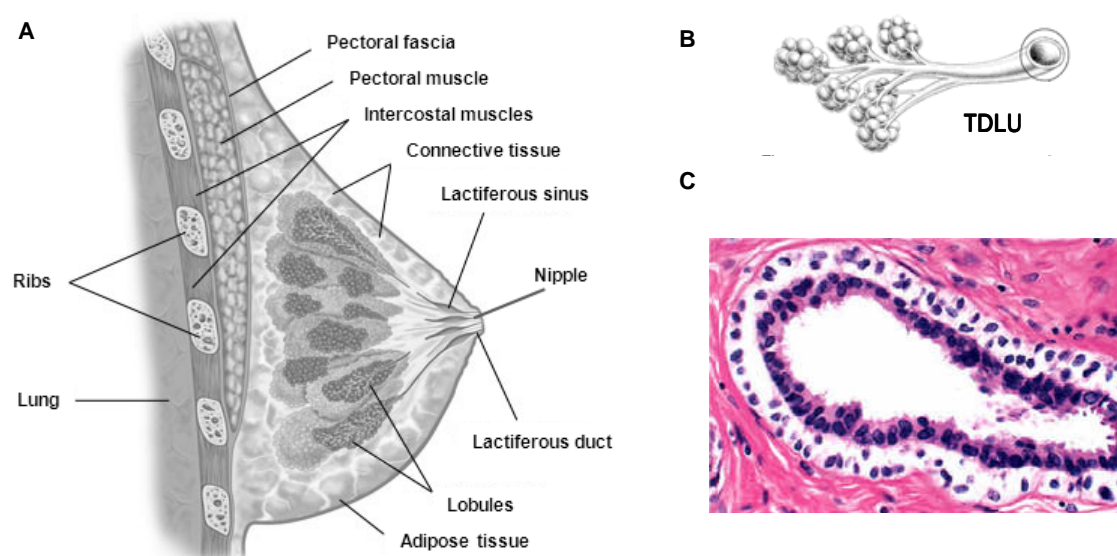

Figure 1 –Schematic representation of a mature breast, showing mammary gland anatomy (adapted from http://www.britannica.com/) (**A**), Diagram of a TDLU – Terminal Ductal Lobular Unit), taken from http://www.my-breast-cancer-guide.com/What-is-breast-cancer.html (**B**), Cross-section of a breast duct, in which the epithelial and myoepithelial cells can be easily identified (Haematoxylin-eosin staining; x1000) (**C**).

Between 6 to 10 large ducts originate at the nipple and successive branching of these ducts leads to a cluster of Terminal Duct Lobular Units (TDLU), the anatomical and functional unit of the breast [3].

In the adult woman the terminal duct branches into an arborescent cluster of small acini to form a lobule [2]. In the normal breast, ducts and lobules are lined by two cell types: a sheath of contractile cells containing myofilaments (myoepithelial or basal cells), which are in contact with the basement membrane, and a second layer of epithelial cells that contours the lumen of the ducts (luminal cells). Myoepithelial cells are responsible for assisting milk ejection during suckling in response to oxytocin and they also play an important role in maintaining the normal structure and function of the lobule and basement membrane; the luminal cells are secretory cells whose function is to produce milk [4]. The majority of breast stroma is composed of dense and fibrous connective tissue admixed with adipose tissue [2]. Thus, the adult human breast is comprised of a network of ducts branching into a cluster of terminal lobular units surrounded by stroma.

However, the gland is not fully formed at birth, it undergoes cyclic changes during reproductive life and it is only with the onset of pregnancy that the breast assumes its complete morphologic maturation and functional activity [2]. During embryonic development, the specialised mesenchyme of the breast fat pad condenses around the epithelium of the breast bud. Via a complex interaction between stromal and epithelial cells, there is invagination of the mammary epithelium into the stroma to form the ductal system [1, 2].

The development of the gland predominantly takes place during puberty, by the main action of two proliferative hormones: oestrogen and progesterone. While oestrogen is responsible for the stimulation of ductal elongation, progesterone mediates the branching [5]. Upon menarche, with the hormonal stimulation, the terminal ducts originate lobules and the interlobular stroma increases in volume. During the reproductive years, the mammary gland undergoes cyclic changes. In the follicular phase of the menstrual cycle, the lobules are quite unchanged. But after ovulation and upon hormonal stimulation, with increasing levels of oestrogen and progesterone, cell proliferation is intensified, inducing a rise in the number of acini per lobule, while epithelial cells become vacuolated and the intralobular stroma develops edema. At menstruation, there is a decline in oestrogen and

progesterone levels, followed by disappearance of the stromal edema and shrinking in the size of the lobules [2].

With the onset of gestation, the mammary gland assumes its final phase of development and lobules expand both in number and size. Consequently, by the end of the pregnancy, the breast is predominantly comprised of lobules connected by a scarce quantity of stroma. Immediately after birth, colostrum is produced by epithelial cells and, within days, as progesterone levels decrease, cells begin to secrete milk. After weaning, the lobules regress and atrophy and the total size of the gland is significantly reduced. However, full mammary regression to the nulliparous size does not take place, as with pregnancy there is a permanent increase in the size and number of lobules [2]. One important aspect of the mammary epithelium is its ability to regenerate, which allows the formation of a new functional structure for each lactating period [1].

Luminal cells can be identified by the expression of cell-type specific cytoskeletal markers, namely a subset of epithelial cytokeratins (CK8, CK18 and CK19), nuclear receptors for the ovarian steroid hormones oestrogen and progesterone (ER and PgR) and low levels of milk proteins. In contrast, the myoepithelium expresses distinct basal epithelial cytokeratins (CK5 and CK14), P-cadherin and α-smooth muscle actin (α-SMA) [6].

It is now accepted that both epithelial and myoepithelial cells arise from a pluripotent stem cell, but this cell has not yet been fully characterised in humans. Adult stem cells are believed to reside within the ducts, since ductal fragments can be serial-passaged *in vitro* [7] and studies using electron microscopy have proposed basal-positioned small electron-lucent cells as likely candidates [8]. Additionally, a combination of molecular markers have been used to isolate human mammary stem cells: EpCAM (epithelial cell adhesion molecule) or ESA (epithelial specific antigen), CD49f and, to a lesser degree, MUC1 (mucin 1). It has also been reported that human mammary epithelial cells, that have the ability to engraft mouse mammary fat pads, express high levels of aldehyde dehydrogenase

1 (ALDH1) [9]. Thus, mammary stem cells display an EpCAM^{low} CD49f^{high} MUC1$^-$ ALDH1$^+$ phenotype, which is suggestive of a basal position within the mammary epithelium as well [8].

1.2. Breast cancer epidemiology

Breast cancer is by far the most frequent cancer among women worldwide, with about 39.0 new cases per 100 000 women / year in 2008. From these, it was expected that 12.5 deaths per 100 000 women / year would occur [10].

The incidence rates are higher in the more developed regions of the world and among these, the areas of western and northern Europe, Australia and New Zealand display the highest number of cases (Figure 2A). Conversely, less developed countries, namely in eastern and middle African regions and south-central and eastern Asia, exhibit the lowest rates. Along the years, there has been a trend for the increase of breast cancer incidence rates in the more developed countries of Europe and North America. However, from the year 2000 onward there was a change in this tendency in some regions: in most Scandinavian countries the incidence rates stabilised and in the United States of America and Australia the rates started to decrease (Figure 2B). Inversely, in the same period, in developing countries such as India or the Republic of Korea the incidence rates seemed to reach an exponential phase [10].

The mortality rates are lower because of the more favourable survival of breast cancer in (high-incidence) developed regions (Figure 2A). As a result, breast cancer ranks as the fifth cause of death from cancer overall, but it is still the most frequent cause of cancer death in women in both developing and developed regions. It is observed that southern and western African regions present the highest rates in the world. Interestingly, Central America, Micronesia and Eastern Asia regions exhibit the lowest percentage of deaths. Since 1990 there has been a tendency for the decrease of

mortality rates in the developed countries, except Japan (Figure 2C). Contrarily to what is observed in the western world, in the emerging nations, the trend still seems to be for a rise in mortality numbers [10].
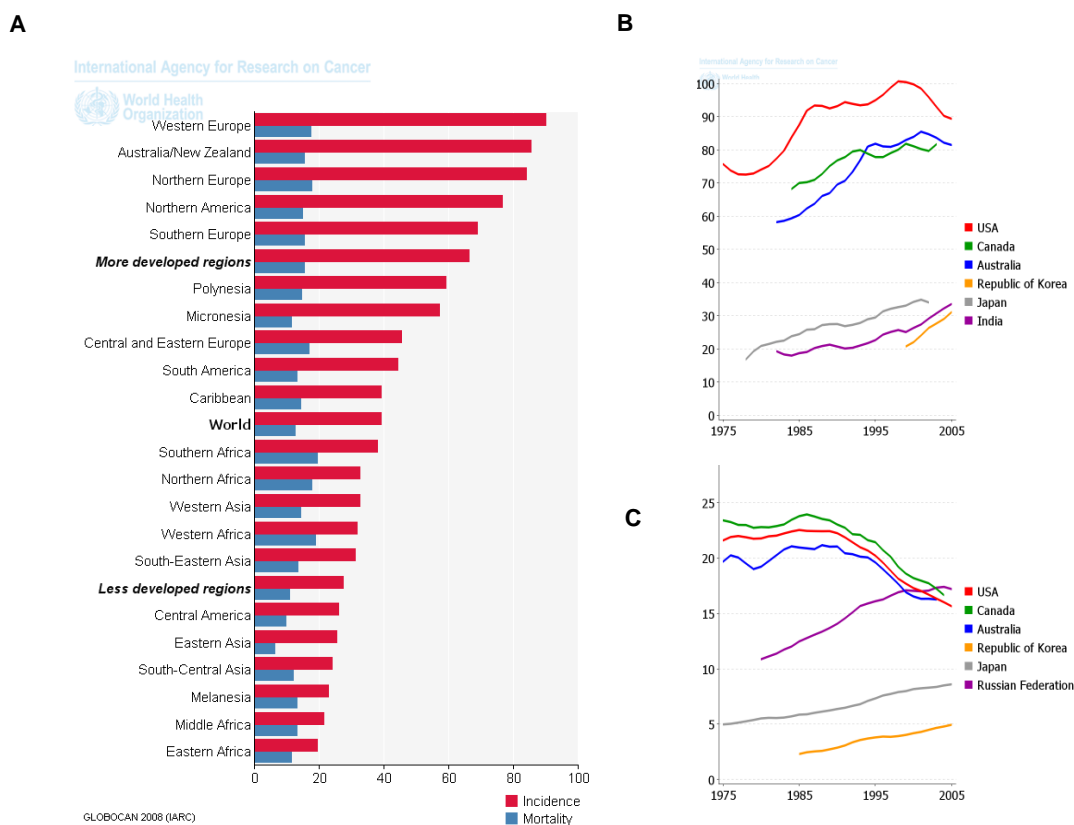

Figure 2 – Breast cancer estimated age-standardised incidence and mortality rates (World) per 100 000 (**A**), Trends in incidence of breast cancer in selected countries: age-standardised rate per 100 000 (**B**), Trends in mortality of breast cancer in selected countries: age-standardised rate per 100 000 (**C**) (taken from [10]).

According to the available data, in Portugal, about 5300 new cases were diagnosed in 2008 and it is estimated that 1500 women died from this disease [10]. Furthermore, it is known that 1% of the diagnosed breast cancers arose in males [11].

1.3. Molecular markers in breast cancer

The two main molecular biomarkers important for therapeutic management in breast cancer are the Oestrogen Receptor (ER) and the Human Epidermal growth factor Receptor type 2 (HER2, also known as c-ErbB2 or *neu* oncogene). The expression assessment of these proteins is extremely important in breast cancer, as ER and HER2 are considered prognostic and predictive markers. Not only do they stratify patients for treatment by identifying cases with different risks of outcome, but they also select patients that are likely to respond to therapy (as discussed below).

ER is a ligand-activated transcription factor, belonging to the superfamily of nuclear receptors for steroid hormones. There are two subtypes of ER: ERα and ERβ, which vary in structure and whose encoding genes lay on different chromosomes [12]. Oestrogen can influence breast carcinogenesis in two different manners: it can act as initiator, causing DNA damage by hydroxylated oestrogen metabolites, or as promoter, inducing growth of transformed cells [13, 14]. Upon cellular diffusion, oestrogen binds to ER, which alters receptor conformation and activates dimerization. The dimers interact with several coactivators or corepressors in order to modulate transcription of target genes [12, 15].

The expression of ER is frequently associated with low histological grade tumours and better prognosis. This is in part due to the existence of directed therapies targeting ER signalling pathways [15, 16]. Current endocrine therapies rely on three known mechanisms of action: antagonising ER function by competitive binding (performed by selective oestrogen receptor modulators and pure antiestrogens), downregulating ER (achieved by pure antiestrogens) and reducing levels of synthesised oestrogen (performed by aromatase inhibitors).

Selective oestrogen receptor modulators (SERMs) are molecules that act like agonists of oestrogen in some tissues, but are antagonists to oestrogen action in others [16, 17]. Tamoxifen is the most

known SERM and is a valuable therapy for patients with ER-positive breast tumours because of its antiestrogenic activity [16, 18]. However, an undesirable side effect of this drug is the ER agonist effect that it induces in the uterus. In the breast, Tamoxifen binds to ER and induces dimerization, but it impairs the binding of the dimers to DNA and inhibits the binding of coactivator proteins. In the recent years, new SERMs have been developed, like Raloxifene, which reduces breast cancer incidence and has no oestrogen-like activity in the uterus [19].

Aromatase inhibitors are molecules that hamper the conversion of adrenal androgens into oestrogen, thereby diminishing the levels of circulating oestrogen and also in the tumour [20]. These inhibitors are currently highly used in clinical practise due to several beneficial lines of evidence, like the fact that they can be effective in postmenopausal women, in which oestrogen levels are extremely low. These molecules are also more effective than Tamoxifen in ER-positive tumours, but particularly when these tumours are HER2 positive as well [21]. Additionally, it has been recently demonstrated an increase in overall survival in patients who had switched from Tamoxifen to aromatase inhibitors [22].

Pure antiestrogens, such as ICI 182,780, are agents that competitively inhibit the binding of oestrogens to ER, prevent dimerization, promote ER degradation and thereby abolish the transcription of target genes [23]. In medical terms, ICI 182,780 has been shown to be efficient in the treatment of metastatic ER-positive breast cancer [24] and an appropriate clinical option in ER-positive and HER2-overexpressing tumours [25]. Moreover, ICI 182,780 exceeds other ER targeted therapies, since no side effects have been observed in premenopausal women with metastatic breast cancer, previously exposed to Tamoxifen and aromatase inhibitors [26].

HER2 is a member of the HER family (or ErbB) of receptor tyrosine kinases, which is involved in proliferation and survival [27]. This family encompasses four growth factor receptors with a high degree of homology: HER1 to HER4, being HER1 usually designated as EGFR (Epidermal Growth

Factor Receptor) [28]. Curiously, HER2 is a ligandless receptor [29]; however, it can be transactivated by Epidermal Growth Factor (EGF)-like ligands or neuregulins, resulting in the formation of heterodimers with the other family members. This heterodimerization between HER2 and the other receptors permits the participation of HER2 in signal transduction.

HER2 overexpression is a frequent event in breast cancer and predicts poor prognosis in patients with primary disease [30, 31]. Gene amplification is the main cause leading to this overexpression in mammary tumours, although activating mutations have also been described in other cancer models [32]. In order to counteract the malignant growth effects induced by HER2 overexpression, there has been an attempt to develop drugs that could effectively block the activity of this transmembrane protein.

The targeting of HER2 was achieved in two ways: inhibition of the extracellular domain using monoclonal antibodies and inhibition of the tyrosine kinase domains through tyrosine kinase inhibitors [33]. Trastuzumab is a humanised murine monoclonal antibody that was the first genomic research-based product approved for cancer therapy [34]. It displays a potent growth inhibitory effect and, since the Trastuzumab introduction in clinical practise, it significantly improved disease-free and overall survival rates in patients with HER2-overexpressing breast carcinomas [35, 36]. A more recent approach has been the design of small molecules that bind to the intracellular kinase domain of HER2, thereby inhibiting its activity. One of such molecules is Lapatinib, which inhibits both EGFR and HER2. Recent reports have shown that the use of Lapatinib, in combination with other agents, leads to survival advantages in patients with HER2-overexpressing metastatic breast cancer [37, 38]. Both, monoclonal antibodies and tyrosine kinase inhibitors, represent powerful therapeutic strategies that have improved the survival rates and quality of life of breast cancer patients.

1.4. Genetic profiling of breast cancer

As previously discussed, breast cancer is the most common type of cancer and is the main cause of death by cancer in women worldwide. Although research for therapies has accomplished some results, more than half of the affected women still undergo relapses [39], probably due to the high degree of heterogeneity of this disease.

Mammary tumours can display various biological features, therapeutic responses and patient outcome and thus, some years ago, expression microarray profiling studies have classified breast carcinomas according to their molecular profile and clinical outcome [30, 31]. This has proven to be a difficult task and for the past years, many reports have been published on genetic profiling of breast cancer [40-45]. The most widely accepted classification has established four main subtypes of mammary tumours (Figure 3): Luminal A, Luminal B, HER2-overexpressing tumours and Triple-negative carcinomas (which comprises normal-like, claudin-low and basal-like carcinomas) [30, 31, 46].
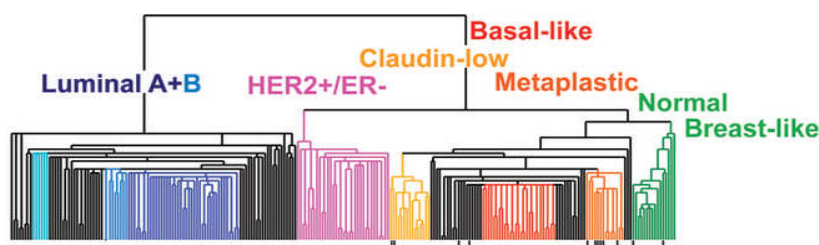

Figure 3 – Classification of breast cancer into specific molecular subtypes (taken from [46]).

Luminal tumours constitute the majority of breast carcinomas (60-75%), are usually positive for ER expression and are generally considered good prognosis carcinomas. Their name comes from the fact that these tumours display high levels of genes expressed by breast luminal cells, like CK8/18 [30]. Luminal A and Luminal B subtypes can be distinguished by some features, namely HER2 expression and proliferation rates: Luminal B tumours are HER2 positive and display higher levels of

proliferation genes, such as Ki-67. Additionally, these tumours express lower levels of ER-associated genes and show worse prognosis than Luminal A tumours [40, 43, 44, 47]. Therapeutic strategies for these subtypes of breast carcinomas involve direct targeting of ER, either by the use of Tamoxifen or the administration of aromatase inhibitors, thereby inhibiting ER mitogenic effects and arresting breast cancer cell growth.

HER2-overexpressing carcinomas comprise about 20% of all mammary tumours [48] and are characterised by lack of expression of ER and high expression levels of HER2 and genes located in the HER2 amplicon on 17q21 [49]. These tumours display poor prognosis [31, 44], but recently they have been targeted with the monoclonal antibodies against HER2 (Trastuzumab) or using tyrosine kinase inhibitors, such as Lapatinib.

Triple-negative carcinomas are a heterogeneous group of tumours that lack ER, HER2 and PgR expression. This group of tumours encompasses normal-like, claudin-low and basal-like carcinomas. The normal-like subgroup displays high levels of expression of genes associated with adipose tissue and other non-epithelial cell types, as well as basal cell genes, whereas presents low expression of luminal cell genes. These tumours usually cluster together with benign mammary lesions and can represent just an artefact of the arrays [44, 50]. Claudin-low tumours are a new subgroup within the triple-negative tumours, distinguished by the downregulation of a cluster of genes involved in tight junctions and cell-cell adhesion, such as Claudins 3, 4 and 7, Occludin and E-cadherin (Epithelial cadherin). Furthermore, these tumours exhibit low to absent expression of luminal differentiation genes, inconsistent expression of basal-like genes, as well as high expression of endothelial cell markers, enrichment for epithelial to mesenchymal transition (EMT) markers, immune response genes and cancer stem cell-like features [41, 46, 51].

Basal-like tumours represent about 15% of invasive breast carcinomas [52] and although the majority of these carcinomas are grade III invasive ductal carcinomas of no special type, they comprise

metaplastic and medullary carcinomas as well [53-55]. Morphologically, these tumours display pushing borders of invasion and central areas of geographical or comedo-type necrosis and lymphocytic infiltrates. Cellular pleomorphism and high nuclear-cytoplasm ratio are common features, as well as high mitotic indexes and frequent presence of apoptotic cells [48]. Concerning the molecular profile of these carcinomas, they are known to display a genetic signature typical of the basal / myoepithelial cells, such as CK5/6, CK14 and CK17, Laminin-5, α6β4-integrin, Caveolin-1 and -2 and P-cadherin (Placental cadherin) [30, 56-58]. Regarding protein expression, there is no consensus on the markers used to identify this subtype of tumours. Studies using immunohistochemistry have proposed, besides the triple-negative phenotype, the expression of multiple markers, which mainly include CK5/6, CK14, CK17, P-cadherin, EGFR, p63, Vimentin, α-smooth muscle actin and c-Kit [57, 59-63]. Basal-like carcinomas are associated with poor prognosis, an aggressive clinical history, development of recurrence within the first 5 years after diagnosis, shorter survival and high mortality rates [48, 64]. Curiously, these tumours present a specific pattern of distant metastasis, with increased frequencies in the lungs and brain [65, 66]. Due to their triple-negative phenotype, these carcinomas do not have a directed therapy and thus, chemotherapy and radiation therapy remain the only options to treat basal-like carcinomas. This has led to intensive research on alternative therapeutic strategies for these tumours.

2. Vitamin D

Vitamin D was first identified in 1919 by Edward Mellanby as a lipid soluble substance with anti-rachitic properties [67]. It belongs to a family of secosteroid hormones, which differ in their side-chain structures and can be classified into five forms: Vitamin D_2 to D_6 [68]. Vitamins D_2 (ergocalciferol) and D_3 (cholecalciferol) can exert biological activity. Humans can get Vitamin D_3 by two main sources: from the diet and from the action of sunlight exposure on the skin; Vitamin D_2 can only be obtained

from food and fortified products. Interestingly, ergocalciferol is just derived from plant sources and cholecalciferol is only originated from animal ones [69]. Few natural foods contain Vitamin D in significant amounts and, among these, fatty fish, eggs and sun-dried mushrooms can be highlighted. In some Western countries, there are food products that are fortified with Vitamin D, namely milk, dairy products and bread [70]. Still, the majority (90-95%) of Vitamin D requirement is obtained through skin production by sunlight (ultraviolet B radiation) [71] and this has caused Vitamin D to be named "the sunshine Vitamin".

2.1. Synthesis and metabolism of Vitamin D

The synthesis of vitamin D is a multistep process involving different organs (Figure 4). When the skin is exposed to solar radiation, 7-dehydrocholesterol, a cholesterol-like precursor present in the dermis and epidermis is photolysed and converted into pre-vitamin D_3. This molecule is then thermically isomerised to Vitamin D_3 [72]. In order to achieve physiological activity, Vitamin D has to be metabolised: this mechanism takes place in the liver and kidney and requires the transport of Vitamin D metabolites into these organs. Because Vitamin D is a lipophilic molecule, with low aqueous solubility, it must be carried bound to plasma proteins. The most important transporter is the Vitamin D Binding Protein (DBP) [73]. Thus, after being synthesised in the skin or absorbed by the enterocytes, Vitamin D enters in circulation bound to DBP.

Once inside the liver, the activation of Vitamin D starts with the hydroxylation of carbon 25 performed by a cytochrome enzyme named 25-hydroxylase (25-OHase) [74], encoded by the gene *CYP27A1*. 25-hydroxyvitamin D_3 or calcidiol, the resulting molecule, is the major circulating form of Vitamin D and is the one that is used to account for the levels of Vitamin D in the body. 25-hydroxyvitamin D_3 is taken up by the cells in the proximal tubules of the kidney by endocytosis, a cellular process that is facilitated by the presence of important proteins, named megalin and cubilin, in the plasma

membrane of these cells. Calcidiol is then further hydroxylated to the hormonally active secosteroid 1α,25-dihydroxyvitamin D_3 (1α,25(OH)$_2D_3$ or calcitriol) [74]. This step occurs through the action of 1α-hydroxylase (1α-OHase / CYP27B1), a mitochondrial enzyme encoded by the gene *CYP27B1*.

Other cell types, such as colon, brain, prostate, skin and breast [75], also express 1α-OHase and can contribute to the circulating levels of Vitamin D. Based on this observation, two distinct pathways of Vitamin D biosynthesis and action have emerged: first, the extrarenally produced Vitamin D, which functions in an autocrine and paracrine manner, generating tissue-specific cell regulatory effects through the local release of Vitamin D; and second, the one in which Vitamin D is metabolised in the kidney, to exert endocrine actions, generating systemic calcemic effects through circulating Vitamin D [76].
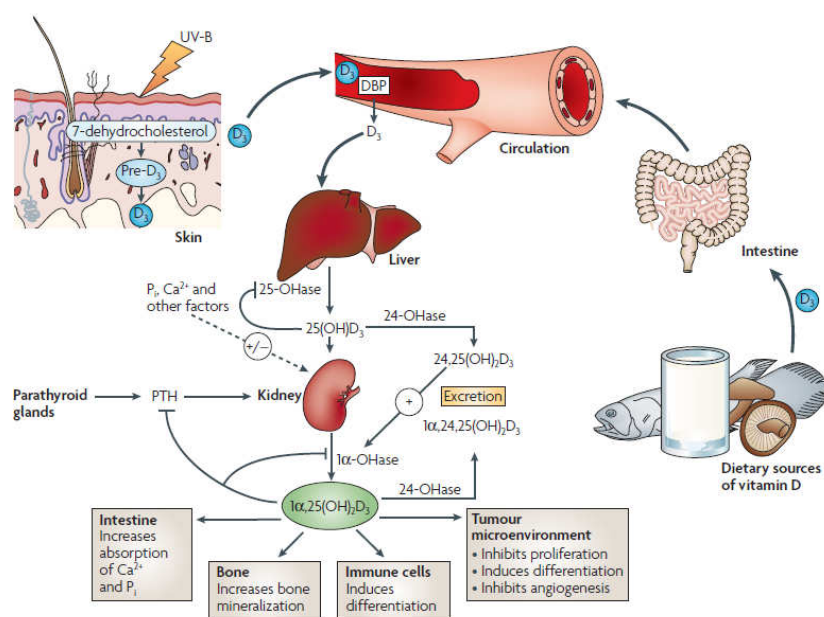

Figure 4 – Synthesis and catabolism of Vitamin D (taken from [77]).

In order to keep the homeostasis of the organism, especially regarding the levels of calcium and phosphate, the amount of circulating Vitamin D has to be tightly regulated. This regulation is achieved by a series of complex feedback mechanisms that limit the risk for Vitamin D intoxication. The

enzyme 25-hydroxyvitamin D 24-hydroxylase (24-OHase / CYP24A1, encoded by the gene *CYP24A1*) plays a key role in this process, being activated by 1α,25-dihydroxyvitamin D_3, whenever there is an increase of the levels of this hormone. This enzyme is responsible for converting 25-hydroxyvitamin D_3 and 1α-hydroxyvitamin D_3 to the biologically inactive metabolites $24,25(OH)_2D_3$ and $1α,24,25(OH)_2D_3$, respectively [74]. These inactive compounds are then excreted. Conversely, if the levels of $24,25(OH)_2D_3$ are elevated, there is then an induction of the synthesis of 1α,25-dihydroxyvitamin D_3. Calcitriol also negatively regulates the parathyroid hormone (PTH), which in turn activates 1α-OHase, thus controlling itself [78]. Moreover, 1α,25-dihydroxyvitamin D_3 can directly repress the activity of 1α-OHase [79, 80]. Other factors, such as the levels of calcium and phosphate, can also downregulate the activity of 1α-OHase and, consequently, decrease the levels of calcitriol [74, 78, 79, 81]. However, the control of the amount of 1α,25-dihydroxyvitamin D_3 can be exerted upstream, in the liver. Calcidiol can negatively regulate its own levels by repressing the activity of 25-OHase.

In this thesis, for simplicity purposes, whenever Vitamin D is stated, it will be referring to the biologically active form (1α,25-dihydroxyvitamin D_3 / $1α,25(OH)_2D_3$), unless otherwise mentioned. The same criterion will be applied to the enzymes 1α-hydroxylase and 25-hydroxyvitamin D 24-hydroxylase, which, further on, will be termed CYP27B1 and CYP24A1, respectively.

2.2. Genomic actions of Vitamin D

After cell internalization, Vitamin D exerts most of its biological activities by binding to a specific high-affinity receptor, the Vitamin D Receptor (VDR), first identified in a breast cancer cell line in 1979 [82]. VDR belongs to the superfamily of nuclear receptors for steroid hormones and regulates gene expression by acting as a ligand-activated transcription factor [83]. VDR binds to Vitamin D through a high-affinity portion of its molecule, named Ligand Binding Domain (LBD), and upon ligand binding,

heterodimerization with the Retinoid X Receptor (RXR) occurs, which is essential for the gene transcription process. The complex Vitamin D-VDR-RXR specifically binds to the promoter regions of target genes containing Vitamin D-Responsive Elements (VDREs) [84]. These are specific DNA sequences, in which two hexanucleotide repeats are intercalated by varying numbers of nucleotides [84]. Nuclear coactivator proteins work synergistically with the VDR to enhance Vitamin D-mediated gene expression. These proteins function as chromatin-modifying enzymes, unfolding and exposing the DNA, and allowing the recruitment of the transcriptional machinery [74]. At the same time, Vitamin D binding to the VDR causes the release of corepressor proteins which normally maintain chromatin in an inactive state [85].

Interestingly, Vitamin D is also capable of inducing transcriptional repression. This process is very similar to the one leading to gene expression and occurs when the Vitamin D-VDR-RXR complex binds to negative VDREs [86, 87]. Recruitment of histone deacetylases (HDACs) is induced, along with dissociation of coactivator proteins, preventing chromatin exposure and binding of transcription factors [88].

2.3. Non-genomic and VDR-independent actions of Vitamin D

Vitamin D is also known to exert rapid effects that are not dependent on gene transcription. Presumably, these effects are mediated by cell surface membrane receptors and two distinct proteins have been implicated in this process: the Membrane-Associated Rapid Response Steroid binding ($1,25D_3$-MARRS) protein and a membrane VDR.

The evidence for the existence of a receptor protein that could bind to Vitamin D, other than the VDR, came from two observations: first, the existence of Vitamin D analogues that can elicit the rapid actions of Vitamin D, but show low levels of affinity to the VDR [89, 90]; and second, the existence of

a Vitamin D binding protein that has been described in the basolateral membrane of rat and chick enterocytes [91]. Some previous studies have shown that $1,25D_3$-MARRS protein plays an important role in Vitamin D-stimulated uptake of phosphate and intracellular calcium flux [92, 93]. $1,25D_3$-MARRS has also been implicated in the activation of Protein Kinase C (PKC) [94, 95], which then can induce the rapid opening of voltage-gated calcium channels, activating consecutively the Rat sarcoma (Ras) cascade in skeletal muscle cells [96]. In breast cancer cells, a recent paper has demonstrated that $1,25D_3$-MARRS is involved in Vitamin D growth inhibition effects: cells transfected with a ribozyme construct designed against human $1,25D_3$-MARRS mRNA underwent greater growth inhibition than the cells transfected with control ribozyme. Furthermore, these $1,25D_3$-MARRS clones were sensitive to lower Vitamin D concentrations than the control clones [97].

Along with the role of $1,25D_3$-MARRS as a Vitamin D binding protein, some data have suggested that also VDR could mediate these non-transcriptional effects. The most striking evidence supporting this idea was the demonstration that the Vitamin D-induced rapid actions are lost in osteoblasts from *Vdr* knockout mice [98]. Moreover, VDR has been identified within caveolae-enriched plasma membrane fractions from various cell types [99]. Although the existence of these Vitamin D-induced non-genomic effects is becoming increasingly clearer, the specific function of these actions still remains to be elucidated. A physiological role has been proposed by the work of Zhou and colleagues [100], which reported a rapid Vitamin D-induced stimulation of intestinal calcium absorption, a process they entitled transcaltachia. Another process presumably regulated by membrane initiated signalling is cell proliferation: it has been demonstrated that, in some cell lines, VDR associates with phosphatase proteins, initiating a cascade of events that end in the inactivation of p70S6 kinase, an enzyme involved in the cell cycle G_1-S transition [101]. This is likely followed by the VDR-mediated transcriptional activity of genes involved in the cell cycle progression (discussed below). Other roles have been proposed for the rapid Vitamin D signalling, namely that it may optimize the genomic effects mediated by VDR. It has been suggested that the crosstalk between these two Vitamin D effects (genomic and non-genomic) is mediated by specific phosphorylation of proteins involved in the

VDR transcriptional complex. Specifically, some studies have proposed that proteins responsible for the chromatin structure remodelling, such as histones, can be phosphorylation targets [102].

3. Vitamin D in the normal mammary gland

3.1. Role of Vitamin D in breast development

The VDR has been shown to be expressed in the normal mammary gland. Since most of Vitamin D actions are intricately dependent upon VDR action, studies conducted to determine the role of this hormone in breast development have been mainly based on the use of *Vdr* knockout mice. Zinser and colleagues (2002) have published an elegant study regarding the role of the Vitamin D signalling pathway in the growth regulation of the mammary gland during pubertal development [103]. They found that *Vdr* knockout female mice displayed more extensive ductal elongation and branching, when compared with their wild-type counterparts. Furthermore, they observed that this enhanced morphogenesis was not associated with the deregulation of cell proliferation and apoptosis pathways. Moreover, they saw that VDR ablation leads to an increase of breast responsiveness to exogenous hormones (oestrogen and progesterone), symbolised by an increase in cell growth [103]. Collectively, these data indicate that VDR has an important impact on breast development and suggest that the Vitamin D signalling pathway participates in the negative growth regulation of the mammary gland. In another study published by the same group [104], it was shown that the lack of VDR did not impair lactation, but *Vdr* deficient mice secreted higher amounts of milk in response to exogenous oxytocin when compared with the wild-type animals. Furthermore, they demonstrated that *Vdr* knockout mice had delayed mammary gland regression after weaning and that this effect was associated with reduced apoptosis in the epithelial cell compartment.

3.2. Vitamin D metabolic / signalling pathways and the mammary gland

Vitamin D has been shown to play an important role in the development and function of the mammary gland and its function seems to be closely related with VDR action. Immunohistochemical studies have demonstrated that the VDR protein is expressed in samples from normal breast tissues [105] and also in breast cancer biopsy specimens [106]. In mice, VDR expression was found to be temporally and spatially distinct. VDR staining was present in the nuclei of epithelial cells (TDLU and ducts), and, within the terminal end bud, expression was observed in luminal epithelial cells (although lower than that observed in the luminal epithelial cells of the ducts), cap cells (infrequently positive) and stromal cells [103]. Over the pubertal time course, data indicate that the number of positive cells and the intensity of the VDR staining is highest in luminal epithelial cells of the ducts from young mice (5 to 7 weeks-old); in contrast, older females (8 to 10 weeks-old) display downregulation of VDR expression, since the pubertal phase of glandular development is largely completed [103]. No results regarding VDR expression in basal / myoepithelial cells are currently available. Additionally, VDR expression is enhanced during pregnancy and lactation and is thought to be mediated by lactogenic hormones [107], indicating a role for Vitamin D in the differentiation of the mammary gland. This finding is consistent with the observation that VDR expression is greater in differentiated cells than in proliferating cells of the breast [103]. Assessment of VDR expression by Real-time PCR has demonstrated that this receptor is upregulated in mammary tumour tissue in comparison with adjacent normal tissue and healthy normal breast tissue [108].

Discrepancies have been observed in reported data concerning the expression of the main enzymes involved in Vitamin D metabolism (CYP27B1 and CYP24A1). Townsend and collaborators (2005) have observed that CYP27B1 and CYP24A1 were expressed and functionally active in non-malignant tissue [109]. However, CYP27B1 was more abundant in breast tumours. These results are in accordance with those obtained in other studies, in which the expression of the VDR and Vitamin D metabolic enzymes was enhanced in breast carcinomas when compared with non-neoplastic tissue.

[110-112]. Additionally, it has also been found that the activity of CYP27B1 was increased in tumour samples compared with normal breast, but conversion of Vitamin D to the inactive metabolite $1\alpha,24,25(OH)_2D_3$ was also significantly higher in tumours [109]. Another study by de Lyra and collaborators [113] has observed no differences in the expression of CYP27B1 and CYP24A1 between non-neoplastic tissue and breast cancer tissue. The evaluation of CYP27B1 expression in mammary tissue by Real-time PCR has shown that this enzyme is upregulated in tumour samples, compared with adjacent non-cancerous normal tissue, but healthy normal breast tissue displayed the highest levels of expression of all types of samples considered [108]. In another work, where the expression of CYP24A1 was assessed in mammary cell lines and tissues (both benign and malignant), the results were conflicting [114]. For cell lines, it has been observed that the expression of CYP24A1 is decreased in tumour cells when detected by Western blot, but increased when detected by Real-time PCR, compared with benign breast cells. In contrast, using breast samples, CYP24A1 expression was lower in malignant tissues, regardless of the detection technique employed. The use of human mammary epithelial cells has demonstrated that the mRNA and protein levels of VDR and CYP27B1 were highly reduced, as well as Vitamin D synthesis, upon oncogenic transformation [115].

4. Vitamin D in breast cancer

4.1 Epidemiological studies

There has been a great amount of information in the literature regarding the protective role of Vitamin D in various cancer models. Two major types of studies have been conducted: first, the ones that were focused on the association between solar radiation and breast cancer incidence rates; and second, the ones that analysed the relationship between Vitamin D intake and breast cancer risk.

Regarding the first set of epidemiological studies, the existence of an inverse association between decreased sunlight exposure and consequent diminished Vitamin D production on the skin and higher breast cancer incidence and mortality has been reported [116-118]. It has been described that white women with breast cancer display lower Vitamin D blood levels than unaffected ones [119]. Additionally, it has been found that early stage breast cancer patients show highest serum levels of Vitamin D than those who have advanced bone metastatic disease [120].

Concerning the relationship between Vitamin D intake and breast cancer risk, some reports have demonstrated that there is an inverse association between the circulating levels of 25-hydroxyvitamin D_3 (the major circulating form of Vitamin D and the one that is used to account for the levels of Vitamin D in the body) and the risk for developing breast cancer [121, 122]. Additionally, the relationship between Vitamin D serum levels and the different subtypes of breast carcinomas has been studied: it has been described that patients harbouring the most aggressive subgroup of mammary tumours (triple-negative) displayed the lowest levels of 25-hydroxyvitamin D_3 [123]. These results have been supported by a recent study, where it was observed that breast cancer patients with suboptimal vitamin D levels were more likely to have tumours with more aggressive profiles and worse prognostic markers [124].

Altogether, these studies demonstrate a protective role for Vitamin D in breast cancer, suggesting that Vitamin D signalling pathway disruption may be a predisposition to develop cancer.

4.2   Antiproliferative effects of Vitamin D

Suppression of cell growth by Vitamin D was first observed in 1981 by Abe and colleagues [125] and paved the way for Vitamin D to be considered as a potential targeted therapy in cancer research.

Since then, numerous studies have been conducted in various cancer models, in order to identify the molecular mediators of such effect.

Using mammary models, it has been demonstrated that, upon treatment with Vitamin D, a change in the expression of several proteins involved in cell cycle regulation occurs, namely cyclins, Cyclin Dependent Kinases (CDK) and CDK inhibitors (CKIs) [77]. Cell cycle was shown to be arrested at the G_0-G_1 transition upon Vitamin D treatment: specifically, it has been reported an increase in the expression of two CKIs, p21 (a functional VDRE was described in the promoter of its gene [126]) and p27, and an impairment in the expression of CDK2, CDK4, cyclin D1, cyclin D3, cyclin A1 and cyclin E1 [127, 128]. This effect leads to the inhibition of CDK activity, as well as to the hypophosphorylation of the retinoblastoma protein, pRB [128, 129]. Along with these effects in cell cycle controlling machinery, a downmodulation of the oncogenic protein c-Myc has also been reported [130], as well as other potential molecular effectors of Vitamin D were described, as Transforming Growth Factor beta-1 (TGF-β1), TGF-β receptor type 2 [131] and Insulin Growth Factor 1 (IGF1) [132-134]. The Vitamin D induction of *BRCA1* (Breast Cancer susceptibility gene 1) has also been inversely correlated with cell proliferation [135], while it has been described that Vitamin D decreases aromatase expression [136] and thereby can modulate ER-positive breast cancer growth. Interestingly, Vitamin D provokes a sharp inhibition of MCF-7 growth, together with a slight induction in the activity of antioxidant enzymes [137]. Moreover, it has also been demonstrated by our group that the anti-proliferative effects of Vitamin D in MCF-7 cells are not dependent on the presence of the VDR [138].

In some cancer models, Vitamin D has been shown to induce the expression of the transcription factor CCAAT Enhancer Binding Protein beta (C/EBPβ), a protein that has been identified as a strong suppressor of the oncogenic cyclin D1 signature in human epithelial tumours [139]. Additionally, it has been proposed that, in some breast cancer cell lines, C/EBPα may be mediating Vitamin D growth inhibitory effects [140]. It was observed that, when C/EBPα-negative MDA-MB-231 breast cancer

cells were transfected with C/EBPα, there was a decrease in cell proliferation, together with the suppression of the antiproliferative effects of Vitamin D in MCF-7 cells with the knockdown of C/EBPα.

In order to study the effects of Vitamin D in mammary carcinogenesis, animal models have been developed. The most widely used models are mice treated with 7,12-dimethylbenzanthracene (DMBA) and N-methyl-N-nitrosourea (MNU). Both carcinogens induce mammary adenocarcinomas in rats with nearly 100% of incidence. The histopathological evaluations revealed very close similarities between these carcinogen-induced tumours in rats and human breast cancer pathology [141]. In mice exposed to DMBA, there was a high amount of VDR expression in cells from carcinogen-induced mammary tumours [142]. This model also allowed the observation of the increased percentage of DMBA-driven preneoplastic breast lesions in glands from *Vdr* knockout mice, compared with wild-type animals. Furthermore, the histopathologic characteristics of mammary tumours that developed in *Vdr* deficient mice were different from those of tumours developing in wild-type animals (primarily myoepithelial tumours). However, it should be noted that *Vdr* knockout animals do not develop cancers in a spontaneous way, but only when exposed to oncogenes or carcinogens. In another report using *Vdr* knockout mice exposed to DMBA, it has been demonstrated that *Vdr* deficient animals have a higher incidence rate of *in situ* hyperplasias (both lobular and alveolar) when compared with their littermates [143]. This study further confirmed previous results [142], since the histologic subtypes of mammary tumours were different among the two groups of animals: in wild-type mice, the majority of tumours were papillary myoepithelial carcinomas whereas in *Vdr* knockout animals the tumours were mainly squamous cell carcinomas. The *Vdr* knockout tumours displayed squamous metaplasia, which suggested that VDR ablation was able to induce transdifferentiation of mammary epithelium into epidermal and squamous structures [143]. Furthermore, high amounts of expression of epidermal markers not usually detected in the mammary gland (CK1 and/or CK6) were detected in tumours arising in *Vdr* deficient animals. Overall, these data suggest that tumours generated in *Vdr* knockout mice exhibit a higher propensity for squamous metaplasia or

transdifferentiation into epidermal structures compared with wild-type mice. Studies with the Mouse Mammary Tumour Virus (MMTV)-*neu* transgenic mouse model were conducted in order to test whether VDR ablation would enhance sensitivity to transformation through a proto-oncogene that is often overexpressed in human breast cancer [144]. Results showed that VDR can be highly expressed in *neu*-induced mouse mammary tumours and in lung metastatic foci. Moreover, loss of either one or both copies of VDR was shown to be associated with increased incidence of preneoplastic lesions and abnormal ductal morphologic features in MMTV-*neu* animals [145]. Interestingly, loss of just one copy of the VDR was enough to significantly increase *neu*-induced mammary tumourigenesis, suggesting that haploinsufficiency of VDR gene can be associated with mammary gland pathologic lesions and sensitisation of the gland to transformation in response to altered growth factor signalling.

In normal breast, it was noticed that *Vdr* knockout mice display lower levels of TCF-4 and, consequently, a diminished β-catenin activity [146]. Furthermore in the same study, it has been described that Vitamin D regulates TCF7L2 promoter in mouse mammary cells and induces the expression of TCF-4 in human colorectal cell lines. Since TCF-4 is known to be a growth inhibitor protein, impairing β-catenin activity and cell growth in colorectal cancer [147], and is now being proposed as a tumour suppressor molecule in breast cancer [148], these data suggest a role for TCF-4 in the antiproliferative effects induced by Vitamin D in mammary tumours.

Collectively, these data (molecular effectors and *in vivo* studies) support an important role for Vitamin D as an anticancer agent (Figure 5). However, additional studies are necessary to further elucidate the role of this hormone as an antiproliferative agent in breast cancer, in order to clearly pinpoint and identify the molecular effectors responsible by this cellular mechanism.
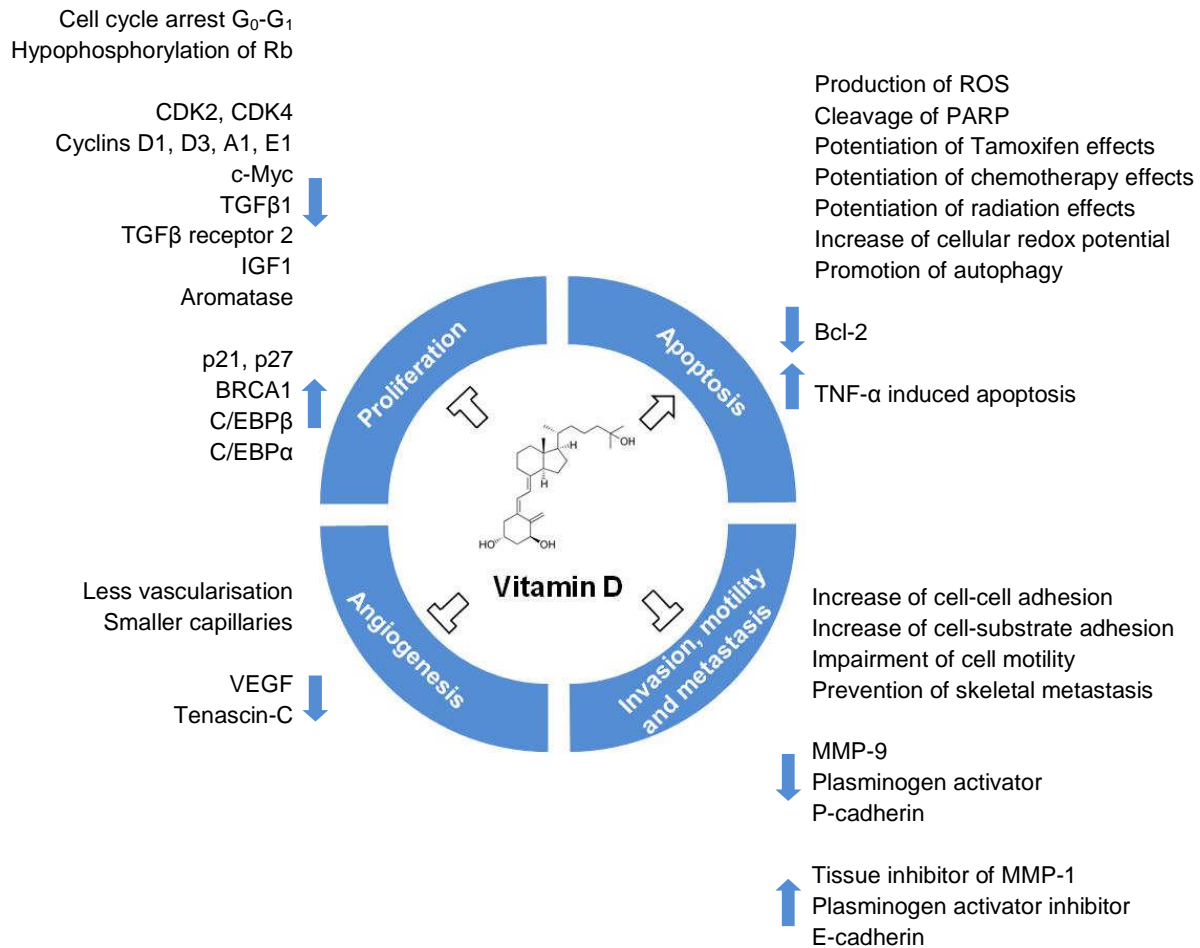
Cell cycle arrest G_0-G_1
Hypophosphorylation of Rb

CDK2, CDK4
Cyclins D1, D3, A1, E1
c-Myc
TGFβ1
TGFβ receptor 2
IGF1
Aromatase

p21, p27
BRCA1
C/EBPβ
C/EBPα

Less vascularisation
Smaller capillaries

VEGF
Tenascin-C

Production of ROS
Cleavage of PARP
Potentiation of Tamoxifen effects
Potentiation of chemotherapy effects
Potentiation of radiation effects
Increase of cellular redox potential
Promotion of autophagy

Bcl-2

TNF-α induced apoptosis

Increase of cell-cell adhesion
Increase of cell-substrate adhesion
Impairment of cell motility
Prevention of skeletal metastasis

MMP-9
Plasminogen activator
P-cadherin

Tissue inhibitor of MMP-1
Plasminogen activator inhibitor
E-cadherin

Vitamin D

Proliferation
Apoptosis
Angiogenesis
Invasion, motility and metastasis

Figure 5 – Vitamin D anti-carcinogenic effects in breast cancer.

4.3   Induction of apoptosis by Vitamin D

Additionally to the antiproliferative effects, Vitamin D has been proposed to have a role in the induction of apoptosis in breast cancer cells, also contributing to the growth-suppressing properties of this hormone (Figure 5).

For example, MCF-7 cells treated with Vitamin D display several features of apoptosis, namely cell shrinkage, chromatin condensation and DNA fragmentation [149]. Reports indicated that Vitamin D downregulates the expression of the antiapoptotic protein Bcl-2 in MCF-7-induced breast tumour xenografts [150], as well as it is able to enhance Tumour Necrosis Factor alpha (TNF-α)-induced apoptosis through caspase-dependent and caspase-independent mechanisms in breast cancer cells [151]. Supporting the role of caspase-independent cell death mediated by Vitamin D, it has been shown that induction of apoptosis in MCF-7 cells was due to disruption of mitochondrial function, which was associated with Bax translocation from the cytosol to the mitochondria, cytochrome C release and production of reactive oxygen species. These mitochondrial effects did not require caspase activation, since these were not blocked by a specific caspase inhibitor [152]. Further studies on the involvement of caspases have shown that treatment of ER-negative breast cancer cells with Vitamin D induces the cleavage of Poly (ADP-Ribose) Polymerase (PARP), a substrate of caspase 3 and caspase 7 [153]. Vitamin D is also able to potentiate and enhance the morphological effects of apoptosis when administered to MCF-7 cells in combination with Tamoxifen [154]. Effects of other anticancer agents are enhanced by the administration of Vitamin D, namely doxorubicin [155], taxol [156] and cisplatin [157]. Additionally, an important study has demonstrated that micromolar concentrations of Vitamin D are able to induce growth inhibition and apoptotic morphology in wild-type and *Vdr* knockout breast cancer cell lines, suggesting that mediation of growth regulatory effects can also be VDR-independent [158].

It has also been shown that Vitamin D-mediated cell death is dependent on calcium signalling [159]. In treated cells, apoptosis was triggered by intracellular calcium increase, as well as by depletion of the endoplasmic reticulum calcium stores. It is widely known that the increase in calcium concentration is associated with the activation of a calcium-dependent cysteine protease, the μ-calpain [160]. Interestingly, it has been found that the calcium/calpain-dependent caspase-12 was expressed and active in Vitamin D-treated cells, but not in normal cells [159]. According to these authors, a model for a novel apoptotic pathway in breast cancer cells treated with Vitamin D was

proposed: the increase in calcium concentration leads to μ-calpain activation, which in turns activates caspase-12, leading to cellular apoptosis [159].

Finally, Vitamin D was still described as a pro-oxidant in breast cancer cells, causing an increase in the overall cellular redox potential [161], which may also be an important mechanism underlying the pro-apoptotic effects of this hormone.

Regarding the role of Vitamin D analogues, VanWeelden and collaborators (1998) reported that these can also inhibit tumour growth by induction of apoptosis in tumour epithelial MCF-7 breast cancer cells [162]. Furthermore, these same analogues have been reported to increase the pro-apoptotic properties of ionising radiation [163] and to promote autophagic cell death [164].

In conclusion, Vitamin D is able to mediate apoptosis in breast cancer cells, but the mechanisms underlying this effect are still not completely elucidated and seem to be cell / tissue specific.

4.4   Vitamin D effects on motility, invasion and metastasis

The formation of metastasis constitutes the main clinical problem of breast cancer patients and it is intricately related to the processes of cell motility and invasion. It has been demonstrated that Vitamin D can also regulate the cellular mechanisms involved in these processes (Figure 5).

Mørk Hansen and collaborators (1994) have demonstrated that Vitamin D has the ability to inhibit the *in vitro* invasive potential of human breast cancer cells [165]. This reduced invasiveness was found to be associated with diminished activity of the metalloproteinase MMP-9, simultaneously with increased tissue inhibitor of MMP-1 activity [166]. Vitamin D has also been implicated in the downregulation of the plasminogen-activator and in the induction of its inhibitor [166]. Moreover, it was reported that

Vitamin D is able to induce cells to be more adhesive to each other (through the induction of expression of E-cadherin and other adhesion molecules), as well as to some substrates, impairing at the same time their *in vitro* mobility [167]. Vitamin D has also been shown to downregulate the expression of P-cadherin [167], an invasion promoter molecule in breast cancer cells [168]. *In vitro* experiments, using Vitamin D analogues, have demonstrated that they can inhibit the invasive potential of mammary cancer cells [169], as well as prevent the formation of skeletal metastasis and prolong survival time in nude mice transplanted with human breast cancer cells [170].

Altogether, these data demonstrate a role for Vitamin D as an agent with the ability to suppress the formation of metastasis by regulating the processes of cell motility and invasion.

4.5   Antiangiogenic properties of Vitamin D

Angiogenesis is an essential process for tumour growth and metastasis. Vitamin D has also been implicated in the suppression of the formation of new blood vessels in tumours (Figure 5).

Using the chick embryo chorioallantoic membrane assay (CAM), Oikawa and collaborators (1991) have observed that low concentrations of an analogue of Vitamin D were able to inhibit angiogenesis [171]. A Vitamin D analogue was also able to inhibit the expression of Vascular Endothelial Growth Factor (VEGF), an inducer of angiogenesis in tumours [172]. Using xenografted mice with VEGF-overexpressing MCF-7 breast cancer cells, it was observed that the administration of Vitamin D produces tumours that appear less vascularised than the controls [173]. Furthermore, the tumours formed in the treated animals displayed smaller capillaries when compared with their littermates, suggesting that Vitamin D may also inhibit vessel growth and maturation. In another study in mammary epithelial cells [174], Vitamin D was reported to be capable of inhibiting the expression of tenascin-C, which is an extracellular protein involved in growth, invasion and with angiogenic-inducing

properties.

These data indicate that Vitamin D could serve as a potential therapy against angiogenic processes in breast cancer, but further studies are necessary in order to identify other molecular mediators of this process.

4.6   Clinical studies

Phase I clinical trials combining Vitamin D with paclitaxel [175] and gefitinib [176] have been completed and were based on the knowledge that most positive preclinical studies used a high-dose and intermittent Vitamin D administration [77]. Still, because of its endocrine role as a regulator of calcium transport in bone metabolism, Vitamin D induces sytemic calcemic effects, making its administration to breast cancer patients severely hampered. This problem has led to the development of Vitamin D analogues with less calcemic effects. These compounds have proven their efficacy as anticancer agents in numerous *in vitro* studies and are now being used in clinical trials.

The importance of synthetic molecules was fully proven when it was demonstrated that these are capable of acting synergistically with chemotherapeutic agents. In a phase I clinical trial using EB1089 (a Vitamin D analogue) in patients with advanced breast and colorectal cancer, no clear antitumour effects were observed; however, 6 patients (2 colorectal and 4 breast cancer) showed disease stabilisation for at least 3 months [177]. In a phase II study, in prostate cancer, it has been shown that DN101 (another Vitamin D analogue) plus docetaxel improved patient survival [178]; however the phase III clinical trial, performed to further evaluate this survival difference, failed to confirm these optimistic results [179]. In line with this study, in a phase II trial, using patients with metastatic breast cancer who had received a high dose of Vitamin D daily, no significant results in the progress of the disease have been achieved [180]. In terms of Vitamin D intoxication, glucocorticoids

can be used to decrease hypercalcemia levels. In addition, it has been reported that dexamethasone significantly improves the anti-tumour efficacy of Vitamin D, both *in vitro* and *in vivo*, through direct effects on VDR [181].

Although the results in clinical trials have not been as promising as the *in vitro* studies, these data provide evidence for a role for Vitamin D analogues as a potential treatment for various types of cancer, if not alone, at least in combination with other anticancer therapeutics.

5.   Vitamin D and Epithelial to Mesenchymal Transition

Some evidence shows that Vitamin D is able to alter the expression of EMT markers. In breast cancer cells, the treatment with Vitamin D induces downregulation of N-cadherin expression, while E-cadherin expression is augmented [167], and in colon carcinoma cells, Vitamin D stimulates the development of a differentiated phenotype through an increase in E-cadherin expression [182]. Furthermore, overexpression of Snail is significantly associated with the downregulation of the VDR in human colonic tumours [183]. Hence, in this section, a small review on the role of EMT in normal development and cancer will be presented.

5.1   EMT in normal development

EMT is an embryonic programme through which epithelial cells acquire mesenchymal traits and it is an essential process during the morphogenesis of multicellular organisms [184]. In a simple manner, during EMT there is disruption of the epithelium, creating a new type of cells, the mesenchymal cells.

At the molecular level, the transition between epithelial to mesenchymal cells begins with the loss of apico-basal polarity along with the dissolution of tight junctions, allowing apical and basolateral membrane components to mix [185]. Other cell-cell junctions, including adherens and gap junctions also disassemble and the underlying basement membrane is degraded [186]. Cell membrane proteins that are responsible for mediating cell-cell adhesion and cell-basement membrane adhesion, like E-cadherin and integrins, respectively, are substituted by N-cadherin (Neural cadherin) and integrins that provide less adhesive properties, thereby priming the cell for the mesenchymal phenotype. Furthermore, there is reorganisation of the cytoskeleton, as the peripheral actin is replaced by stress fibres, while Vimentin takes the place of the cytokeratin intermediate filaments. Altogether, these alterations change the cell from a cubical to a spindle shape. Finally, the cell acquires the ability to invade and move into the extracellular matrix devoid of any cell-cell contacts [185]. Overall, EMT is characterised by a downregulation of epithelial markers, particularly E-cadherin, together with an upregulation of mesenchymal markers, particularly Vimentin and N-cadherin (the so-called cadherin switch), accompanied by an increase in cell migration and invasion (Figure 6).
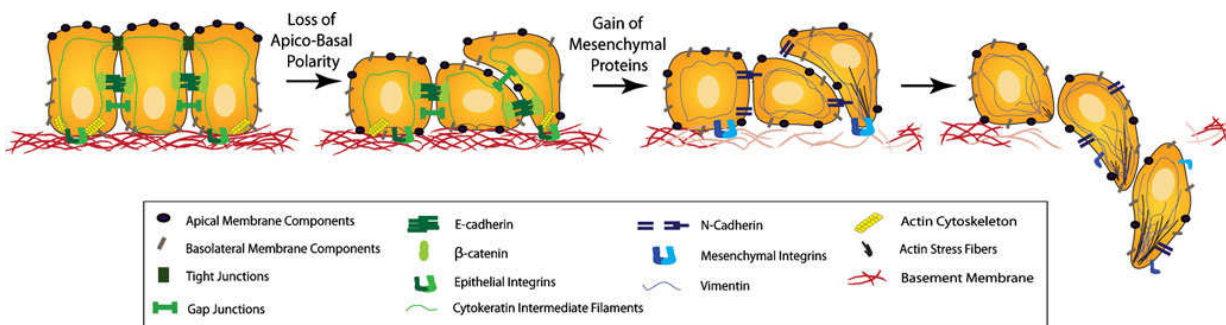

Figure 6 – Schematic representation of the molecular events underlying EMT (taken from [185]).

During development, EMT is a dynamic process present in many phases, such as gastrulation, neurulation and neural crest formation [187, 188]. Additionally, EMT can also occur during

organogenesis; for example, cells are reported to undergo EMT in the formation of the heart valves and myogenesis [189, 190].

The earliest occurrence of EMT is in gastrulation, which originates the primary mesenchymal cells and is responsible for the formation of the three primitive germ layers. Hence, there is disruption of the basement membrane underlying the primitive ectoderm [191] and upregulation of Snail by Fibroblast Growth Factor (FGF), which destabilises epithelial connections to neighbouring cells through the repression of E-cadherin [192]. The cells that migrate inwards can either maintain their mesenchymal traits and originate the mesoderm, or undergo Mesenchymal to Epithelial Transition (MET) and generate the endoderm. During neurulation, there is also EMT, with the formation of a neural crest cell population [188]. The molecular participant responsible for this process is Snail [193], which is thought to be induced by various signalling pathways, namely Bone Morphogenetic Proteins (BMPs), Wnt and FGF [194]. For the formation of cardiac valves, TGF-β, BMP, β-catenin and Slug [195-198], secreted by the myocardium, activate endocardial cells to undergo EMT and invade the basement membrane. These cells remain mesenchymal and mediate the development of the heart valves.

During mammary gland morphogenesis, there is also an incomplete process of EMT, named epithelial plasticity [185]. As previously discussed, during puberty there is extensive branching, along with ductal elongation and formation of the Terminal End Buds (TEB, which are the rodent equivalent to the human TDLUs). The TEB is the predominant site of branching [199] and this process is regulated by some EMT inducible cues, such as MMPs [200]. Unlike other organs, in the mammary gland, the elongation of the ducts is also dependent on proliferation, instead of the invasive capacity of cap cells [201]. But still, these cells display signs of epithelial plasticity, such as the loss of apico-basal polarity, while TEB cells secrete MMP-3, leading to the thinning of the basement membrane [200]. Furthermore, other evidences point to the induction of epithelial plasticity by EMT modulators in the mammary gland development. Using organotypic cultures, it has been demonstrated that cells at

the sites of branching induce the expression of Vimentin and MMP-3 [202]. Importantly, branching induced by MMP-3 occurred in the absence of added growth factors in these organoid cultures. These authors speculate that MMP-3 activity can lead to a transient EMT that may be necessary for the invasion of the mammary gland ductal tree in the fat pad. Thus, at the leading edge of ductal migration, epithelial cells may exhibit mesenchymal-like characteristics allowing them to invade as an organised structure [200]. Moreover, it was demonstrated a significant increase in the expression of Snail and Twist in TEB compared with mature ducts [203]. Wnt / β-catenin signalling pathway is also important during mammary development, as it has been observed that the expression of Wnt-1 under the control of the MMTV promoter induces hyperbranching and early alveolar differentiation [204]. Overall, these data provide compelling evidence to show that, although the mammary gland development does not depend on the full activation of the EMT programme, some cells do undergo epithelial plasticity and acquire features of a non-epithelial nature.

5.2   EMT in cancer

EMT is a key player in tumour progression, including in breast cancer [205-207]. It has been demonstrated that the invasive and metastatic behaviour of breast cancer cell lines is tightly associated with features typical of EMT, such as increased expression of Vimentin, diminished expression of epithelial cytokeratins and reduced or absent expression of E-cadherin [208]. These observations have led to the hypothesis that the acquisition of EMT traits in cancer cells may help to explain the formation of metastasis in distant sites from the primary tumour (Figure 7).

However, there is still much controversy surrounding this subject. One of the most challenging arguments against it is the fact that absolute evidence of EMT is lacking in most cancers. However, this can be easily explained, since tumour cells undergoing EMT may be phenotypically identical to fibroblasts. Moreover, there is no histopathologic demonstration of EMT at the site of metastasis, as

most lesions display an epithelial phenotype, which has also been contributing to the scepticism of some. The most probable explanation for this is that, at the secondary site, tumour cells undergo MET and colonise the new environment [185]. Hence, it seems that EMT is a flexible and dynamic process during cancer progression.
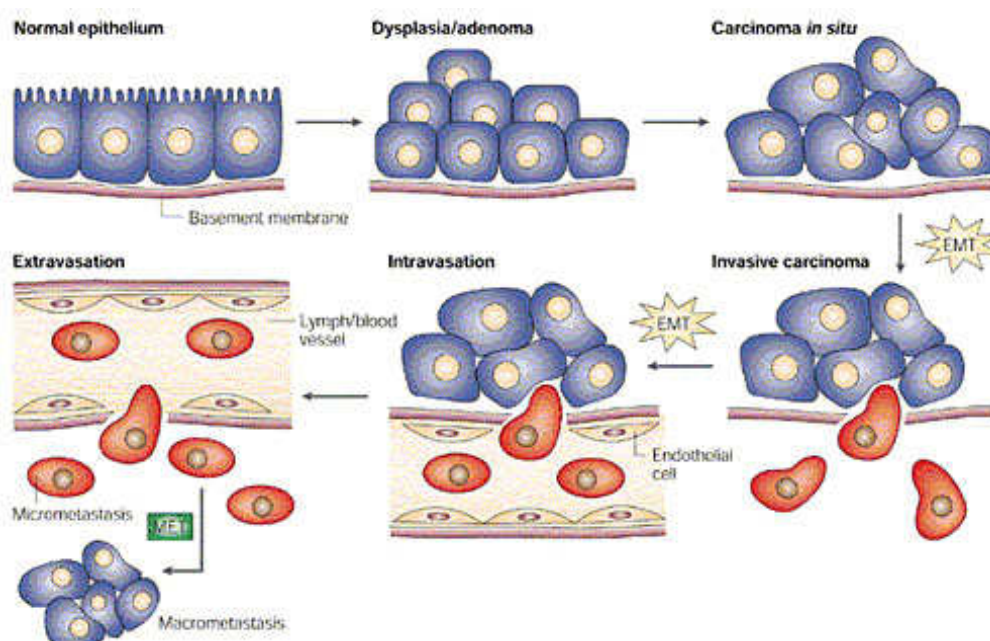

Figure 7 – EMT and MET allow the invasive and metastatic capacities of cancer cells (taken from http://www.ucdenver.edu/academics/colleges/medicalschool/departments/CellDevelopmentalBiology/facultyrese arch/PublishingImages/Research/prekeris_fig-2.jpg).

Despite this debate, recent evidence shows that some breast cancer subtypes are prone to undergo EMT. For example, the triple-negative carcinomas display the most aggressive phenotype and poor outcome [45], as previously discussed, and interestingly, it has been demonstrated that basal-like carcinomas and claudin-low tumours often exhibit EMT features [46, 209]. Additionally, applying the molecular classification of mammary tumours to breast cancer cell lines shows that cells grouped in the basal B subtype present mesenchymal traits [210]. These results have been further confirmed by other works in breast cancer cell lines, where observations demonstrate that the basal-like phenotype is associated with the upregulation of Vimentin, N-cadherin, Slug, Twist, and reduced expression of

E-cadherin [211, 212]. Correlating with these findings, it has also been found that the EMT programme is associated with a cancer stem cell phenotype [213, 214]. Additionally, there are data that provide evidence for enrichment in cells with mesenchymal properties and cancer stem-cell features after conventional therapy [215]. This finding may have clinical implications, since current therapeutic approaches against cancer are directed at common tumour cells, in order to cause tumour regression, but apparently, are leaving cancer stem cells unharmed [216]. This may explain, in part, the aggressive behaviour and poor outcome that are associated with claudin-low and basal-like breast carcinomas.

Various proteins and signalling pathways have been implicated in the modulation of EMT in cancer: from these, Snail, Slug and Twist, as well as TGF-β and β-catenin can be highlighted. Snail, Slug and Twist, apart from their role in E-cadherin repression [217-219], are involved in the modulation of other aspects of EMT. For instance, Snail and Slug regulate the stability of tight junctions, the expression of proteases and proteins in gap junctions and the dismantlement of desmosomes [220-223]. These transcription factors are under the control of EMT inducible stimuli, which include TGF-β and Wnt / β-catenin signalling [224, 225]. Additional studies have allowed the establishment of significant associations between Snail, Slug and Twist expression and poor prognosis in breast cancer. Thus, Snail expression correlates with lymph node metastasis and with decreased relapse-free survival [226, 227], whereas Slug expression has been associated with metastasis development [228]. Moreover, the expression of Twist significantly associated with invasive lobular carcinomas and diminished disease-free survival [219, 228]. TGF-β is also a master regulator of EMT and treatment of cells with this cytokine leads to a myriad of effects, namely loss of apico-basal polarity, as well as decrease in cell-cell adhesion, represented by diminished expression of E-cadherin and β-catenin, increased expression of mesenchymal cytoskeleton proteins, including Vimentin and matrix metalloproteinases [224, 229-231]. It has also the ability to induce the repression of E-cadherin and tight junction proteins, namely Occludin and Claudin 3, through the induction of Snail [232]. In later stages of tumourigenesis, TGF-β promotes metastatic spread, probably due to its capacity as

regulator of EMT [233, 234]. In animal models, TGF-β is markedly associated with an aggressive phenotype of tumours, like in mice overexpressing the *neu*-oncogene, where TGF-β signalling promotes pulmonary metastasis [235]. In human mammary tumours, TGF-β has been significantly associated with poor patient outcome [236, 237] and with the establishment of bone metastases [238]. Concerning the importance of the Wnt / β-catenin signalling pathway in EMT, it has been demonstrated that the activation of this signalling cascade induces the expression of Snail and Vimentin [225, 239], also leading to EMT in mammary cell line models [225, 240]. In breast cancer patients, the presence of nuclear β-catenin has been significantly correlated with poor prognosis [241, 242]. Altogether, these results point to an important role of EMT in the promotion of cancer invasion and metastasis.

Rationale and Aims

Breast cancer is the leading cause of death by cancer in women. It is a heterogeneous disease, with different molecular subtypes that are associated with diverse biological behaviours, various responses to therapy and clinical outcome. Although some of these molecular subgroups have a targeted therapy, the most aggressive tumours, the triple-negative basal-like carcinomas, still lack a molecular target. This has led to intensive research in order to find the best immunohistochemical criterion to identify triple-negative basal-like carcinomas, as well as potential therapeutic approaches for this particular type of tumours.

The large amount of data in the literature clearly highlight the importance of Vitamin D ($1\alpha,25(OH)_2D_3$) not only in its traditional role in bone metabolism as a regulator of calcium and phosphate levels, but also as a potential modulator of cancer features. In various cancer models it has been demonstrated that there is deregulation of $1\alpha,25(OH)_2D_3$ signalling and metabolic pathways.

1. General aim

The general aim of this work was to establish an immunohistochemical surrogate panel for the identification of triple-negative basal-like carcinomas in formalin-fixed paraffin-embedded material, as well as to study the role of $1\alpha,25(OH)_2D_3$ on carcinogenesis and breast cancer progression. In order to achieve this objective, the following specific tasks have been addressed.

2. Specific tasks

Immunohistochemical study for the identification of triple-negative basal-like breast carcinomas in a series of formalin-fixed paraffin-embedded invasive breast carcinomas

In order to establish an immunohistochemical surrogate panel for the identification of triple-negative basal-like carcinomas, we characterised a series of invasive breast tumours for the expression of several markers (ER, PgR, HER2, EGFR, CK5, CK14, P-cadherin, Vimentin and p63) by means of Immunohistochemistry in Tissue Microarrays (TMAs). These markers were used to classify breast tumours in the general molecular subtypes. Additionally, we investigated the value of these proteins in the identification of triple-negative basal-like tumours and the relation between their expression and the overall survival of patients.

Expression of $1\alpha,25(OH)_2D_3$ main partners (VDR, CYP27B1 and CYP24A1) in normal and neoplastic breast tissue

In order to evaluate the importance of $1\alpha,25(OH)_2D_3$ role in the mammary gland and in breast cancer, we assessed the expression of the main partners of $1\alpha,25(OH)_2D_3$ signalling (VDR) and metabolic (CYP27B1 and CYP24A1) pathways in breast tissue, using a series comprising normal mammary tissue, benign mammary lesions, carcinomas *in situ* and invasive breast carcinomas. This study was conducted using Immunohistochemistry in Tissue Microarrays.

<u>Evaluation of the *in vitro* effects of 1α,25(OH)$_2$D$_3$ in human triple-negative basal-like breast cancer cell lines</u>

With the intention of testing 1α,25(OH)$_2$D$_3$ as a potential therapy for triple-negative basal-like breast cancer, we have decided to test its effects on breast cancer cell lines with a triple-negative basal-like phenotype. We have evaluated differences in the expression of several proteins by Western Blotting and quantitative Real-time PCR.

Material and Methods

1. Material

Paper 1 – A series of 301 formalin-fixed paraffin-embedded cases of invasive breast carcinomas were consecutively recovered from the archives of the Pathology Department of Hospital Divino Espírito Santo in Ponta Delgada, Portugal and from the General Hospital of UNIMED in Araçatuba, Brazil. The cases were collected between 1994 and 2005.

Papers 1 and 2 – A series of 161 formalin-fixed paraffin-embedded cases of invasive breast tumours were consecutively retrieved from the archives of the Pathology Department of the Federal University of Santa Catarina in Florianópolis, Brazil. The cases were collected between 1994 and 2004.

Paper 2 – A series of 379 formalin-fixed paraffin-embedded benign lesions of the breast and 189 formalin-fixed paraffin embedded cases of ductal carcinomas *in situ* were consecutively collected from the archives of the Pathology Department of General Hospital of UNIMED in Araçatuba, Brazil. This series of 189 ductal carcinomas *in situ* contained in the same block the matched invasive carcinomas. The cases of ductal carcinomas *in situ* and invasive ductal tumours were collected between 1994 and 2004, while the series of benign lesions was collected between 2002 and 2006.

Paper 3 – A series of 12 formalin-fixed paraffin-embedded metaplastic breast carcinomas were consecutively retrieved from the archives of the Pathology Department of the Federal University of São Paulo, Brazil and from the Federal University of Santa Catarina in Florianópolis, Brazil. The cases were collected between 1994 and 2009.

Papers 2 and 3 – A series of human breast cancer cell lines: MDA-MB-231, Hs578T, BT-549 and MCF-7 (commercially available from ATCC) were grown in complete Gibco® Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, USA), in the presence of 10% foetal bovine serum (Lonza, Switzerland) and 1% Penicillin/Streptomycin (Invitrogen) and maintained at 37ºC with 5% of CO_2.

2. Methods

Tissue Microarray construction

For the Tissue Microarrays (TMAs) used in papers 1 and 2, representative areas of the different lesions were carefully selected on the H&E (hematoxylin and eosin)-stained sections by pathologists and marked on individual paraffin blocks. Two tissue cores (2 mm in diameter) were obtained from each selected specimen and precisely deposited into a recipient paraffin block, using a Tissue Microarray workstation (TMA builder, LabVision, USA). Several TMA blocks were constructed (69 for the invasive breast carcinomas, 22 for the ductal carcinomas *in situ* and 17 for the benign lesions), each containing 24 tissue cores, arranged in a 4×6 sector. In each TMA block, at least 3 non-neoplastic breast tissue cores were also included as controls and 1 core of a non-breast sample (we have used testicular tissue) was used to orientate the block.

To homogenise the paraffin of the receptor block and the paraffin of the cores extracted from the donor blocks, the TMAs were kept at 37ºC for 3 hours. After construction, 2-µm tissue sections were cut and adhered to Superfrost Plus glass slides (Thermo Scientific, Germany). An H&E-stained section from each block was reviewed to confirm the presence of morphological representative areas of the original lesions.

These studies have been conducted under the national regulative law for the usage of biological specimens from tumour banks, where the samples are exclusively available for research purposes in the case of retrospective studies.

Immunohistochemistry

Immunohistochemical staining was performed using the streptavidin-biotin-peroxidase technique (LabVision Corporation) or the HRP labelled polymer (ImmunoLogic, The Netherlands for VDR and Dako, USA for CK14, E-cadherin, EGFR and P-cadherin) in each set of glass slides comprising the TMAs. Epitope retrieval was performed using a dilution of 1:100 of citrate buffer, pH=6.0 (Vector Laboratories, USA) at 98°C for 30 minutes, or using a dilution of 1:10 of EDTA, pH=9.0 (Dako) also for 30 minutes at 98ºC, or by proteolytic enzyme digestion using a solution of pepsin A in distilled water (4 g/L, Sigma) for 30 minutes at 37ºC. The antigen retrieval times, antibodies, dilutions and suppliers are listed in Table 1. After the respective antigen retrieval and washes in a phosphate buffer solution (PBS), endogenous peroxidase activity was blocked with a 3% hydrogen peroxide solution (Panreac, Spain) in methanol (Sigma-Aldrich, USA) for 10 minutes. The slides were incubated in a blocking serum (LabVision, USA) for 15 minutes and then incubated with the respective primary antibodies. Primary antibody incubation was performed for 30 minutes, 1 hour or 2 hours at room temperature or overnight at 4ºC. After washes, the slides were incubated with secondary antibody associated with HRP-labelled (horseradish peroxidase) polymer or incubated with biotinylated secondary antibody (Labvision), followed by streptavidin-conjugated peroxidase (Labvision) during 15 minutes, washed and revealed with DAB (Dako). Tissues were then counterstained with Mayer's haematoxylin, dehydrated and cover-slipped using a permanent mounting solution (Zymed, USA).

Positive controls were included in each run, in order to guarantee the reliability of the assays. Paraffin sections of skin tissue have been used as positive controls for CK14 and E-cadherin, and appendix

tissue was used for Vimentin. Normal prostate tissue was used as positive control for CK5, while normal breast was used for p63 and P-cadherin. Samples of squamous cell carcinoma of the skin were utilised as positive control for EGFR expression, whereas for ER, HER2 and PgR we have used previously tested and strongly positive breast carcinomas. Paraffin sections of a basal cell carcinoma of the skin, normal colon and normal liver were used as positive controls for assessing VDR, CYP27B1 and CYP24A1 expression, respectively. Normal breast ducts and lobules present in many of the selected areas were also used as internal controls, as well as the non-neoplastic breast tissue cores included in each array.

The evaluation of the immunohistochemical results was performed by two pathologists. VDR nuclear expression was evaluated using the H-score method: intensity ranked from 1 to 3 (1 – weak, 2 – moderate, 3 – strong), and extension ranked from 1 to 10 (1 – 0-10% cells, 2 – 11-20% cells and so on, until a maximum score of 10) [243]. The scores for intensity and extension were multiplied and the following criterion was applied: the cases were considered negative when ranging from 1 to 4; samples ranking from 5 to 30 were considered to be positive. Given the lack of previous reports for the immunohistochemical evaluation of the CYP27B1 and CYP24A1, we considered the cases to be positive only when cytoplasmic staining was observed. The other markers were scored as described in previous studies from our group [60, 244-246]. ER, PgR and p63 were considered positive whenever more than 10% of the neoplastic cells showed nuclear staining; similarly, the same cut-off was used for CK5, CK14 and Vimentin cytoplasmic staining, as well as for E-cadherin and P-cadherin membrane staining. Membrane expression for HER2 and EGFR was evaluated according to the DakoCytomation HercepTest® scoring system.

Hormone receptor (ER and PgR) positive tumours were considered Luminal A and B whether or not they overexpressed HER2, respectively [60, 245, 247-249]. Breast carcinomas were considered HER2-overexpressing tumours whenever the immunohistochemical reaction was classified as 3+ or when gene amplification was confirmed by Chromogenic *In Situ* hybridization (CISH) in the 2+ cases,

as described in other works [250]. Cases lacking hormone receptors expression with overexpression of HER2 were classified as HER2-overexpressing tumours. Triple-negative cases with immunoreactivity for EGFR and/or CK5 were considered basal-like breast carcinomas according to the gold standard criterion of Nielsen [61] and cases without expression of the five biomarkers (ER, PgR, HER2, EGFR and CK5) were considered unclassified. When the immunoreactivity for the additional basal markers, namely P-cadherin, CK14 and Vimentin was used, the positive cases for at least one of these markers were considered as basal-like breast carcinomas (P-cadherin and/or CK14 and/or Vimentin).

Table 1 – Antibodies and conditions used for Immunohistochemistry (to be continued in the next page).

Antibody	Clone	Manufacturer	Antigen retrieval	Primary antibody		Detection system
				Dilution	Incubation	
CK5	XM26	Neomarkers, USA	Tris-EDTA solution	1:50	1 hour	Streptavidin-biotin-peroxidase
CK14	LL002	Novocastra, UK	Tris-EDTA solution	1:400	1 hour	HRP-labelled polymer
CYP24A1	C18	Santa Cruz, USA	Citrate buffer	1:75	overnight	Streptavidin-biotin-peroxidase
CYP27B1	C12	Santa Cruz, USA	Citrate buffer	1:200	1 hour	Streptavidin-biotin-peroxidase
E-cadherin	24E10	Cell Signaling, USA	Tris-EDTA solution	1:50	1 hour	HRP-labelled polymer
EGFR	31G7	Zymed, USA	Pepsin A solution	1:100	1 hour	HRP-labelled polymer
ER	SP1	Neomarkers, USA	Citrate buffer	1:150	30 minutes	Streptavidin-biotin-peroxidase
HER2	SP3	Neomarkers, USA	Citrate buffer	1:80	30 minutes	Streptavidin-biotin-peroxidase
p63	4A4	Neomarkers, USA	Citrate buffer	1:150	1 hour	Streptavidin-biotin-peroxidase

Table 1 – Antibodies and conditions used for Immunohistochemistry (continued from the previous page).

Antibody	Clone	Manufacturer	Antigen retrieval	Primary antibody		Detection system
				Dilution	Incubation	
P-cadherin	56	BD Biosciences, USA	Tris-EDTA solution	1:50	1 hour	HRP-labelled polymer
PgR	SP2	Neomarkers, USA	Citrate buffer	1:300	30 minutes	Streptavidin-biotin-peroxidase
VDR	9A7γE10.4	Calbiochem, Germany	Pepsin A solution	1:50	overnight	HRP-labelled polymer
Vimentin	V9	Dako, USA	Citrate buffer	1:150	30 minutes	Streptavidin-biotin-peroxidase

Cell culture and treatments

All breast cancer cells were grown in complete Gibco® Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, USA) in the presence of 10% foetal bovine serum (Invitrogen) and 1% Penicillin/Streptomycin (Invitrogen).

Treatments with $1\alpha,25(OH)_2D_3$ 100 nM (Cayman Chemical, USA), 5-aza-2-deoxycytidine 5 μM (5-aza-dC, Sigma, Germany), DMSO (dimethyl sulfoxide, vehicle for 5-aza-dC and TSA) and ethanol (vehicle for $1\alpha,25(OH)_2D_3$) were performed for 72 hours, while the treatment with Trichostatin A 100 nM (TSA, Sigma) was performed only in the last 16 hours. Every 24 hours, culture medium was changed and a new treatment was performed. Treatment with PTH (parathyroid hormone) (Sigma-Aldrich, Germany) 100 nM and water (vehicle) were performed for 4h.

Protein extraction

Total protein lysates were prepared from cultured cells, using catenin lysis buffer [1% (v/v) Triton X-100 (Sigma, Germany) and 1% (v/v) NP-40 (Sigma) in deionised PBS – phosphate buffered saline], supplemented with 1:7 proteases inhibitors cocktail (Roche Diagnostics GmbH, Germany). Cells were washed twice with PBS and were allowed to lyse in 700 µl of catenin lysis buffer, for 10 minutes, at 4°C. Cell lysates were submitted to vortex 3 times and centrifuged at 14000 rpm and 4°C, during 10 minutes. Supernatants were collected and protein concentration was determined using the Bradford assay (Bio-Rad protein quantification system, USA).

Western blotting

Proteins were dissolved in sample buffer [Laemmli with 5% (v/v) 2-β-mercaptoethanol and 5% (v/v) bromophenol blue] and boiled for 5 minutes at 95°C. Equal amount of protein samples were separated by an 8% SDS-PAGE and proteins were transferred into nitrocellulose membranes (GE Healthcare Life Sciences, UK) at 100 V for 90 minutes.

For immunostaining, membranes were blocked for non-specific binding with 5% (w/v) non-fat dry milk, in PBS containing 0.5% (v/v) Tween-20. The membranes were subsequently incubated with the primary antibodies for 1 hour, 1 hour and 30 minutes or overnight, followed by four 5 minutes washes in PBS/Tween-20. The antibodies, dilutions and suppliers are listed in Table 2. Next, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:1000, Santa Cruz, USA) for 60 minutes. The membranes were then washed six more times for 5 minutes, and proteins were detected using ECL reagent (GE Healthcare Life Sciences) as a substrate. Blots were then exposed to an autoradiographic film.

Table 2 – Antibodies and conditions used for Western blotting.

Antibody	Clone	Manufacturer	Primary antibody		Secondary antibody
			Dilution	Incubation	
α-tubulin	DM1A	Sigma, Germany	1:10000	1 hour	Mouse
β-actin	I19	Santa Cruz, USA	1:1000	1 hour	Goat
CYP27B1	C12	Santa Cruz, USA	1:200	overnight	Goat
CYP24A1	C18	Santa Cruz, USA	1:200	overnight	Goat
E-cadherin	24E10	Cell Signaling, USA	1:1000	1 hour	Rabbit
VDR	9A7γE10.4	Calbiochem, Germany	1:400	overnight	Rat

RNA extraction, cDNA synthesis and quantitative Real-Time PCR

RNA was extracted from breast cancer cells using TRIzol® reagent (Invitrogen), according to the manufacturer's protocol. After extraction, RNA was quantified using NanoDrop spectrophotometer (Thermo Scientific, USA). cDNA was synthesised from 1 μg of RNA, using the Omniscript Reverse Transcription kit (Qiagen, Germany), following the manufacturer's instructions. Finally, real-time PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems, USA), using 1 μL of cDNA and in accordance to the manufacturer's protocol. The TaqMan Gene Expression Assays used were Hs01023895_m1 (for *CDH1*, Applied Biosystems) and TaqMan PreDeveloped Assay Reagents Human GAPDH (for *GAPDH,* Applied Biosystems). Reactions were performed using standard cycle parameters on a 7500 Fast Real-Time PCR System (Applied Biosystems). Relative transcript levels were determined using human GAPDH as an internal reference. Differences between samples were determined using the Quantitation – Relative Standard Curve method. All reactions were done in triplicate and expressed as mean of the values from three separate experiments.

DNA extraction and promoter methylation assay

DNA was extracted from breast cancer cell lines using ULTRAPrep Genomic DNA Blood & Cell Culture Kit (AHN Biotechnologie, Germany), according to the manufacturer's instructions. After extraction, DNA was quantified using the NanoDrop spectrophotometer (Thermo Scientific, USA). Bisulfite treatment was performed on 300 ng of DNA, using the EpiTect Bisulfite kit (Qiagen, Germany) following the manufacturer's guidelines and afterwards the DNA was quantified using the NanoDrop spectrophotometer (Thermo Scientific).

The samples were then amplified by PCR. For a final volume of 15 µL of PCR reaction mix, 120 ng of bisulfite-modified DNA was added to the PCR mix containing 7.5 µL of multiplex mix (Multiplex PCR Kit, Qiagen), 0.3 µL of primers (10 µM) (see Table 3) and DNase-free water. The samples were placed in a MyCycler™ thermal cycler (Bio-Rad), under the following conditions: the initial denaturation step at 96°C for 15 minutes; 96°C for 30 seconds, 62°C for 1 minute and 30 seconds and 72°C for 1 minute and 30 seconds (3 cycles); 96°C for 30 seconds, 60°C for 1 minute and 30 seconds and 72°C for 1 minute and 30 seconds (3 cycles); 96°C for 30 seconds, 58°C for 1 minute and 30 seconds and 72°C for 1 minute and 30 seconds (30 cycles) and a final extension step at 72°C for 10 minutes. PCR products were then loaded into a 2% (w/v) SeaKem® LE agarose (Lonza) gel and, in cases where DNA could not be detected, a reamplification PCR was performed. For a final volume of 20 µL of PCR reaction mix, 1 µL of amplified DNA was added to the PCR mix containing a 10.0 µL of multiplex mix, 0.4 µL of primers (10 µM) (see Table 3) and DNase-free water. The samples were placed in a MyCycler™ thermal cycler (Bio-Rad), under the following conditions: the initial denaturation step at 96°C for 15 minutes; 96°C for 30 seconds, 62°C for 1 minute and 30 seconds and 72°C for 1 minute and 30 seconds (3 cycles); 96°C for 30 seconds, 61°C for 1 minute and 30 seconds and 72°C for 1 minute and 30 seconds (3 cycles); 96°C for 30 seconds, 59°C for 1 minute

and 30 seconds and 72ºC for 1 minute and 30 seconds (30 cycles) and a final extension step at 72ºC for 10 minutes. 5 µL of DNA were purified in a MyCycler™ thermal cycler (Bio-Rad) using the enzymes 0.5 µL of ExoNuclease I (New England BioLabs, USA, 20000 U/mL) and 1 µL of FastAP™ Thermosensitive Alkaline Phosphatase (Fermentas, USA, 1 U/µL), under the following conditions: 15 minutes at 37ºC and 15 minutes at 85ºC. Afterwards, a sequencing PCR reaction was performed: for a final volume of 5 µL, 1 µL of purified DNA has been used, along with 1 µL of BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), 1.6 µL of Sequencing Buffer (Applied Biosystems), water and 0.6 µL of a CpG flanking primer (Sigma) – a different mix has been made for each primer (see Table 3). The samples were put in a MyCycler™ thermal cycler (Bio-Rad), under the following conditions: the initial denaturation step at 96ºC for 2 minutes; 96ºC for 30 seconds, 54ºC for 15 seconds and 60ºC for 2 minutes (35 cycles); and a final extension step at 60ºC for 10 minutes.

Table 3 – Sequences of the primers used for *CDH1* promoter methylation analysis (F – Forward, R – Reverse).

CpG flanking primers	Primer Sequence	PCR product size	Type of PCR
F	GGTAGGTGAATTTTTAGTTAAT	226 bp	Amplification
R	ACTCCAAAAACCCATAACTAAC		
F	GGGGTTTATTTGGTTGTAGTTA	176 bp	Reamplification
R	ACTCCAAAAACCCATAACTAAC		
F	GGGGTTTATTTGGTTGTAGTTA	-	Sequencing
R	ACTCCAAAAACCCATAACTAAC		

Finally, the samples were further purified using Sephadex™ G-50 Fine (GE Healthcare): a volume of 500 µL of Sephadex™ solution in distilled water (66,6g/L) was added to previously washed in distilled water columns inside collection tubes and centrifuged for 4 minutes at 4400 rpm. The columns were

then placed in clean 1.5 mL tubes and the samples were loaded into the Sephadex^{TM} columns and centrifuged for 4 minutes at 4400 rpm. The columns were discarded and 12 µL of Hi-Di^{TM} Formamide (Applied Biosystems) were added to the samples. Finally, the samples were analysed in a 3130xl Genetic Analyzer (Applied Biosystems).

Immunofluorescence

Cells were seeded in coverslips in complete DMEM medium, in the presence of 10% foetal bovine serum and 1% Penicillin/Streptomycin. Afterwards, cells were washed three times with PBS for 5 minutes and fixed with formaldehyde 4% (v/v) for 30 minutes. Coverslips were further washed three times with PBS for 5 minutes, followed by incubation with 50 mM NH$_4$Cl in PBS for 10 minutes. Following another set of three 5 minutes washes with PBS, coverslips were incubated with Triton X-100 0.2% (v/v) for 5 minutes and washed with PBS three times for 5 minutes. Subsequently, these were blocked for non-specific binding with BSA 5% in PBS, containing 0.5% (v/v) Tween-20, for 30 minutes and incubated with the primary antibody for E-cadherin (Zymed, clone HECD1, 1:100) for 1 hour. After three 5 minutes washes with PBS, coverslips were incubated with a goat anti-mouse secondary antibody (Alexa Fluor 594, 1:500, Invitrogen), washed with PBS for 3 times 5 minutes and mounted using Vectashield with DAPI (Vector Laboratories, USA).

Transfection with siRNA for VDR

MDA-MB-231 cells (2.5 x 10^5 cells) were plated in complete DMEM medium, in the presence of 10% foetal bovine serum and 1% Penicillin/Streptomycin, in 6 well-plates for 24 hours. For each well, 150 nmol of siRNA against VDR (Hs_VDR_8 FlexiTube siRNA, Qiagen, Germany) was mixed with 117.5 µL of Opti-MEM$^®$ medium (Invitrogen) for 5 minutes and, at the same time, 4 µL of Lipofectamine^{TM}

2000 (Invitrogen) was mixed with 121 µL of Opti-MEM$^®$ medium for 5 minutes. Next, the diluted siRNA against VDR was mixed with the diluted Lipofectamine^{TM} 2000 for 20 minutes at room temperature. Meanwhile, cell medium (complete DMEM) was replaced by 750 µL of DMEM medium without foetal bovine serum and antibiotics and 250 µL of the siRNA plus Lipofectamine was carefully added to the cells. After 5 hours of incubation, cell medium was replaced by complete DMEM medium, in the presence of 10% foetal bovine serum and 1% Penicillin/Streptomycin and the cells were treated with 1α,25(OH)$_2$D$_3$ 100 nM and ethanol. The evaluation of siRNA efficiency occurred 48 hours after transfection.

Statistical analysis

For the immunohistochemistry results, the Statview 5.0 software package (SAS Institute, USA) was used for all statistical analysis. Correlations between discrete variables were performed using the chi-square test and analysis of variance was employed to search for associations between continuous and discrete variables. In all analyses, a p value < 0.05 was considered to be statistically significant.

For all other experiments, statistical analyses were performed with Student's t-test. Differences with p values < 0.05 were considered to be statistically significant. All the presented results are representative of at least three independent experiments, unless stated otherwise.

In order to determine which were the most sensitive and specific biomarkers to identify basal-like breast carcinomas, the sensitivity and the specificity of the antibodies used were calculated. Sensitivity measurement was defined by the quotient between the true positive (TrueP) cases and the sum of the true positive and the false negative (FalseN) cases [sensitivity = TrueP / (TrueP + FalseN)]. Specificity was measured in a similar way, by the quotient between the true negative (TrueN) cases with the sum of the true negatives and the false positives (FalseP) [specificity = TrueN

/ (TrueN + FalseP)]. Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were calculated as follows: PPV = TrueP / (TrueP + FalseP) and NPV = TrueN / (TrueN + FalseN). As described before, ER/PgR/HER2 negative tumours that express CK5/6 and/or EGFR were considered basal-like breast carcinomas [61]. Consequently, TrueP and TrueN cases were the basal-like breast carcinomas tumours that were positive or negative, respectively, to the marker or pair of markers in analysis. Inversely, FalseP and FalseN were non-basal-like breast carcinomas positive or negative to the basal markers in study. Follow-up information was available for 282 of the 387 cases and a maximum cut-off of 77 months was considered. Survival curves were estimated by the Kaplan-Meier method using log-rank test to assess significant differences for overall survival, using the SPSS statistics 17.0 (SPSS Inc., Chicago, IL, USA) software program.

Results

1. Immunohistochemical study for the identification of triple-negative basal-like carcinomas in a series of invasive breast tumours

Paper related with this chapter:

Introduction

Breast cancer is frequently designated as a heterogeneous disease with divergent biological behaviours. cDNA microarray studies have provided an improvement in cellular and molecular understanding of breast cancer, identifying distinct subtypes of breast carcinomas with different molecular signatures and clinical outcomes [30, 31, 44, 63, 251]. The basal-like subtype has definitely drawn the attention of the scientific community. These tumours are characterised by a triple-negative phenotype, lacking the expression of receptors ER, PgR and HER2. Basal-like breast carcinomas are associated with aggressive tumour behaviour and shorter overall survival when compared with the Luminal and HER2-overexpressing subtypes and there is an enthusiastic search for molecular markers expressed in basal-like breast carcinomas that could be used as targets to therapy [61].

Nowadays, gene expression profiles or cDNA microarrays studies are considered the "gold standard" methods for the identification of breast carcinomas with a basal-like phenotype, since these technologies were the first to identify basal-like breast carcinomas as a distinct subgroup with a specific molecular signature [30] and clinical identity [31, 44, 252]. However, gene expression profiling is expensive, is not easily applicable as a routine laboratory diagnostic tool in large scale clinical-pathological analysis and has limited value in retrospective studies using formalin-fixed paraffin-embedded tissues [52, 253]. Thus, the idea of developing an immunohistochemical-based assay for the identification of basal-like breast carcinomas is appealing. The characteristic protein expression of tumours would be a useful surrogate of gene expression profiles, and the immunohistochemical profile would help to standardise research and uniformly identify a group of tumours with a basal-like transcriptional program [52].

However, the most appropriate panel of antibodies to be used, in order to identify breast carcinomas with a basal-like phenotype, has not reached a consensus yet. The triple-negative phenotype criterion is used by some authors who assume that triple-negative tumours and basal-like breast carcinomas

are synonymous [248, 254]. Other authors use high molecular weight cytokeratins alone (CK5/6, CK14 or CK17) to identify basal-like breast carcinomas, claiming that basal-like breast carcinomas and triple-negative tumours are different entities [65, 255-257]. In addition, since basal-like breast carcinomas express proteins that are characteristic from the basal / myoepihelial outer layer of the mammary gland, such as EGFR, p63, P-cadherin, Calponin, CD10, S100 and α-SMA [59, 61, 258, 259], some definitions of basal-like breast carcinomas associate the lack of expression of ER, PgR and HER2 with the immunoreactivity for some of these basal markers that were already correlated with the basal-like phenotype and poor prognosis [60, 61, 260].

In this study, we aim to refine the immunohistochemical criterion to identify basal-like breast carcinomas by analysing the sensitivity and the specificity of the main basal markers that have been described, namely CK5, EGFR, P-cadherin, CK14, Vimentin and p63 and suggest possible additional markers for basal-like breast carcinomas identification, especially in CK5- and EGFR-negative breast carcinomas.

Results

Classification of a series of invasive breast carcinomas according to the molecular profile

In this series of 387 breast carcinomas, 223 out of 387 (57.6%) and 144 out of 387 (37.2%) cases were ER and PgR positive, respectively, and 65 out of 387 (16.8%) overexpressed HER2. Using the triple-negative criterion, this series comprised 109 (28.2%) triple-negative and 278 (71.8%) non-triple-negative tumours. Considering the molecular subtypes of breast cancer, 213 (55%) cases were Luminal A, 13 (3.4%) were Luminal B and 52 (13.4%) were HER2-overexpressing tumours. According to the criterion of Nielsen, 37 (9.6%) cases presented a basal-like phenotype and 72

(18.6%) were considered "unclassified" by this criterion. Figure 8 shows the immunohistochemical staining for CK5, EGFR, P-cadherin, Vimentin and CK14 in basal-like breast carcinomas.
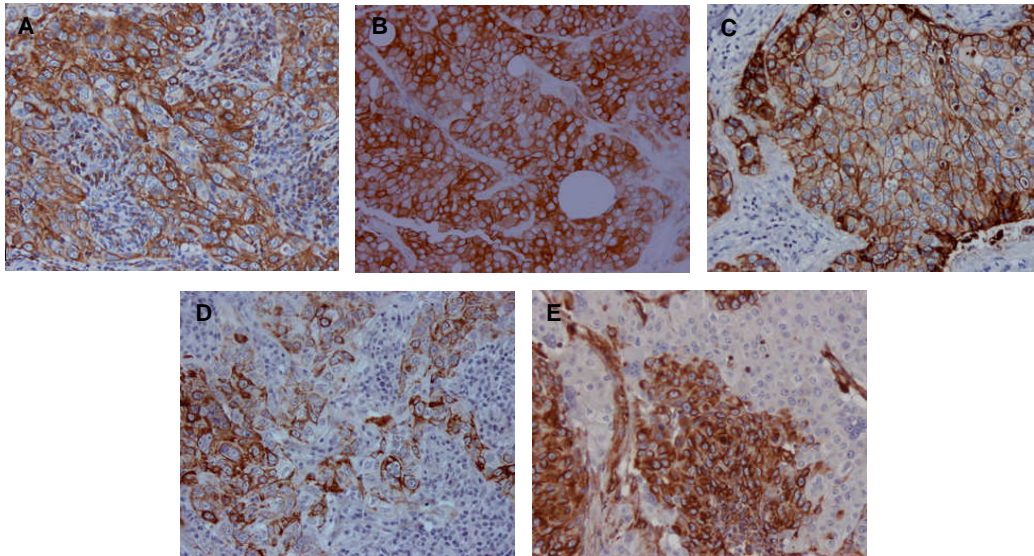

Figure 8 – Expression of CK5 (**A**), EGFR (**B**), P-cadherin (**C**), CK14 (**D**) and Vimentin (**E**) in basal-like breast carcinomas. (x 200)

Identification of potential biomarkers for basal-like breast carcinomas

We have analysed the associations between CK5, EGFR, P-cadherin, CK14, p63 and Vimentin and the basal-like breast carcinoma *versus* non-basal-like breast carcinoma (Table 4). As expected, the markers were significantly associated with the basal-like phenotype (p < 0.0001), with the exception of p63 (p = 0.5403).

Table 4 – Association between the expression of CK5, EGFR, P-cadherin, CK14, p63 and Vimentin with basal-like and non-basal-like breast carcinomas.

		n	Basal-like n (%)	Non-basal-like n (%)	p value
		387	37 (9.6)	350 (90.4)	
CK5	+	89	34 (91.9)	55 (15.7)	< 0.0001
	-	298	3 (8.1)	295 (84.3)	
EGFR	+	21	11 (29.7)	10 (2.9)	< 0.0001
	-	366	26 (70.3)	340 (97.1)	
P-cadherin	+	123	25 (67.6)	98 (28.0)	< 0.0001
	-	264	12 (32.4)	252 (72.0)	
CK14	+	17	12 (32.4)	5 (1.4)	< 0.0001
	-	370	25 (67.6)	345 (98.6)	
p63	+	14	2 (5.4)	12 (3.4)	0.5403
	-	373	35 (94.6)	338 (96.6)	
Vimentin	+	63	17 (45.9)	46 (13.1)	< 0.0001
	-	324	20 (54.1)	304 (86.9)	

Afterwards, the sensitivity, specificity, positive predictive value and negative predictive value of each biomarker for the identification of basal-like breast carcinomas were calculated (Table 5), except for p63 which was not even related with basal-like phenotype. CK5 was the most sensitive biomarker (91.9%), followed by P-cadherin (67.6%). CK14 and EGFR were the most specific markers, presenting 98.6% and 97.1% of specificity, respectively, and Vimentin was also shown to be very specific (86.9%).

Table 5 – Sensitivity, Specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of the immunohistochemical method for the basal markers studied to discriminate basal-like carcinomas.

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
CK5	91.9	84.3	38.2	99.0
EGFR	29.7	97.1	52.4	92.9
P-cadherin	67.6	72.0	20.3	95.5
CK14	32.4	98.6	70.6	93.2
Vimentin	45.9	86.9	27.0	93.8

In order to find the best combination of basal markers with the ability to identify basal-like breast carcinomas, we have evaluated the most sensitive and the most specific markers in pairs (CK5, P-cadherin with CK14, EGFR or Vimentin). Since P-cadherin presented good sensitivity and specificity values, we have also evaluated its association with CK5 (Table 6). The statistical associations considered cases that were positive for both markers (+/+), positive for at least one marker (+/- or -/+) or negative for both (-/-).

Table 6 – Association between the expression of pairs of basal markers with basal-like and non-basal-like breast carcinomas.

		n	Basal n (%)	Non-basal n (%)	p value
CK5 / EGFR	+/+	11	8 (21.6)	3 (0.8)	< 0.0001
	At least one +	88	29 (78.4)	59 (16.9)	
	-/-	288	0 (0)	288 (82.3)	
CK5 / CK14	+/+	11	11 (29.7)	0 (0)	< 0.0001
	At least one +	83	23 (62.2)	60 (17.1)	
	-/-	293	3 (8.1)	290 (82.9)	
CK5 / Vimentin	+/+	24	16 (43.2)	8 (2.3)	< 0.0001
	At least one +	104	19 (51.4)	85 (24.3)	
	-/-	259	2 (5.4)	257 (73.4)	
P-cadherin / EGFR	+/+	13	8 (21.6)	5 (1.4)	< 0.0001
	At least one +	118	20 (54.1)	98 (28.0)	
	-/-	256	9 (24.3)	247 (70.6)	
P-cadherin / CK14	+/+	12	9 (24.3)	3 (0.9)	< 0.0001
	At least one +	116	19 (51.4)	97 (27.7)	
	-/-	259	9 (24.3)	250 (71.4)	
P-cadherin / Vimentin	+/+	41	11 (29.7)	30 (8.6)	< 0.0001
	At least one +	104	20 (54.1)	84 (24)	
	-/-	242	6 (16.2)	236 (67.4)	
P-cadherin / CK5	+/+	38	23 (62.2)	15 (4.3)	< 0.0001
	At least one +	136	13 (35.1)	123 (35.1)	
	-/-	213	1 (2.7)	212 (60.6)	

Table 7 shows the percentages of sensitivity, specificity, positive predictive value and negative predictive value for the several pairs of markers. In these analyses, we considered as true positive the cases that were +/+ and positive for at least one of the markers in the subgroup of basal-like breast carcinomas previously distinguished by the criterion of Nielsen, and as false positive the cases that were positive for the two markers and the ones expressing at least one marker in non-basal-like tumours. True negative and false negative were the -/- cases in non-basal-like and in basal-like breast carcinomas, respectively. All the associations were statistically significant ($p < 0.0001$). The pair CK5/EGFR presented, as expected, the highest values of sensitivity and specificity, 100% and 82.3%, respectively. However, concerning sensitivity, the pairs CK5 / CK14, P-cadherin / CK5 and CK5 / Vimentin showed similar values to the "gold standard" CK5 / EGFR pair, with 91.9%, 97.3% and 94.6% of sensitivity, respectively. The specificity of CK5 / CK14 combination (82.9%) was approximately equal to the one presented by CK5 / EGFR (82.3%).

Table 7 – Sensitivity, specificity, PPV and NPV of the immunohistochemical method for the pairs of basal markers studied to discriminate basal-like carcinomas.

Pairs	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
CK5 / EGFR	100	82.3	11.4	100
CK5 / CK14	91.9	82.9	10.5	99
CK5 / Vimentin	94.6	73.4	12.0	99.2
P-cadherin / EGFR	75.7	70.6	10.2	96.5
P-cadherin / CK14	75.7	71.4	10.1	96.5
P-cadherin / Vimentin	83.8	67.4	11.6	97.5
P-cadherin / CK5	97.3	60.6	14.5	99.5

In the basal-like breast carcinomas group, when analysing the number of cases that were +/+ and

positive for at least one of the markers of the pair, against the -/- cases, it is possible to observe that only one basal-like breast carcinoma was negative for both markers in the P-cadherin / CK5 pair (Table 8). The CK5 / Vimentin pair missed the expression in 2 cases, while CK5 / CK14 did not stain three basal-like breast carcinomas. All the other pairs were positive in basal-like breast carcinomas for the two markers, or for at least one of them, in at least 75.7% of breast carcinomas with basal-like phenotype.

Table 8 – Analysis of the distribution of expression of the pairs of markers in basal-like breast carcinomas.

Pairs		Basal-like n (%)
CK5 / EGFR	+/+ and at least one +	37 (100)
	-/-	0 (0)
CK5 / CK14	+/+ and at least one +	34 (91.9)
	-/-	3 (9.1)
CK5 / Vimentin	+/+ and at least one +	35 (94.6)
	-/-	2 (5.4)
P-cadherin / EGFR	+/+ and at least one +	28 (75.7)
	-/-	9 (24.3)
P-cadherin / CK14	+/+ and at least one +	28 (75.7)
	-/-	9 (24.3)
P-cadherin / Vimentin	+/+ and at least one +	31 (83.8)
	-/-	6 (16.2)
P-cadherin / CK5	+/+ and at least one +	36 (97.3)
	-/-	1 (2.7)

More importantly, given the sensitivity of P-cadherin and the specificity of CK14 and Vimentin, we have also analysed their expression among the triple-negative / CK5- and EGFR-negative tumours ("unclassified" by the criterion of Nielsen). In 38/72 (52.8%) cases, none of the biomarkers was expressed; however, in the other 34/72 cases (47.2%), there was the expression of, at least, one of the biomarkers. P-cadherin was present in 29 (40.3%), Vimentin in 18 (25%) and CK14 in 5 (6.9%) of these tumours (Table 9). In a more detailed analysis, 15 cases were positive only for P-cadherin, while only one and three cases were positive for CK14 and for Vimentin alone, respectively (Figure 9).

Table 9 – Expression of P-cadherin, Vimentin and CK14 in the 72 triple-negative tumours also negative for CK5 and EGFR.

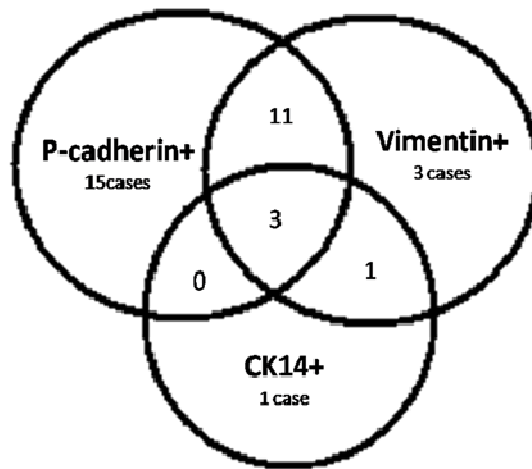|		Triple-negative / CK5- and EGFR-negative (n=72) (%)
P-cadherin	+	29 (40.3)
	-	43 (59.7)
Vimentin	+	18 (25.0)
	-	54 (75.0)
CK14	+	5 (6.9)
	-	67 (93.1)

Figure 9 – Distribution of P-cadherin, Vimentin and CK14 expression in triple-negative tumours that were negative for CK5 and EGFR.

Interestingly, if we consider as basal-like breast carcinomas these triple-negative / CK5- and EGFR-negative "unclassified" cases that presented immunoreactivity for P-cadherin, CK14 and/or Vimentin [basal-like breast carcinomas (P-cadherin- and/or CK14- and/or Vimentin-positive)], this series presents 71/387 (18%) of basal-like breast carcinomas. Basal-like breast carcinomas defined by triple-negative / CK5- and/or EGFR-positive and basal-like breast carcinomas defined as triple-negative, CK5- / EGFR-negative and immunoreactivity for P-cadherin and/or CK14 and/or Vimentin were analysed separately. These two differently defined basal-like breast carcinomas presented a similar percentage of high histological grade tumours [56% and 47% in basal-like breast carcinomas (CK5- and/or EGFR-positive) and in basal-like breast carcinomas (P-cadherin- and/or CK14- and/or Vimentin-positive), respectively], (Table 10).

Table 10 – Distribution of histological grade among triple-negative breast carcinomas of the studied series.

Triple-negative tumours	Histological grade		
	I	II	III
Basal-like breast carcinomas (CK5- and/or EGFR-positive) (n=34*)	3 (9%)	12 (35%)	19 (56%)
Basal-like breast carcinomas (P-cadherin- and/or CK14- and/or Vimentin-positive) (n=32*)	2 (6%)	15 (47%)	15 (47%)
Unclassified (triple-negative,CK5-, EGFR-, P-cadherin-, CK14- and Vimentin-negative) (n=37)	17 (46%)	15 (40%)	5 (14%)

Basal-like breast carcinomas (CK5- and/or EGFR-positive) are the triple-negative tumours that were positive for CK5 and/or EGFR and basal-like breast carcinomas (P-cadherin- and/or CK14- and/or Vimentin-positive) are the triple-negative / CK5- and EGFR-negative tumours immunoreactive for one of the additional markers in study: P-cadherin, CK14 and Vimentin.

*: Histological grade of some cases could not be assessed because the patients were submitted to preoperative chemotherapy.

We have also looked for differences in the overall survival of these groups of tumours: the overall survival was similar for the two groups as it can be seen in Figure 10.
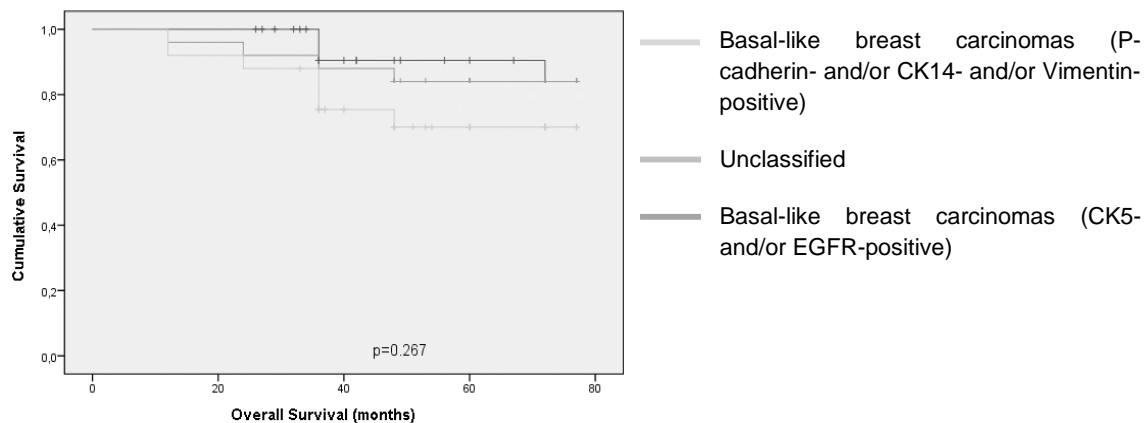
Figure 10 – Kaplan-Meier survival curves for overall survival of the triple-negative breast carcinoma patients' cohort, with a 77 months cut-off. Basal-like breast carcinomas defined by triple-negative / CK5- and/or EGFR-positive [Basal-like breast carcinomas (CK5- and/or EGFR-positive)], Basal-like breast carcinomas defined as triple-negative, CK5- / EGFR-negative and immunoreactivity for P-cadherin and/or CK14 and/or Vimentin [Basal-like breast carcinomas (P-cad- and/or CK14- and/or Vimentin-positive)] and tumours that were negative for all the basal markers in study were analysed (Unclassified), p=0.267 (not statistically significant).

Discussion

The need for a more precise diagnosis of breast cancer that converges with the clinical outcome and the choice of the most appropriate therapy has motivated studies in different areas of breast cancer research. The cDNA microarray technology is a "gold standard" method for the recognition of the basal-like phenotype, but from a practical point of view, we need to translate these results to an accessible method. It is undeniable that the basal-like breast carcinomas immunohistochemistry definition requires cDNA microarray validation, since these tumours were first identified by this technique [30, 59]. However, from the pathologists and oncologists point of view, the lack of molecular targets for therapy in this subgroup of patients indicates the urgent need for an easier and less expensive way to identify basal-like breast carcinoma patients. Based on this, there is an attempt to establish an immunohistochemical surrogate panel, easily applied on formalin-fixed paraffin-

embedded samples, which identifies a pool of breast cancer patients who may require more aggressive systemic therapy and that would be the most appropriate subjects for clinical trials, specifically targeting this molecular subgroup of breast cancer. However, there is still no consensual definition about the ideal immunohstochemical panel of biomarkers to distinguish the basal-like phenotype. In fact, many different panels have been used, in which CK5, EGFR, P-cadherin, CK14 and Vimentin are included. Due to this diversity of criteria, a wide range of percentages of basal-like breast carcinomas are described in the several studied series [62, 63, 65, 251, 256, 257, 261-275]. Nielsen *et al.* (2004) have demonstrated that CK5 and EGFR could reliably discriminate basal-like breast carcinomas that were identified by gene expression profiling, considering these two basal markers the "gold standard" immunohistochemical panel of antibodies to the identification of basal-like breast carcinomas, together with ER and HER2 lack of expression [61]. Recently, Cheang *et al.* (2008) have compared two basal-like breast carcinomas immuno-panels and concluded that the ER-/PR-/HER2- and expression of CK5 and/or EGFR provides the more accurate definition of basal-like breast carcinomas and can better predict breast cancer patients survival [253].

However, we cannot assure which are the best antibodies to be included in a daily practise panel for the recognition of the basal-like phenotype in breast carcinomas: should we look for the most sensitive or the most specific ones? None of these markers are actually pathognomonic of a basal-like phenotype, since they are variably expressed in the other subgroups of breast carcinomas, which support the search for "ideal" biomarkers to be used in the anatomic pathology workup and with clinical relevance.

We demonstrate herein that P-cadherin, Vimentin or CK14 may possibly be useful biomarkers to include in immunohistochemical panels for distinguishing basal-like breast carcinomas. P-cadherin reveals consistent values of sensitivity and specificity, while Vimentin and CK14 presented high specificity values. The three markers were able to reliably recognise the basal-like phenotype, especially when associated to CK5.

The presence of P-cadherin, an adhesion molecule expressed in myoepithelial cells of the normal mammary gland, was already described in invasive and in *in situ* breast carcinomas with worst prognosis, namely in those with high histological grade and basal phenotype [245, 276-279]. The role of P-cadherin in breast carcinogenesis has been one of the main fields of interest of our research group and we have observed that this molecule presents an inverse correlation with hormone receptors [276-279] and a direct correlation with EGFR [277], HER2 and high proliferation rates, strengthening the value of P-cadherin as a poor prognostic indicator in breast cancer [276, 278-280]. The expression of P-cadherin in neoplastic cells has already been related to a histogenetic origin in cap cells or to the acquisition of a stem cell-like phenotype, suggesting that P-cadherin-expressing tumours could be associated to a stem cell origin [276, 279, 281]. Recently, it has been suggested that basal-like breast carcinomas may be genuine stem / early progenitor cell tumours of the mammary gland, relating their origin to a more undifferentiated type of precursor cells [282]. Also, Rakha *et al.* (2009) have demonstrated more evidence of the features of dual-lineage differentiation / stem cell phenotype of basal-like breast carcinomas by showing a higher frequency of CK19 expression in this type of tumour [283].

CK14 does not show a differential presence in breast carcinomas with basal-like phenotype identified by cDNA microarray technology, but this cytokeratin is frequently associated with poor prognosis [267] and with the morphological features observed in basal-like breast carcinomas [284]. For this reason, CK14 has been included in the immunopanel used to identify basal-like breast carcinomas by several other authors [55, 63, 251, 260, 270].

Vimentin is an intermediate filament protein whose expression in normal mammary gland is also restricted to the myoepithelial / basal layer. Its expression has been associated with high histological grade, lack of ER, p53 mutations, high proliferation rates [285-290] and expression of CK5/6 and EGFR [291, 292]. Vimentin-expressing carcinomas have been observed in association with sporadic

and familial basal-like breast carcinomas and with a specific pattern of metastasis similar to basal-like breast carcinomas [274]. Like P-cadherin, Vimentin was also described to be differentially expressed by basal-like breast carcinomas identified by gene expression profiles, being proposed to integrate the panel of antibodies for the identification of basal-like breast carcinomas [59].

Our results show that P-cadherin, CK14 and Vimentin, together with CK5, can identify almost all basal-like breast carcinomas that were classified as such using the most widely accepted immunohistochemical panel to classify basal-like breast carcinomas: ER-/PR-/HER2-negativity and CK5- and/or EGFR-positivity.

Triple-negative phenotype by immunohistochemistry is one of the characteristic features of basal-like breast carcinomas and several authors claim that basal-like tumours are almost all triple-negative tumours [254, 293]. Kreike *et al.* (2007), in a series of 97 triple-negative cases, has observed that 90% of these tumours have a basal-like phenotype by cDNA microarray analysis [254]. However, the lack of expression of ER, PgR and HER2 as the sole criterion to identify these tumours is risky [294] because there are technical limitations when dealing with formalin-fixed paraffin-embedded tissue samples, which reinforces the need for a more suitable panel.

There is a significant overlapping of features shared by triple-negative and basal-like breast carcinomas in what concerns, for example, the prevalence of these types of cancer in younger patients, in African-American women [295], their presentation as interval cancers, a similar pattern of recurrence [296, 297], the more aggressive behaviour comparing with other types of breast cancer [52] and the biological and clinical similarity between sporadic triple-negative and basal-like breast tumours with breast carcinomas arising from BRCA1 mutation carriers [52]. However, several studies claim that this overlap is not complete [298, 299]. It is known that triple-negative carcinomas with a basal-like phenotype have a significant shorter disease-free survival than triple-negative carcinomas without expression of basal markers [62, 297] and that germline BRCA1 mutation carriers are more

probably found in triple-negative tumours expressing CK5/6 and/or EGFR than in triple-negative carcinomas with no expression of these basal markers [283, 300]. It has also been observed in gene expression profiles that the triple-negative group is composed by other subgroups of tumours with different outcomes, namely the normal breast-like tumours [30, 31, 40, 44, 46, 247, 301] and a recently described subgroup of claudin-low tumours [46, 51]. The existence of triple-negative tumours that do not react immunohistochemically with any of the basal markers routinely used has been described, and variably designated as non-basal triple-negative, unclassified, undetermined, null phenotype [302] or TN3BKE- (Triple Negative 3 Basal Keratins- and EGFR-negative) [283]. It seems extremely important to distinguish basal-like breast carcinomas from the whole triple-negative group, reducing the triple-negative heterogeneity, since their biological behaviour appears to be different. The elucidation of this heterogeneity would enable patients to benefit from their differential recognition [52, 62, 283, 294, 299, 302, 303]. This distinction is also important because triple-negative tumours defined by immunohistochemistry tend to be clinically considered as basal-like breast carcinomas and selected for clinical trials [298], probably misleading the effect of the drugs in the clinical trials.

It is interesting to emphasise that among the analysed triple-negative / CK5- and EGFR-negative tumours that were also negative for P-cadherin, CK14 and Vimentin, approximately 50% of these cases presented low histological grade. P-cadherin was expressed alone in a higher number (15 cases) of triple-negative / CK5- and EGFR-negative tumours, compared with CK14 (1 case) and Vimentin (3 cases). When P-cadherin, CK14 and Vimentin expression are considered along with CK5 and EGFR for the basal-like breast carcinomas identification, 34 cases are added to the 37 already identified basal-like breast carcinomas (CK5- and/or EGFR-positive) and the percentage of basal-like tumours in the pool of triple-negative cases of our series rounds 65% (71/109). This rate is similar to the one identified by Bertucci [298], where 70% of immunohistochemical triple-negative tumours presented a basal-like phenotype by gene expression profiles. It is worth noticing that using P-cadherin, CK14 and Vimentin to recruit basal-like breast carcinomas from the pool of tumours that could not be classified using only CK5 and EGFR as basal-like makers, these newly identified basal-

like breast carcinomas are clinically similar to the basal-like tumours identified by the criterion of Nielsen, since the majority of the cases presented high histological grade and there are no significant differences in what concerns overall survival of the patients.

Although CK5 and EGFR have been consistently used to recognise basal-like breast carcinomas, P-cadherin, CK14 and Vimentin could also be recruited for an immunohistochemical recognition of basal-like breast carcinomas [59, 60, 245, 246, 274, 281]. Our results showed that these three markers can reliably identify the basal-like phenotype, especially when associated to CK5, and can be alternative options in this setting. We also demonstrate that P-cadherin, due to its high sensitivity, can recognise possible basal-like breast carcinomas among the immunohistochemical triple-negative tumours, probably identifying patients with poor prognosis that can benefit from this differential recognition. Pathologists have faced continuous changes in the diagnostic approach of breast cancer and, regarding its classification, it is still controversial whether or not the histological classification should be replaced by the "molecular" taxonomy. Therefore, it is essential to move towards a standardised methodology to establish an immunohistochemical panel of biomarkers to the most appropriate recognition of basal-like breast carcinomas.

2. Expression of $1\alpha,25(OH)_2D_3$ main partners (VDR, CYP27B1 and CYP24A1) in normal and neoplastic breast tissue

Paper related with this chapter:

Lopes N, Sousa B, Martins D, Gomes M, Vieira D, Veronese LA, Milanezi F, Paredes J, Costa JL, Schmitt F. Alterations in Vitamin D signalling and metabolic pathways in breast cancer progression: a study of VDR, CYP27B1 and CYP24A1 expression in benign and malignant breast lesions. *BMC Cancer*. 10:483-492, 2010.

Introduction

Breast cancer is one of the major causes of death by cancer in women worldwide [304]. Ultimately, cancer results from alterations in the control of the complex balance of proliferation, differentiation and programmed cell death [305] and these processes appear to be regulated by intrinsic and extrinsic factors, like niche signals, hormonal and dietary aspects, among others [306, 307].

Vitamin D ($1\alpha,25(OH)_2D_3$) is a lipid soluble substance that belongs to the family of secosteroid hormones. Apart from its role in bone metabolism, subsequent studies have widened the range of functions for $1\alpha,25(OH)_2D_3$ in the field of cancer research. Specifically, it has been demonstrated the capacity to modulate cancer features, namely proliferation and differentiation, apoptosis, angiogenesis, invasion and metastasis [77, 165, 173, 308].

$1\alpha,25(OH)_2D_3$ exerts most of its biological activities by binding to a specific high-affinity receptor, the VDR, which regulates gene expression by acting as a ligand-activated transcription factor [83]. Because the VDR is expressed in the mammary gland and $1\alpha,25(OH)_2D_3$ has been shown to display anticarcinogenic properties, this hormone has emerged as a promising targeted therapy. But, in order to keep the homeostasis of the organism, the amount of circulating $1\alpha,25(OH)_2D_3$ has to be tightly regulated. This is a very complex process, in which the main components are the enzymes CYP27B1 (mediates the synthesis of $1\alpha,25(OH)_2D_3$) and CYP24A1 (is responsible for the catabolism of $1\alpha,25(OH)_2D_3$) [74]. It has been observed that both CYP27B1 and CYP24A1 are upregulated in breast tumours when compared with normal tissue. However, deregulated expression of CYP24A1 seems to abrogate the effects of CYP27B1, resulting in the degradation of $1\alpha,25(OH)_2D_3$ to less active metabolites [109]. In contrast, a recent paper has demonstrated that CYP27B1 mRNA in breast tumours is decreased in comparison with normal mammary tissue [108].

The main purpose of this work was to perform an immunohistochemical study of the expression of the

VDR, CYP27B1 and CYP24A1 in a comprehensive series of human breast tissues comprised of normal breast, benign mammary lesions, carcinomas *in situ* and invasive breast carcinomas.

Results

<u>VDR, CYP27B1 and CYP24A1 immunohistochemical staining</u>

The expression patterns of the VDR, CYP27B1 and CYP24A1 have been evaluated by immunohistochemistry in 947 breast tissue samples arranged in 79 TMAs. From this set of cases, some samples could not be assessed due to the fact that either the core had fallen out or it did not have enough biological material to study.

In all TMAs, positive and negative cases were obtained for each protein. The immunostainings for these markers had been previously validated in whole tissue sections with an overall agreement of 90%. A panel with representative immunostainings for each protein in different breast tissues is shown in Figure 11. We have observed that the VDR displays nuclear staining, as would be expected from a nuclear receptor which acts as a transcription factor. Considering CYP27B1 and CYP24A1 expression, nothing has ever been described on their expression status in the mammary gland, as far as we know. This is the first report showing the expression of these two enzymes in breast lesions. These proteins present cytoplasmic and granular staining, which could reflect their mitochondrial localisation. All proteins (VDR, CYP27B1 and CYP24A1) have been found to be expressed in all lesions studied and also in the normal breast tissue, although at different levels.
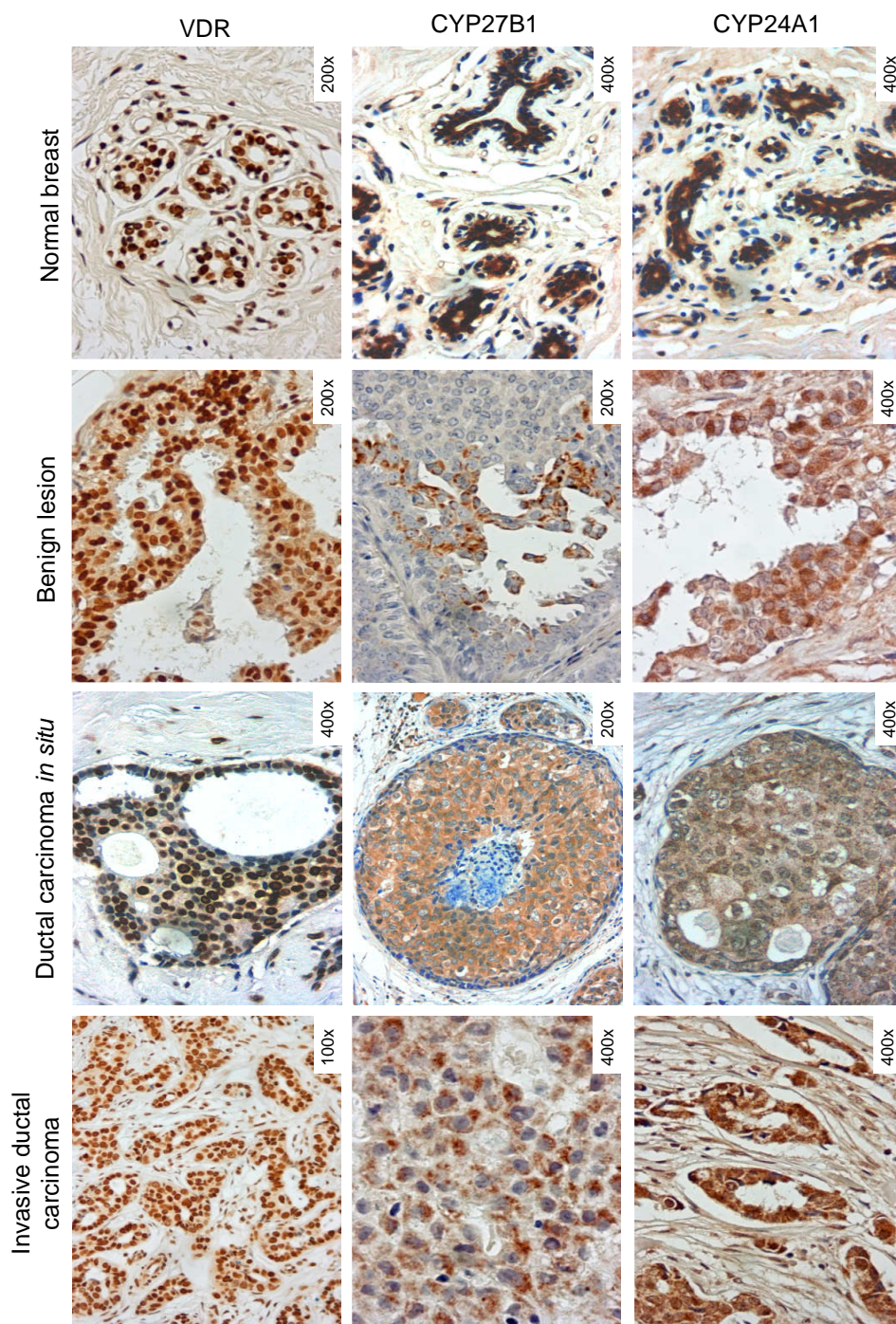
Figure 11 – Immunohistochemical staining for the VDR, CYP27B1 and CYP24A1 in the different types of breast tissue.

Expression of the VDR, CYP27B1 and CYP24A1 in benign lesions of the mammary gland

In order to study the VDR, CYP27B1 and CYP24A1 expression in benign lesions of the mammary gland, we have evaluated 379 cases arranged in 17 TMAs. The series consisted of a variety of breast lesions, namely usual and atypical ductal hyperplasias (UDH represent 20.1%, corresponding to 76 samples; while ADH represent 5.4%, corresponding to 21 samples), columnar cell lesions (CCL – 25.6% of cases, corresponding to 97 samples), papillomatosis (16.9% of cases, corresponding to 64 samples) and adenosis (17.2% of cases, corresponding to 65 samples). The percentage of immunoreactive cases for the VDR was very high (93.5%, corresponding to 259 cases out of 277). Regarding the expression of CYP27B1, we have observed 55.8% of positive cases, corresponding to 173 lesions out of 310. Concerning CYP24A1 expression, we have detected 62 positive cases out of 327 samples (19.0%). Amongst all lesions, ADH cases were overall less immunoreactive to the three proteins. We have correlated the histological classification of the benign lesions with the VDR, CYP27B1 and CYP24A1 expression, but no significant associations have been found (see Table 11 for further details).

Table 11 – VDR, CYP27B1 and CYP24A1 expression in the various types of benign breast lesions (ns – not significant).

	VDR		CYP27B1		CYP24A1	
	+ (%)	- (%)	+ (%)	- (%)	+ (%)	- (%)
Usual ductal hyperplasia	84 (92.3)	7 (7.7)	57 (55.9)	45 (44.1)	23 (20.5)	89 (79.5)
Atypical ductal hyperplasia	9 (81.8)	2 (18.2)	4 (36.4)	7 (63.6)	1 (7.1)	13 (92.9)
Columnar cell lesions	63 (95.5)	3 (4.5)	43 (55.8)	34 (44.2)	13 (16.5)	66 (83.5)
Papillomatosis	45 (95.7)	2 (4.3)	30 (56.6)	23 (43.4)	9 (17.0)	44 (83.0)
Adenosis	49 (92.5)	4 (7.5)	32 (55.2)	26 (44.8)	13 (22.0)	46 (78.0)
p value	**ns**		**ns**		**ns**	

Expression of the VDR, CYP27B1 and CYP24A1 in breast ductal carcinomas *in situ*

A fully characterised series of 189 breast ductal carcinomas *in situ* (DCIS) arranged in 22 TMAs was assessed for the expression patterns of VDR, CYP27B1 and CYP24A1. For the VDR, we have observed that 62 cases out of 131 cases (47.3%) displayed staining for this protein. Concerning CYP27B1 expression, we have encountered positive staining in 66.4% of the cases (91 out of 137 samples); whereas CYP24A1 expression was observed in 56.0% of the tumours (70 out of 125 cases).

We have also assessed the expression of other breast cancer biomarkers in our cohort (ER, HER2 and PgR and basal markers as defined by our group [60] and others [61]) and looked for the existence of correlations between the expression of the $1\alpha,25(OH)_2D_3$ partners and these molecular markers (Table 12). ER expression has been observed in 117 cases (61.9%), HER2 protein was present in 37 cases (15.6%) and PgR expression was detected in 90 cases (47.6%). We have also tested our series for basal markers and have obtained the following results: EGFR expression is present in 10 cases (5.3%), CK5 is positive in 15 cases (7.9%) and P-cadherin was observed in 36 samples (19.0%). Expression of the VDR correlated positively with ER status (p = 0.0227), with a higher percentage of VDR-positive cases among the ER-positive tumours – 74.2% (46 out of 62 cases). Additionally, we have seen that there is an inverse correlation between the expression of the VDR and P-cadherin (p = 0.0078). CYP27B1 expression only presented an inverse correlation (p = 0.0295) with EGFR expression, but the number of cases positive for EGFR was very low. No statistically significant associations have been observed between CYP24A1 expression and the markers studied.

Table 12 – VDR, CYP27B1 and CYP24A1 and other breast cancer biomarkers expression in ductal carcinomas *in situ* (ns – not significant).

		VDR		CYP27B1		CYP24A1	
		+ (%)	- (%)	+ (%)	- (%)	+ (%)	- (%)
ER	+ (%)	46 (35.1)	38 (29.0)	58 (42.3)	29 (21.2)	41 (32.8)	36 (28.8)
	- (%)	16 (12.2)	31 (23.7)	33 (24.1)	17 (12.4)	29 (23.2)	19 (15.2)
	p value	0.0227		ns		ns	
HER2	+ (%)	9 (6.9)	14 (10.7)	18 (13.1)	7 (5.1)	9 (7.2)	12 (9.6)
	- (%)	53 (40.5)	55 (42.0)	73 (53.3)	39 (28.5)	61 (48.8)	43 (34.4)
	p value	ns		ns		ns	
PgR	+ (%)	35 (26.7)	30 (22.9)	49 (35.8)	18 (13.1)	38 (30.4)	22 (17.6)
	- (%)	27 (20.6)	39 (29.8)	42 (30.7)	28 (20.4)	32 (25.6)	33 (26.4)
	p value	ns		ns		ns	
CK5	+ (%)	3 (2.3)	8 (6.1)	7 (5.1)	4 (2.9)	8 (6.4)	4 (3.2)
	- (%)	59 (45.0)	61 (46.6)	84 (61.3)	42 (30.7)	62 (49.6)	51 (40.8)
	p value	ns		ns		ns	
EGFR	+ (%)	1 (0.8)	5 (3.8)	2 (1.5)	5 (3.7)	5 (4.0)	3 (2.4)
	- (%)	61 (46.6)	64 (48.9)	89 (65.0)	41 (29.9)	65 (52.0)	52 (41.6)
	p value	ns		0.0295		ns	
P-cad	+ (%)	4 (3.1)	16 (12.2)	14 (10.2)	12 (8.8)	16 (12.8)	7 (5.6)
	- (%)	58 (44.3)	53 (40.5)	77 (56.2)	34 (24.8)	54 (43.2)	48 (38.4)
	p value	0.0078		ns		ns	

The information concerning the histological grade of the *in situ* tumours of this series was available and has been correlated with the expression of VDR, CYP27B1 and CYP24A1 (Table 13). We have found no significant associations between the expression of the $1\alpha,25(OH)_2D_3$ related proteins and this clinicopathological feature.

Table 13 – VDR, CYP27B1 and CYP24A1 expression and the histological grade in ductal carcinomas *in situ* (ns – not significant, inter – intermediate).

		VDR		CYP27B1		CYP24A1	
		+ (%)	- (%)	+ (%)	- (%)	+ (%)	- (%)
Histological grade	high (%)	18 (13.7)	21 (16.0)	32 (23.4)	14 (10.2)	21 (16.8)	18 (14.4)
	inter (%)	21 (16.0)	33 (25.2)	31 (22.6)	20 (14.6)	25 (20.0)	20 (16.0)
	low (%)	20 (15.3)	18 (13.7)	28 (20.4)	12 (8.8)	24 (19.2)	17 (13.6)
	p value	ns		ns		ns	

Expression of the VDR, CYP27B1 and CYP24A1 in invasive mammary carcinomas

We have evaluated 350 cases of invasive breast carcinomas arranged in 40 TMAs. The cohort corresponds to 189 cases of the series for which there was an *in situ* component in the adjacent area of the invasive tumour and an additional series of 161 cases of invasive breast carcinomas. Positive staining for the VDR has been observed in 56.2% of the cases (172 out of 306 cases). Regarding CYP27B1 expression, 44.6% of cases were positive (123 out of 276 samples), whereas 53.7% of cases (151 out of 281 tumours) presented positivity for CYP24A1.

Next, we searched for associations between the expression of $1\alpha,25(OH)_2D_3$ partners and the expression of the molecular markers mentioned in the previous section (Table 14). We have obtained 197 cases (56.3%) positive for ER, 70 cases (20%) for HER2 and 143 cases (40.9%) for PgR. As for basal markers, we have observed that 13 cases (3.7%) were positive for EGFR expression, 48 cases (13.7%) presented positivity for CK5 and 93 cases (26.6%) stained for P-cadherin.

A statistically significant association was observed between the cases that are positive for VDR and positive cases for ER ($p = 0.0002$). Additionally, the cases that are positive for VDR have also been significantly correlated with the cases that are negative for HER 2 ($p = 0.0238$), but this is probably due to the low number of positive cases for HER2 in our series of mammary carcinomas. CYP27B1 expression presented no significant associations with any of the markers analysed. PgR was the only marker that displayed an inverse correlation with CYP24A1: specifically, cases positive for PgR were mostly negative for CYP24A1 ($p = 0.0485$).

Table 14 – VDR, CYP27B1 and CYP24A1 and other breast cancer biomarkers expression in invasive breast tumours (ns – not significant).

		VDR		CYP27B1		CYP24A1	
		+ (%)	- (%)	+ (%)	- (%)	+ (%)	- (%)
ER	+ (%)	114 (37.3)	60 (19.6)	70 (25.4)	86 (31.2)	93 (33.1)	66 (23.5)
	- (%)	58 (19.0)	74 (24.2)	53 (19.2)	67 (24.3)	58 (20.6)	64 (22.8)
	p value	**0.0002**		ns		ns	
HER2	+ (%)	26 (8.6)	34 (11.3)	31 (11.4)	25 (9.2)	29 (10.4)	30 (10.8)
	- (%)	144 (47.7)	98 (32.5)	90 (33.1)	126 (46.3)	121 (43.5)	98 (35.3)
	p value	**0.0238**		ns		ns	
PgR	+ (%)	71 (23.3)	59 (19.3)	52 (18.8)	64 (23.2)	71 (25.3)	46 (16.4)
	- (%)	100 (32.8)	75 (24.6)	71 (25.7)	89 (32.2)	80 (28.5)	84 (29.9)
	p value	ns		ns		**0.0485**	
CK5	+ (%)	27 (8.8)	19 (6.2)	15 (5.4)	24 (8.7)	27 (9.6)	16 (5.7)
	- (%)	145 (47.4)	115 (37.6)	108 (39.1)	129 (46.7)	124 (44.1)	114 (40.6)
	p value	ns		ns		ns	
EGFR	+ (%)	4 (1.3)	7 (2.3)	4 (1.5)	6 (2.2)	6 (2.1)	3 (1.1)
	- (%)	166 (54.8)	126 (41.6)	118 (43.1)	146 (53.3)	145 (51.8)	126 (45.0)
	p value	ns		ns		ns	
P-cad	+ (%)	42 (13.8)	40 (13.1)	30 (10.9)	42 (15.2)	40 (14.3)	37 (13.2)
	- (%)	129 (42.3)	94 (30.8)	93 (33.7)	111 (40.2)	110 (39.3)	93 (33.2)
	p value	ns		ns		ns	

Since the data regarding tumour size, histological grade and lymph node invasion was available in

our series, we also tested them for associations with the expression of VDR, CYP27B1 and CYP24A1, but we have not observed any significant correlations (Table 15).

Table 15 – VDR, CYP27B1 and CYP24A1 expression and some clinicopathological data in invasive carcinomas (ns – not significant).

		VDR		CYP27B1		CYP24A1	
		+ (%)	- (%)	+ (%)	- (%)	+ (%)	- (%)
Histological grade	I (%)	68 (23.1)	57 (19.3)	47 (17.8)	62 (23.5)	61 (22.4)	53 (19.5)
	II (%)	64 (21.7)	58 (19.7)	59 (22.3)	55 (20.8)	65 (23.9)	50 (18.4)
	III (%)	26 (8.8)	22 (7.5)	11 (4.2)	30 (11.4)	20 (7.4)	23 (8.5)
	p value	ns		ns		ns	
Tumour size	Average size (cm)	3.25	3.99	3.25	3.62	3.48	3.47
	p value	ns		ns		ns	
Lymph node invasion	Yes	61 (51.7)	24 (20.3)	26 (25.2)	49 (47.6)	44 (39.6)	35 (31.5)
	No	22 (18.6)	11 (9.3)	11 (10.7)	17 (16.5)	14 (12.6)	18 (16.2)
	p value	ns		ns		ns	

The series of 189 tumours with both components (carcinomas *in situ* and the corresponding invasive tumour) allowed the evaluation of the expression of the VDR, CYP27B1 and CYP24A1 simultaneously in the two types of tumours (Table 16). The results obtained show that the three proteins (VDR, CYP27B1 and CYP24A1) display a statistically significant correlation of expression between the two sections (carcinomas *in situ* and the matching invasive tumour). Thus, positive cases in the *in situ* component are also positive in the invasive component and the same is observed for the negative cases.

Table 16 – VDR, CYP27B1 and CYP24A1 expression in tumours that display both the *in situ* and the invasive component in the same histological section.

Invasive component			*In situ* component								
			VDR		**CYP27B1**			**CYP24A1**			
			+ (%)	- (%)			+ (%)	- (%)			+ (%)	- (%)
	VDR	+ (%)	39 (35.1)	5 (4.5)	**CYP27B1**	+ (%)	44 (39.6)	10 (9.0)	**CYP24A1**	+ (%)	42 (44.2)	8 (8.4)
		- (%)	10 (9.0)	57 (51.4)		- (%)	27 (24.3)	30 (27.0)		- (%)	11 (11.6)	34 (35.8)
p value			< 0.001			0.002				< 0.001		

Expression of the VDR, CYP27B1 and CYP24A1 according to the type of breast lesion

The frequencies of protein expression of the VDR, CYP27B1 and CYP24A1 in the different mammary tissues are shown in Figure 12. The normal mammary gland, as expected, is positive for the expression of the VDR in all cases studied (100%). The majority of the samples also displays immunostaining for CYP27B1 (63.6%) and, in contrast, the levels of expression of CYP24A1 are low (29.6%). The VDR is also highly expressed in benign lesions (93.5%) with a reduction in the percentage of positive cases in DCIS (47.3%) and in invasive carcinomas (56.2%). CYP27B1 expression does not vary greatly between the different breast lesions. It is observed that the positive cases decrease from 66.4% in carcinomas *in situ* to 44.6% in invasive carcinomas, while approximately half of the benign lesions (55.8%) present staining for CYP27B1. In contrast, the expression of CYP24A1 is increased in carcinomas (56.0% in DCIS and 53.7% in invasive carcinomas) compared with the benign lesions (19.0%), which are mostly negative.
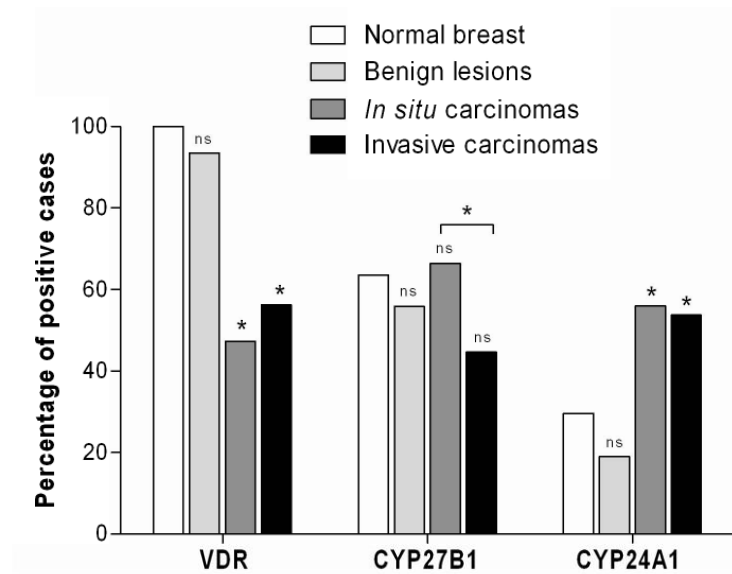
Figure 12 – Percentage of positive cases for VDR, CYP27B1 and CYP24A1 expression in the various types of breast samples studied (Statistical analysis shown uses normal breast as reference. An additional result is presented comparing the number of CYP27B1 positive cases between *in situ* and invasive carcinomas [ns – not significant; * $p < 0.05$]).

Validation of the immunohistochemistry results for CYP27B1 and CYP24A1

The antibodies used for CYP27B1 and CYP24A1 had not been previously described to assess immunohistochemical results, so we have validated their use for this technique, using western blotting (for which they were designed). As presented in Figure 13, the treatment with $1\alpha,25(OH)_2D_3$ induces an increase in the expression of CYP24A1 and the treatment with PTH upregulates CYP27B1 expression in MDA-MB-231 breast cancer cells. We have demonstrated that these antibodies recognise the respective proteins by western blotting, thereby validating our results by immunohistochemistry.
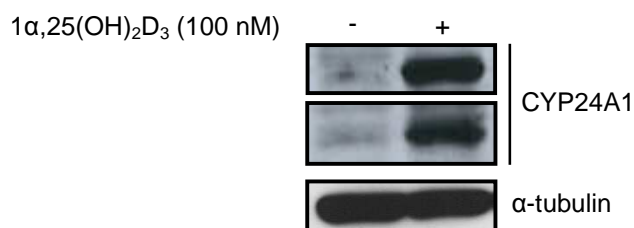
1α,25(OH)$_2$D$_3$ (100 nM)   -   +

CYP24A1

α-tubulin
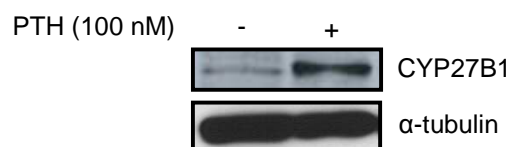
Figure 13 – CYP27B1 and CYP24A1 expression is upregulated in MDA-MB-231 breast cancer cells, upon treatment with PTH and 1α,25(OH)$_2$D$_3$, respectively.

Discussion

1α,25(OH)$_2$D$_3$ mediates anti-proliferative and pro-differentiation signalling in various epithelial tissues, including the mammary gland [77]. Therefore, it is reasonable to assume that disruption of 1α,25(OH)$_2$D$_3$ signalling and metabolic pathways may occur during tumour development. To explore this hypothesis, we have evaluated a cohort of 947 samples of human breast tissues for the presence of VDR, CYP27B1 and CYP24A1. Specifically, our series consisted of normal breast tissue (29 cases), preneoplastic benign mammary lesions (379 cases), ductal carcinomas *in situ* (189 cases) and invasive breast carcinomas (350 cases). To the best of our knowledge, this is the first time that the expression of the VDR, CYP27B1 and CYP24A1 has been evaluated in histological sections of mammary lesions.

The three proteins have been found to be expressed in all breast tissues, although at different levels. VDR presented a nuclear localisation, as it would be expected for a nuclear receptor, while CYP27B1 and CYP24A1 enzymes displayed cytoplasmic staining with a granular pattern, which is consistent with their mitochondrial localisation. The immunohistochemical results were further validated and confirmed using western blotting.

Some studies have demonstrated that the VDR protein is expressed in samples from normal breast tissues [105] and also in breast cancer biopsy specimens [106]. Our results have shown that the VDR is expressed in carcinomas. However, the percentage of positive cases that we have obtained (47.3% in carcinomas *in situ* and 56.2% in invasive carcinomas) is lower than the 80% to 90% that had been previously described in the literature [309, 310]. This discrepancy can be explained by the development of new detection techniques and the use of different scoring methods. In this study, we have used the H-Score, the current method employed for other nuclear receptors, like ER [243], whereas in previous studies the presence of any staining was marked as positive. As far as we know, our study is the first to investigate the immunohistochemical expression of the VDR in a range of benign lesions and ductal carcinomas *in situ* of the mammary gland. The percentage of positive cases for the VDR is higher in benign lesions than in invasive tumours (93.5% and 56.2%, respectively), while the DCIS display the lowest value of all (47.3%) and this difference (between benign and malignant lesions) is significant ($p < 0.0001$). There are some studies showing higher levels of VDR in tumour tissues [108, 311], but this discrepancy can be attributed to the use of different evaluation techniques.

The most significant finding is the correlation between the expression of the VDR and the ER in both *in situ* and invasive carcinomas. In fact, a large number of cases co-express the ER and the VDR and this is not a surprising result, because both are nuclear steroid hormonal receptors [83]. It is thought that one of the VDR functions is to counteract oestrogen-mediated proliferation and maintain differentiation [142]. Indeed, data support the concept that the anti-tumour effects of $1\alpha,25(OH)_2D_3$ and its analogues on ER-positive human breast cancer cells are mediated through the downregulation of the ER itself and the attenuation of oestrogen responses, such as breast cancer cell growth [312, 313]. Thus, being the VDR mostly expressed in ER-positive carcinomas, $1\alpha,25(OH)_2D_3$ or its analogues may become an alternative therapy for these tumours in cases of resistance to ER-targeted therapy.

For the enzymes involved in $1\alpha,25(OH)_2D_3$ metabolism, as far as we know, no studies have been conducted to evaluate their expression in breast cancer tissue by immunohistochemistry. However, some authors have assessed the levels of protein expression of CYP27B1 and CYP24A1 in colon samples, using this methodology [314, 315]. They have observed that CYP27B1 is present at equally high levels in normal colonic epithelium and colorectal cancer, irrespective of differentiation [315]. For CYP24A1 it has been shown that increasing amounts of this enzyme are present in normal colon tissue and pre-malignant lesions. In cancer, the expression of CYP24A1 decreases as a function of tumour cell dedifferentiation [314]. Another report [108] has demonstrated that, in breast, CYP27B1 mRNA expression was significantly downregulated in adjacent non-cancerous tissue from women with breast cancer in comparison with individuals without cancer. Additionally, it has been shown that the expression of mRNA for CYP27B1 and the VDR was higher in carcinomas versus non-neoplastic tissue [109]. Considering differences in expression in the different breast tissue types (benign and malignant), we have observed an increased expression of CYP24A1 and a decreased expression of CYP27B1 with malignant progression. In fact, for CYP27B1 we have observed that 55.8% of the preneoplastic lesions express this protein and this percentage is decreased in invasive tumours (44.6%), while *in situ* carcinomas display the highest value (66.4%) and these differences are statistically significant. In contrast, CYP24A1 is augmented more than 2.5 fold in invasive tumours (53.7%), compared with benign breast lesions (19.0%) and this difference is significant ($p < 0.0001$). The *in situ* carcinomas exhibit the highest percentage of positive cases (56.0%). These observations are consistent with the results of Townsend and colleagues [109], which have demonstrated that there was an upregulation of CYP24A1 mRNA in breast tumour tissue, in comparison with normal breast. It has also been described that the *CYP24A1* gene is amplified in breast cancer [111]. In contrast, another study has found no differences in the expression of the VDR, CYP27B1 and CYP24A1 mRNA in breast cancer and non-neoplastic mammary tissue [113]. These contradictory results may be explained by recent reports where it is described that VDR and CYP24A1 are under the post-transcriptional control of miRNAs [316, 317]. In general, data available are conflicting and

one should be cautious when interpreting the results of the levels of mRNA of CYP27B1 and CYP24A1 as indicators of the tissue status. Instead, the protein levels should be employed. To further address this issue, the activity of the proteins together with the metabolite levels in the tissues should be measured. Although currently challenging, this must be evaluated in the future.

Breast cancer is a process that evolves through the accumulation of (epi)genetic events that drive uncontrolled proliferation and resistance to apoptosis. $1\alpha,25(OH)_2D_3$ is known for its capacity to modulate proliferation and induce apoptosis [77]. Consequently, malignant cells would need to develop mechanisms to deregulate $1\alpha,25(OH)_2D_3$ metabolic and signalling pathways in order to allow tumour development [115]. Furthermore, it has been suggested that the $1\alpha,25(OH)_2D_3$ produced in non-renal tissues is not released into the blood stream, but instead acts locally [318]. Therefore, the amount of $1\alpha,25(OH)_2D_3$ available in the tissue depends on the relative amounts of CYP27B1 (synthesis) and CYP24A1 (catabolism). Accordingly, our results show a deregulation of these two enzymes in the different stages of breast carcinogenesis. The crucial step of transformation introduces a clear unbalance in the $1\alpha,25(OH)_2D_3$ signalling and metabolic pathways. A reduction in the expression of the VDR in carcinomas indicates lower sensitivity of the tissue to $1\alpha,25(OH)_2D_3$ control. Furthermore, a strong increase in CYP24A1 positive cases points to an enhanced ability of the cells to degrade this hormone. In contrast, the stable levels of CYP27B1 throughout the transformation process, with only a small decrease in invasive carcinomas, may reflect a lower capacity to metabolise $1\alpha,25(OH)_2D_3$ into its active form.

3. Evaluation of the *in vitro* effects of 1α,25(OH)₂D₃ in triple-negative basal-like human breast cancer cell lines

Paper related with this chapter:

Introduction

Breast cancer is a heterogeneous disease, comprised of diverse molecular subtypes associated with different biological behaviours and clinical outcomes [30, 31]. Among all breast cancer subgroups, the triple-negative basal-like is the most aggressive type, presents poor patient outcome [31], and it comprises a rare cluster of carcinomas entitled metaplastic tumours [55, 319, 320]. Our group and others have demonstrated that metaplastic carcinomas are distinguished by high levels of expression of classical basal-like markers, such as CK5/6, CK14, EGFR, Vimentin and P-cadherin, as well as E-cadherin downregulation [55, 259, 321]. Furthermore, patients harbouring metaplastic tumours display worse prognosis, exhibiting lower rates of disease-free survival than those with invasive ductal carcinomas [322, 323]. Due to their triple-negative phenotype, metaplastic carcinomas do not have a directed therapy and thus, chemotherapy and radiation therapy remain the only options to treat this cluster of carcinomas. This has led to intensive research on alternative therapeutic strategies for these tumours.

$1\alpha,25(OH)_2D_3$ (the biologically active form of Vitamin D) is a steroid hormone that exerts most of its biological activities by binding to a specific high-affinity receptor, the Vitamin D Receptor (VDR) [82]. We have previously reported that about 56% of invasive breast carcinomas express VDR and, among these, 56% of the cases classified as triple-negative basal-like tumours are positive for VDR expression [324], suggesting that they may be responsive to the anti-carcinogenic properties of $1\alpha,25(OH)_2D_3$. In several cancer models, $1\alpha,25(OH)_2D_3$ was shown to participate in cell growth regulation and cell differentiation [77]. In breast cancer cells, it has been demonstrated that $1\alpha,25(OH)_2D_3$ is able to induce cells to be more adhesive to each other, as well as to some substrates, through an increase in the expression of endogenous E-cadherin and other adhesion molecules [167]. Additionally, $1\alpha,25(OH)_2D_3$ promotes the differentiation of colon cancer cells by inducing the expression of E-cadherin in VDR-expressing cells [182] and a similar result was obtained in prostate cancer cells with an analogue of $1\alpha,25(OH)_2D_3$ [325].

All these data provide good evidence on the ability of $1\alpha,25(OH)_2D_3$ as an epithelial differentiation-inducing agent. Therefore, our purpose was to study if VDR could be a potential therapeutic target for the metaplastic triple-negative breast carcinomas. Additionally, we aimed at evaluating the *in vitro* effects of $1\alpha,25(OH)_2D_3$ as a differentiating agent in triple-negative breast cancer cell lines.

Results

Metaplastic breast carcinomas are positive for VDR expression

We have evaluated a series of 12 metaplastic breast carcinomas for the expression of VDR and we have observed that 8 cases (66.7%) were positive for VDR expression (Figure 14). This finding suggests that some of these triple-negative basal-like tumours could be responsive to the treatment with $1\alpha,25(OH)_2D_3$.

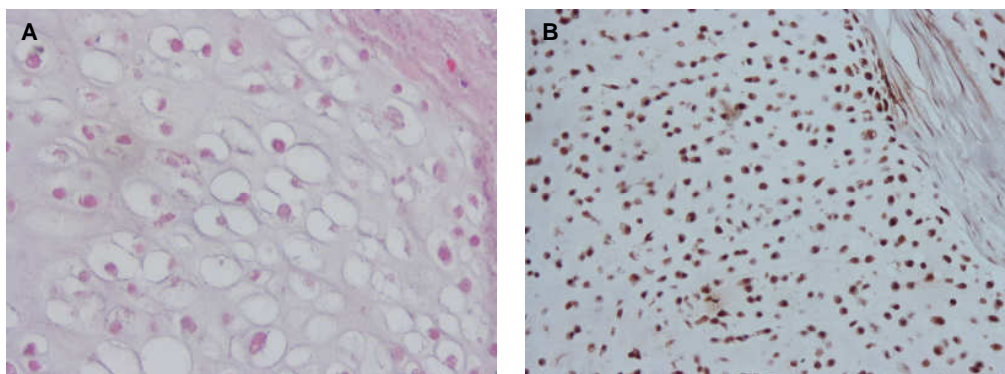**Figure 14** – H&E, magnification 630x (**A**) and VDR, magnification 400x (**B**) staining in a case of metaplastic breast carcinoma.

These 12 metaplastic breast carcinomas had been previously characterised for the expression of E-cadherin and Vimentin (Table 17). The majority of the cases were negative for E-cadherin expression (8 out of 12 samples, corresponding to 66.7%) and 83.3% of the tumours exhibited Vimentin expression (10 out of 12 cases). This finding shows that these tumours are indeed undifferentiated and that they could benefit from the differentiation-inducing properties of $1\alpha,25(OH)_2D_3$ treatment.

Table 17 – Immunohistochemical staining for VDR, E-cadherin and Vimentin in the metaplastic carcinomas.

Case	VDR	E-cadherin	Vimentin
1	Positive	Negative	Positive
2	Positive	Positive	Negative
3	Positive	Negative	Positive
4	Negative	Negative	Positive
5	Positive	Negative	Positive
6	Positive	Negative	Positive
7	Negative	Negative	Positive
8	Negative	Negative	Positive
9	Positive	Negative	Positive
10	Negative	Positive	Positive
11	Positive	Positive	Positive
12	Positive	Positive	Negative

Triple-negative breast cancer cell lines are positive for VDR expression

In order to find out if $1\alpha,25(OH)_2D_3$ could indeed be a possible therapy for metaplastic breast

carcinomas, as well as for other triple-negative basal-like breast tumours, we decided to check if mesenchymal triple-negative breast cancer cell lines (MDA-MB-231, Hs578T and BT-549) were positive for VDR expression by Western blotting. We have observed that all three cell lines studied were positive for VDR expression. MDA-MB-231 cells and BT-549 seem to be more sensitive to $1\alpha,25(OH)_2D_3$, as in these cells there was a clear increase in VDR expression upon hormonal treatment (Figure 15).
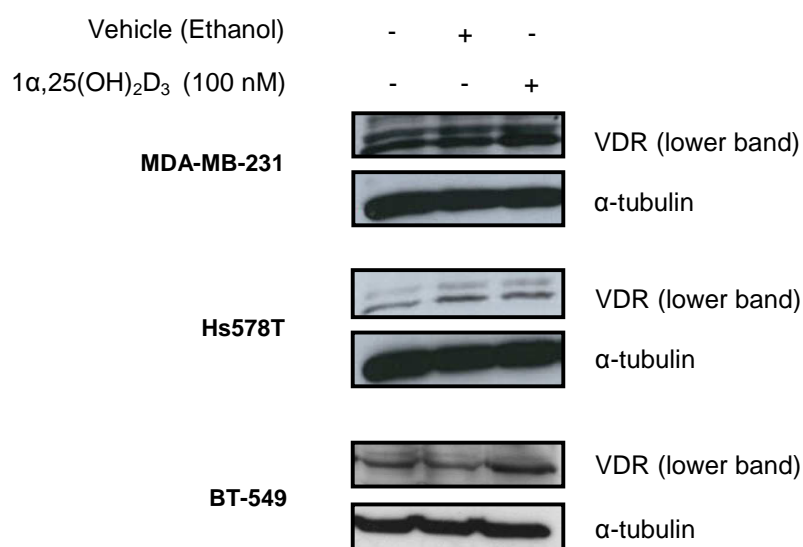

Figure 15 – MDA-MB-231, Hs578T and BT-549 breast cancer cell lines are positive for VDR expression.

$1\alpha,25(OH)_2D_3$ induces the expression of epithelial E-cadherin differentiation marker in triple-negative breast cancer cells and is important for the correct *in vitro* protein localisation in MDA-MB-231 breast cancer cells

Triple-negative breast cancer cells have been treated with $1\alpha,25(OH)_2D_3$ for 72 hours and the expression of E-cadherin was assessed by Western blotting. We have observed that there was a *de*

novo expression of E-cadherin upon treatment with 1α,25(OH)$_2$D$_3$ in MDA-MB-231 cells (Figure 16A). Thus, we evaluated if that expression was dependent on the time of treatment and on the dose of 1α,25(OH)$_2$D$_3$. Cells were then treated with 1α,25(OH)$_2$D$_3$ 100 nM for 12h, 24h and 48h. As it is presented in Figure 16B, the expression of E-cadherin was dependent on the time of treatment. Protein expression was first detected at 24h of treatment and it increased with time. To assess the influence of 1α,25(OH)$_2$D$_3$ dose on E-cadherin expression, MDA-MB-231 cells were treated with increasing 1α,25(OH)$_2$D$_3$ doses for 72 hours. We have observed that E-cadherin expression was dependent on the dose of 1α,25(OH)$_2$D$_3$ and that E-cadherin protein expression was already identified using a very low dose as 1 nM. Also, the expression of E-cadherin was augmented with increasing doses of 1α,25(OH)$_2$D$_3$ (Figure 16C).

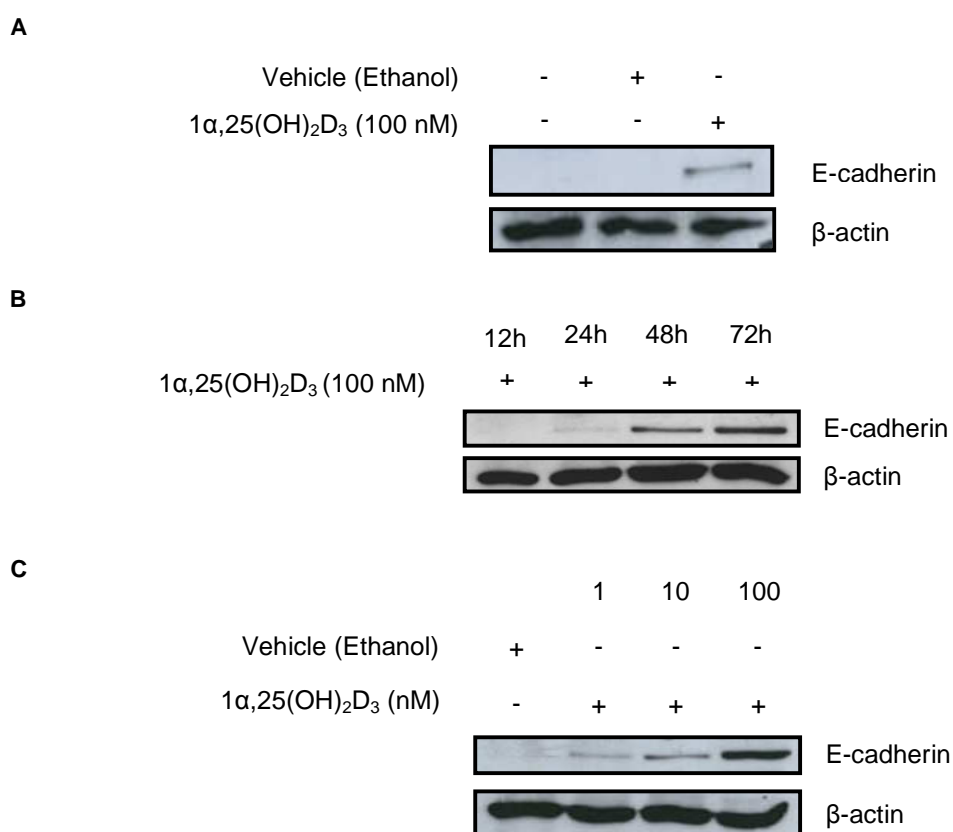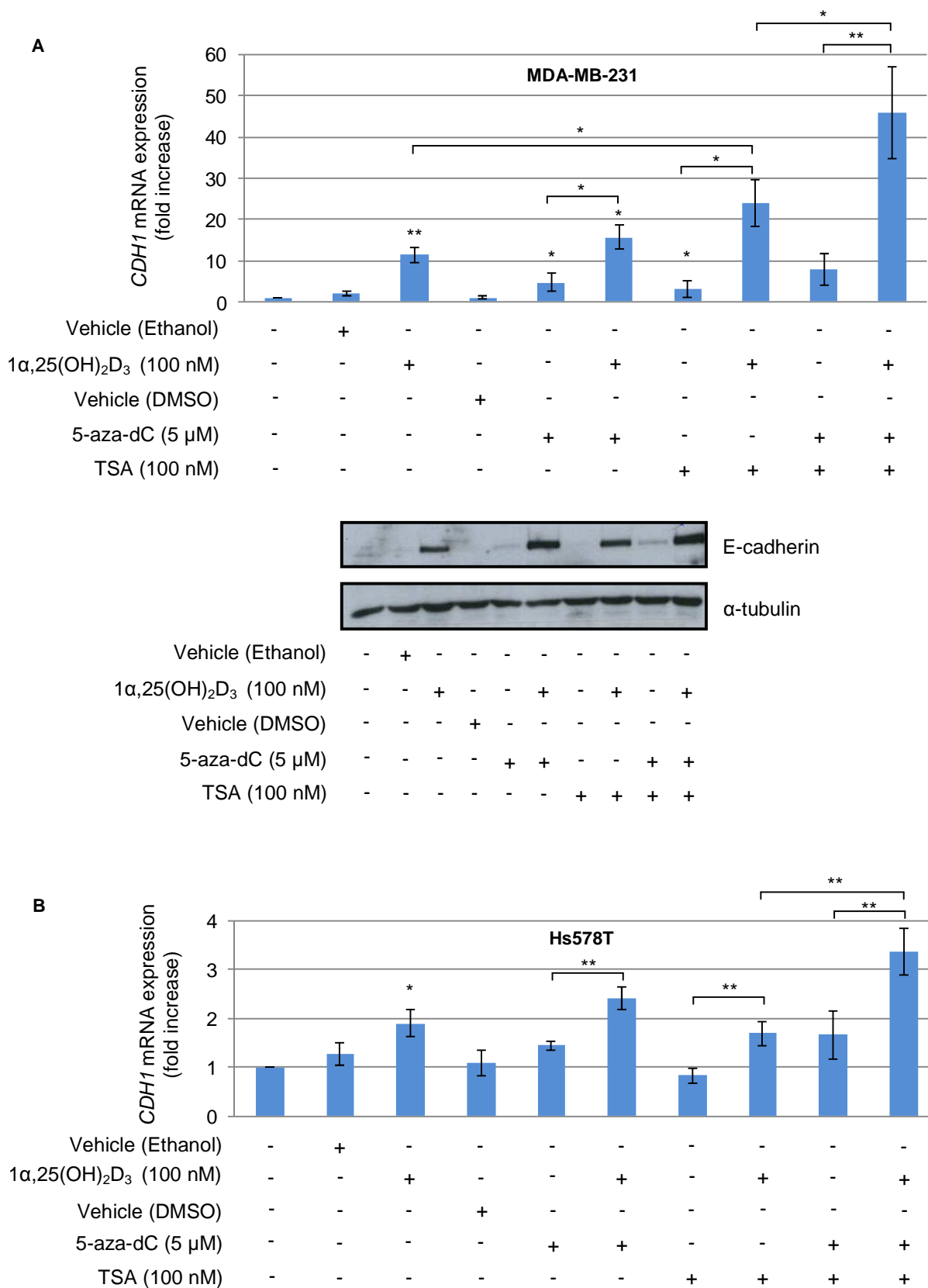**Figure 16** – 1α,25(OH)$_2$D$_3$ induces E-cadherin *de novo* expression in MDA-MB-231 breast cancer cells (**A**) and this effect is time- (**B**) and dose-dependent (**C**).

Since it has been described that the mechanism by which *CDH1* is silenced in these triple-negative breast cancer cells is promoter methylation [326], we have studied the effect of the demethylating agent 5-aza-2-deoxycytidine (5-aza-dC) and the histone deacetylation (HDAC) inhibitor agent TSA, in addition to $1\alpha,25(OH)_2D_3$.

By quantitative real-time PCR in MDA-MB-231 cells, we have observed that $1\alpha,25(OH)_2D_3$ was a potent inducer of *CDH1* mRNA expression, displaying more than 10-fold of induction, compared with the control ($p < 0.001$) – Figure 17A. Furthermore, the level of expression induced by $1\alpha,25(OH)_2D_3$ was 2-fold higher than the one produced by 5-aza-dC alone and 3-fold higher than the one induced by TSA alone. However, both agents display an additive effect to $1\alpha,25(OH)_2D_3$, being the highest levels of expression induced when the three drugs are combined. These results have also been confirmed by protein expression (Figure 17A).

Additionally, we have evaluated the effect of $1\alpha,25(OH)_2D_3$, 5-aza-dC and TSA in the other triple-negative breast cancer cell lines and negative for E-cadherin expression, in which *CDH1* is also silenced by promoter methylation: Hs578T and BT-549. In these cell lines, $1\alpha,25(OH)_2D_3$ was unable to induce E-cadherin expression on its own. However, in Hs578T cells there was a significant induction of *CDH1* expression upon $1\alpha,25(OH)_2D_3$ treatment (Figure 17B). Furthermore, in both cell lines there was induction of *CDH1* mRNA expression upon the treatment with 5-aza-dC. Interestingly, $1\alpha,25(OH)_2D_3$ seems to display an additive effect when administered with both 5-aza-dC and TSA. Again, the highest levels of *CDH1* expression are achieved whenever all agents are added together and, in this case, BT-549 cells are more responsive than Hs578T cells, which corroborates the previous results obtained with VDR expression. Furthermore, these results have been confirmed by protein expression (Figure 17C).

A

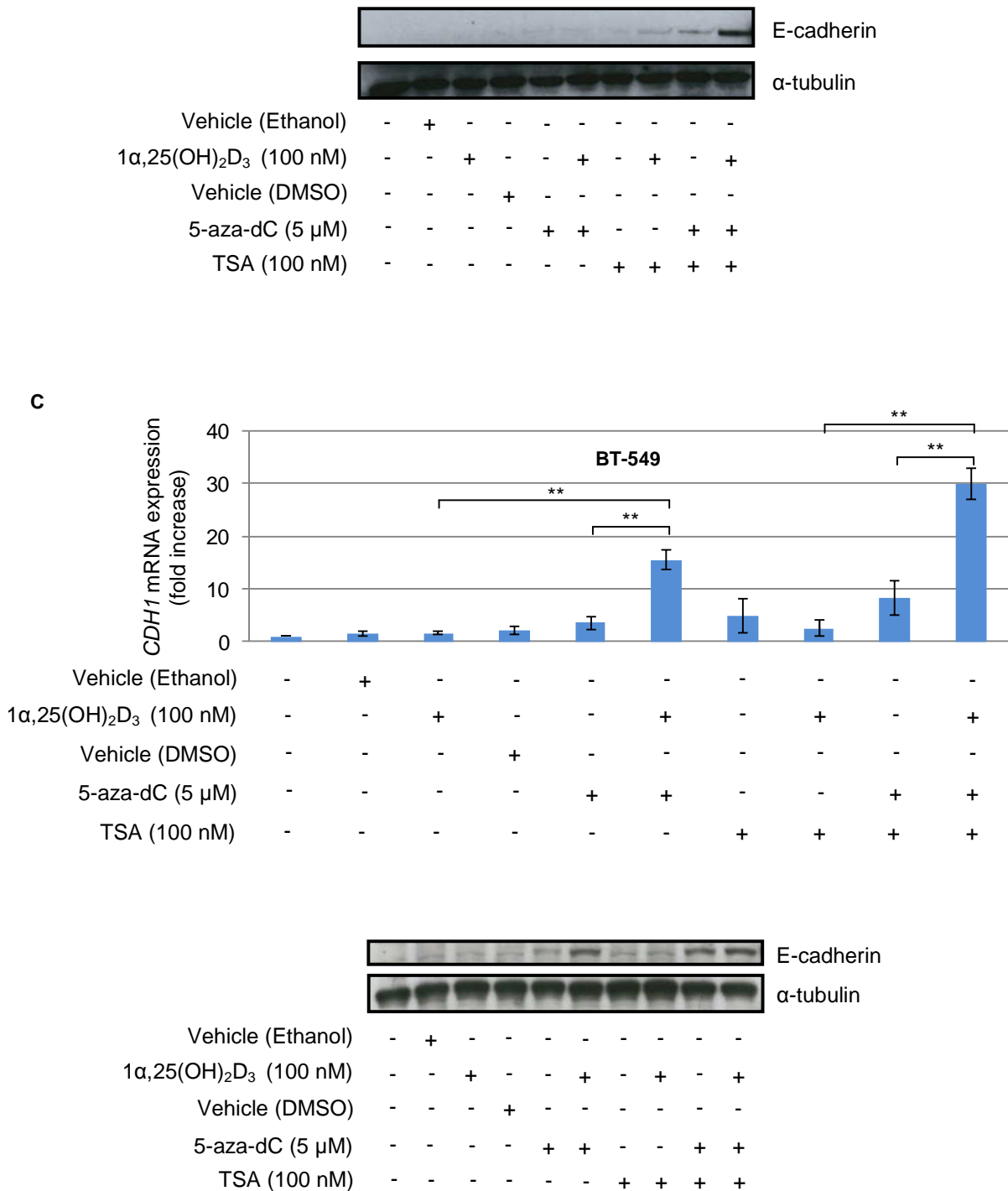

B

Vehicle (Ethanol)	-	+	-	-	-	-	-	-	-	-
1α,25(OH)$_2$D$_3$ (100 nM)	-	-	+	-	-	+	-	+	-	+
Vehicle (DMSO)	-	-	-	+	-	-	-	-	-	-
5-aza-dC (5 µM)	-	-	-	-	+	+	-	-	+	+
TSA (100 nM)	-	-	-	-	-	-	+	+	+	+

C

Vehicle (Ethanol)	-	+	-	-	-	-	-	-	-	-
1α,25(OH)$_2$D$_3$ (100 nM)	-	-	+	-	-	+	-	+	-	+
Vehicle (DMSO)	-	-	-	+	-	-	-	-	-	-
5-aza-dC (5 µM)	-	-	-	-	+	+	-	-	+	+
TSA (100 nM)	-	-	-	-	-	-	+	+	+	+

Vehicle (Ethanol)	-	+	-	-	-	-	-	-	-	-
1α,25(OH)$_2$D$_3$ (100 nM)	-	-	+	-	-	+	-	+	-	+
Vehicle (DMSO)	-	-	-	+	-	-	-	-	-	-
5-aza-dC (5 µM)	-	-	-	-	+	+	-	-	+	+
TSA (100 nM)	-	-	-	-	-	-	+	+	+	+

Figure 17 – 1α,25(OH)$_2$D$_3$, 5-aza-dC and TSA induce *CDH1* mRNA expression and E-cadherin expression in MDA-MB-231 (**A**), Hs578T (**B**) and BT-549 (**C**) breast cancer cells (* $p < 0.005$, ** $p < 0.001$).

We also wanted to check if the protein that has been expressed was functional and if it could be playing its role as an adhesion molecule. For that, we have performed immunofluorescence. As it can be observed in Figure 18, upon treatment with $1\alpha,25(OH)_2D_3$, MDA-MB-231 cells exhibited expression of E-cadherin at the plasma membrane, suggesting a functional adhesion molecule. In contrast, the expression of E-cadherin induced by 5-aza-dC alone was granular and dispersed throughout the cytoplasm, which is suggestive of a non-functional protein. However, when these cells were treated with both agents, there was a rescue of E-cadherin expression to the membrane, hinting that $1\alpha,25(OH)_2D_3$ is indeed inducing not only the expression of E-cadherin (like 5-aza-dC), but, apparently, is also important for the correct localisation of the protein as an adhesion molecule.
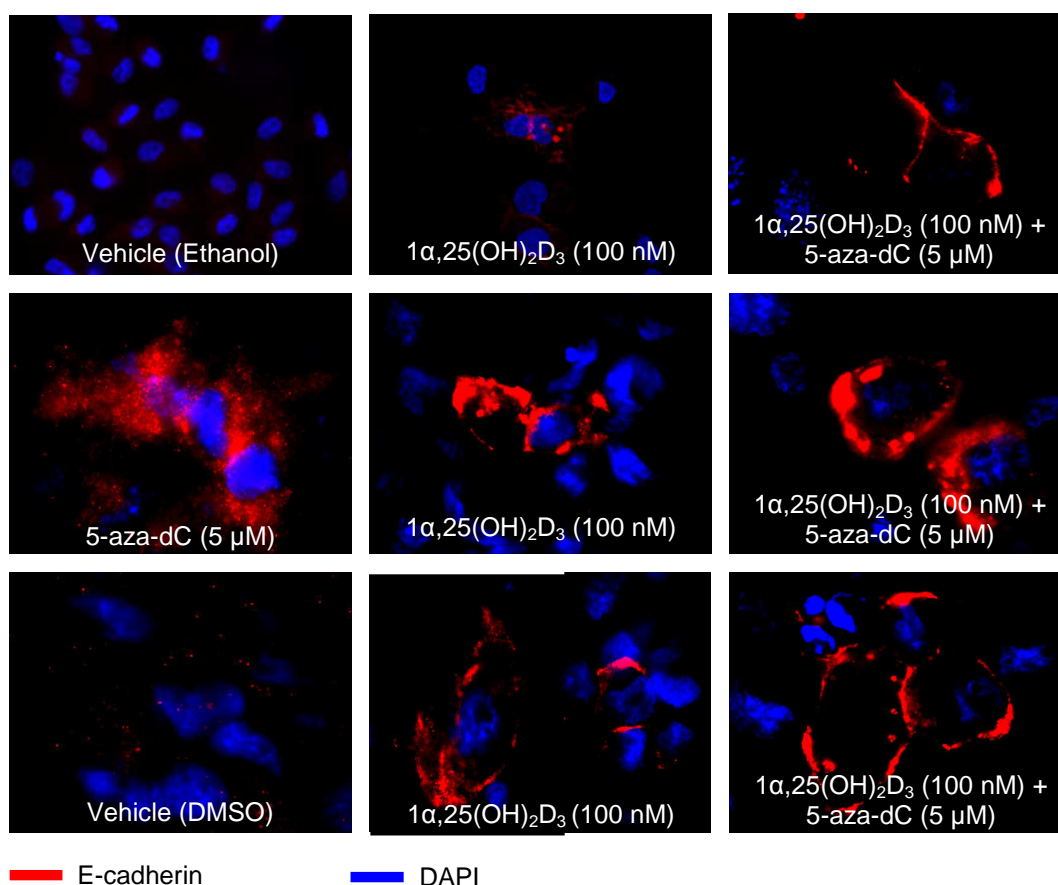

Figure 18 – $1\alpha,25(OH)_2D_3$ induces E-cadherin expression and seems to be important for its correct membrane localisation in MDA-MB-231 breast cancer cells.

The 1α,25(OH)$_2$D$_3$-induced expression of E-cadherin is mediated by the VDR

In order to assess if this 1α,25(OH)$_2$D$_3$-induced expression of E-cadherin could be mediated by the VDR, we have performed siRNA for the VDR. Since 1α,25(OH)$_2$D$_3$ only induced E-cadherin expression at the protein level in MDA-MB-231 cells, the experiments using *VDR* knockdown have only been conducted in this cell line.

When we silenced VDR expression by siRNA in MDA-MB-231 cells, we have observed that E-cadherin expression upon 1α,25(OH)$_2$D$_3$ treatment is abrogated (Figure 19). This suggests that E-cadherin expression is dependent on the presence of the VDR.

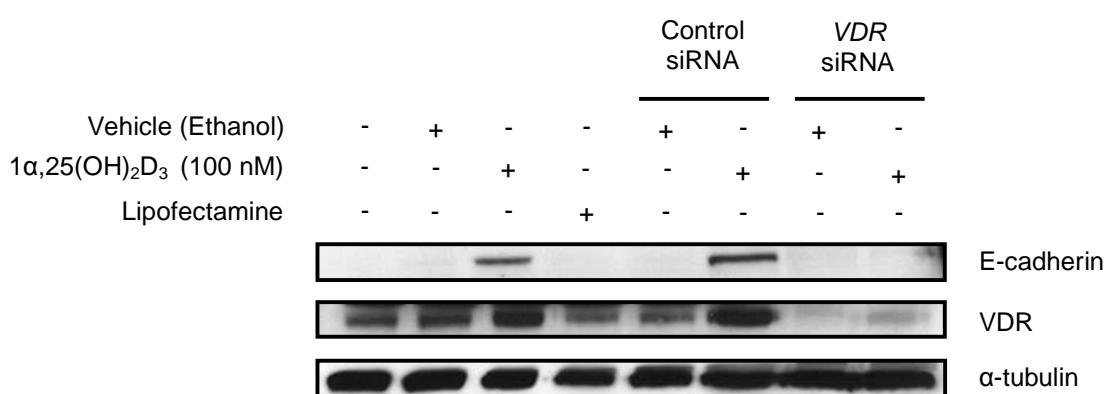|					Control siRNA		VDR siRNA	
Vehicle (Ethanol)	-	+	-	-	+	-	+	-
1α,25(OH)$_2$D$_3$ (100 nM)	-	-	+	-	-	+	-	+
Lipofectamine	-	-	-	+	-	-	-	-

E-cadherin
VDR
α-tubulin

Figure 19 – E-cadherin expression induced by 1α,25(OH)$_2$D$_3$ is abrogated with *VDR* knockdown by siRNA in MDA-MB-231 cell line.

1α,25(OH)$_2$D$_3$ induces E-cadherin expression through *CDH1* promoter demethylation

Finally, we wanted to uncover the mechanism by which 1α,25(OH)$_2$D$_3$ is inducing E-cadherin expression in MDA-MB-231 breast cancer cells. Since it has been demonstrated that *CDH1* is silenced in these cells as a result of promoter methylation [326], we have decided to perform methylation assays in order to confirm this hypothesis.

Indeed, we have observed that upon 1α,25(OH)$_2$D$_3$ treatment there is partial demethylation of *CDH1* promoter in MDA-MB-231 cells (Figure 20). Demethylation has been detected in 7 CpG sites out of the 12 CpG sites analysed, suggesting that 1α,25(OH)$_2$D$_3$ can work as a demethylating agent in MDA-MB-231 breast cancer cells.

A

B
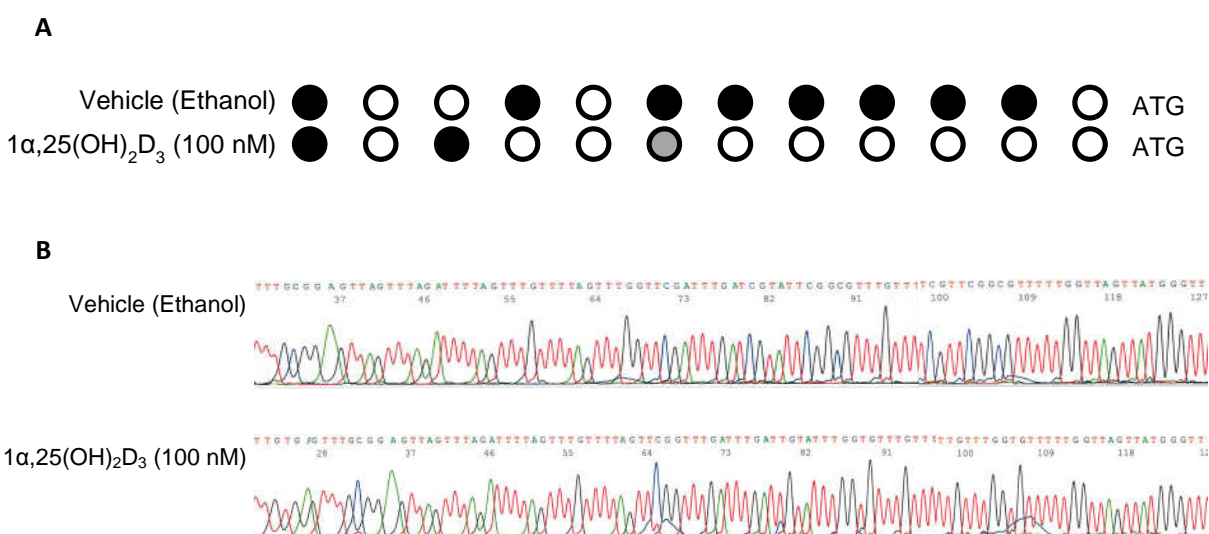

Figure 20 – 1α,25(OH)$_2$D$_3$ induces partial demethylation of *CDH1* promoter in MDA-MB-231 breast cancer cells ● – methylation, ◐ – hemimethylation, ○ – demethylation (**A**). Example of a demethylated CpG site (**B**).

Discussion

Several lines of evidence have demonstrated that 1α,25(OH)$_2$D$_3$ can act as a differentiation inducer agent in cancer cells [77]. We wanted to evaluate if 1α,25(OH)$_2$D$_3$ could be a potential therapeutic approach for the metaplastic triple-negative breast carcinomas. In order to do that, we have assessed the expression of VDR in metaplastic tumours and we have tested the 1α,25(OH)$_2$D$_3$ effects in mesenchymal triple-negative breast cancer cells, since they are representative of this aggressive

subtype of mammary carcinomas with a poor outcome, that lacks targeted therapy [210, 212].

We have observed that the majority of metaplastic breast carcinomas are positive for VDR expression, which suggests that they may be responsive to the treatment with $1\alpha,25(OH)_2D_3$. Afterwards, we have used an *in vitro* model representative of this rare type of tumours and we have seen that $1\alpha,25(OH)_2D_3$ induced the *de novo* E-cadherin expression in the triple-negative MDA-MB-231 breast cancer cell line. This is an important finding, given the major role of E-cadherin as a tumour suppressor protein in lobular breast carcinomas and other cancer models [327, 328] and because downregulation of E-cadherin is required to initiate metastatic growth of breast cancer [329]. Furthermore, we have demonstrated that this effect is dependent on the time of treatment and the amount of $1\alpha,25(OH)_2D_3$ given to the cells. As far as we know, this is the first study demonstrating the *de novo* induction of E-cadherin expression in breast cancer cells by $1\alpha,25(OH)_2D_3$ due to *CDH1* promotor demethylation, although it has been reported that $1\alpha,25(OH)_2D_3$ can augment the expression of endogenous E-cadherin in mammary tumour cells [167]. In addition, it has been demonstrated that an analogue of $1\alpha,25(OH)_2D_3$ can increase the expression of E-cadherin in prostate cancer cells [325]. In colon carcinoma cells, $1\alpha,25(OH)_2D_3$ is also known to promote differentiation by inducing the expression of E-cadherin and other adhesion proteins (Occludin, ZO-1, ZO-2 and Vinculin), and this effect was only observed in VDR positive cells [182]. Likewise, we have shown that, in MDA-MB-231 cells, E-cadherin expression is dependent on the presence of the VDR, suggesting that the VDR is mediating this effect.

In MDA-MB-231 cells, *CDH1* expression is silenced due to promoter methylation [326], so we decided to study the effects of the demethylating agent 5-aza-dC. An interesting finding was that the levels of *CDH1* expression upon $1\alpha,25(OH)_2D_3$ treatment in MDA-MB-231 cells were 2- and 3-fold higher than the ones induced by 5-aza-dC and the HDAC inhibitor agent TSA, respectively, and that the combination of $1\alpha,25(OH)_2D_3$ with either of these molecules promoted an additive effect. These results were further confirmed by protein expression. In gastric cancer cells, it was shown that

1α,25(OH)$_2$D$_3$ can work in synergy with 5-aza-dC and TSA [330], thus supporting the effect that we have obtained. Additional evidence in colon cancer demonstrates that, in cells with silenced HDAC3, upon treatment with 1α,25(OH)$_2$D$_3$, there is an increase in E-cadherin expression [331], a result that mimics what we observed in MDA-MB-231 cells upon treatment with TSA and 1α,25(OH)$_2$D$_3$. In the other cells tested (Hs578T and BT-549) the results were not so encouraging when 1α,25(OH)$_2$D$_3$ was used alone; however, *CDH1* / E-cadherin expression was detectable when the cells were treated with 1α,25(OH)$_2$D$_3$ together with 5-aza-dC or TSA.

Also remarkable was the observation that the treatment with 1α,25(OH)$_2$D$_3$ promoted the correct localisation of E-cadherin at the cell membrane in MDA-MB-231 cells, unlike the treatment with 5-aza-dC, that induced a granular and dispersed pattern of expression. This result also resembles the observations in colon carcinoma, where, upon 1α,25(OH)$_2$D$_3$ treatment, there was E-cadherin expression at the cell membrane [182]. But this effect of 1α,25(OH)$_2$D$_3$ on E-cadherin induction is not exclusive of disease settings, as in normal keratinocytes the treatment with 1α,25(OH)$_2$D$_3$ stimulates the assembly of adherens junctions, assessed by translocation of E-cadherin to the cell membrane [332]. Surprisingly, when MDA-MB-231 breast cancer cells were treated with both drugs (1α,25(OH)$_2$D$_3$ and 5-aza-dC), the effect induced by 1α,25(OH)$_2$D$_3$ prevailed over the 5-aza-dC-induced effect and there was a rescue of E-cadherin expression back to the membrane, hinting that 1α,25(OH)$_2$D$_3$ is indeed inducing not only the expression of E-cadherin, but, apparently, is also important for the correct membrane localisation of the protein as a cell-cell adhesion molecule. Unlike the results we have obtained, it was demonstrated that 5-aza-dc was necessary to sensitise leukaemia cells to differentiate in response to 1α,25(OH)$_2$D$_3$ treatment [333]. In Hs578T and BT-549 cells, the treatment with 1α,25(OH)$_2$D$_3$ did not promote the correct localisation of E-cadherin at the cell membrane; instead, the protein exhibit a granular pattern, dispersed throughout the cytoplasm and similar to the effect induced by 5-aza-dC.

We have also demonstrated that the treatment with 1α,25(OH)$_2$D$_3$ promotes partial *CDH1* promoter

demethylation and this is an original finding, since it has never been described that $1\alpha,25(OH)_2D_3$ can induce demethylation. To the extent of our knowledge, only one study correlated $1\alpha,25(OH)_2D_3$ with methylation and reported that $1\alpha,25(OH)_2D_3$ can induce methylation of *CYP27B1* (the enzyme responsible for its synthesis) and, thus, silence its expression [334]. In colon cancer cells, where $1\alpha,25(OH)_2D_3$ induces E-cadherin expression, a new mechanism involving phosphoinositide signalling has been recently proposed [335], as Kouchi *et al.* have demonstrated that phosphatidylinositol 5-phosphate 4-kinase type II beta is required for E-cadherin expression upon $1\alpha,25(OH)_2D_3$ treatment. Also in colonic cancer cells, a novel mechanism on the involvement of $1\alpha,25(OH)_2D_3$ in epigenetic events has been reported: the knockdown of KDM6B/JMJD3, a histone demethylase induced by $1\alpha,25(OH)_2D_3$, downregulated E-cadherin expression [336]. Studies addressing the importance of these mediators in breast cancer are still lacking.

In summary, we have observed that the majority of metaplastic carcinomas is positive for VDR expression, hinting that this rare type of aggressive cancers may be responsive to the anti-tumour effects of $1\alpha,25(OH)_2D_3$. Furthermore, we have demonstrated that $1\alpha,25(OH)_2D_3$ induces the *de novo* expression of the epithelial differentiation marker E-cadherin in the highly metastatic, triple-negative MDA-MB-231 breast cancer cell line. To the best of our knowledge, this is the first report of the *de novo* induction of E-cadherin in breast cancer cells by $1\alpha,25(OH)_2D_3$ due to *CDH1* promoter demethylation. Therefore, our study reveals a novel mechanism for the action of $1\alpha,25(OH)_2D_3$ in breast cancer cells: the demethylation of *CDH1*. The induction of differentiation promoted by $1\alpha,25(OH)_2D_3$ in metaplastic, triple-negative basal-like breast cancer may decrease the aggressiveness of this subtype of mammary carcinomas and improve patient outcome, but further studies are necessary to confirm this hypothesis.

Concluding Remarks

The main conclusions of the present study are the following:

- P-cadherin, Vimentin and CK14 are useful biomarkers for the identification of a subset of breast carcinomas (triple-negative basal-like tumours), due to their consistent values of sensitivity and specificity.

- There is deregulation of the Vitamin D signalling and metabolic pathways in breast cancer, favouring tumour progression. It seems that, during mammary malignant transformation, tumour cells lose their ability to synthesise the active form of Vitamin D and respond to VDR-mediated Vitamin D effects, while increasing their ability to degrade this hormone.

- $1\alpha,25(OH)_2D_3$ induces the expression of the epithelial differentiation marker E-cadherin in the triple-negative and metastatic MDA-MB-231 breast cancer cell line in a time- and dose-dependent manner. The induction of E-cadherin by $1\alpha,25(OH)_2D_3$ is dependent on the presence of the VDR, since the effect is abrogated upon VDR silencing and is due to *CDH1* promoter demethylation.

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Original Articles

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P-cadherin, Vimentin and CK14 for identification of basal-like phenotype in breast carcinomas:

an immunohistochemical study

P-cadherin, Vimentin and CK14 for identification of basal-like phenotype in breast carcinomas: an immunohistochemical study

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Summary. Introduction: The most suitable immuno-histochemical criterion to identify basal-like breast carcinomas (BLBC), a molecular subgroup of breast cancer associated with poor prognosis, is the triple negative phenotype along with CK5 and/or EGFR immunoreactivity. However, several putative basal markers have been suggested as alternatives to identify BLBC with more accuracy. Experimental Design: The expression of CK5, EGFR, P-cadherin, CK14, Vimentin and p63 were evaluated in 462 invasive breast carcinomas to determine their sensitivity and specificity for BLBC identification. Results: P-cadherin and CK5 showed higher sensitivity values, while EGFR, Vimentin and CK14 were the most specific markers. The combination of CK5 with P-cadherin, Vimentin or CK14 proved to be a reliable option for distinguishing the basal phenotype, compared to the "gold standard" pair CK5/EGFR. Furthermore, P-cadherin was still able to recognize a large number of putative BLBC among the "unclassified" group (ER-/PR-/HER2-/CK5-/EGFR-). Conclusions: P-cadherin, Vimentin and CK14 can recognize BLBC already identified in triple negative/ CK5 and/or EGFR+ tumors, and due to P-cadherin sensitivity for BLBC identification this marker can reliably recruit a large number of breast carcinomas with basal phenotype among immunohistochemistry triple negative/ CK5 and/or EGFR - pool of tumors. Although they need GEP validation, our results can introduce the idea of these markers as additional options in the daily workup of breast pathology laboratories to identify BLBC.

Key words: Basal-like breast cancer, P-cadherin, CK14, Vimentin

Introduction

In the European Union, breast cancer is the most incident form of cancer in women, with an estimated 429.900 cases diagnosed per year (28.9% of all incident cases in women) (Ferlay et al., 2007; Milanezi et al., 2008). Breast cancer is frequently designated as a heterogeneous disease with divergent biological behaviors. cDNA microarray studies have provided an improvement in cellular and molecular understanding of breast cancer, identifying distinct subtypes of breast carcinomas with different molecular signatures and clinical outcomes (Perou et al., 2000; Sorlie et al., 2001, 2003; Rakha et al., 2006a,b). The basal-like subtype has definitely drawn the attention of the scientific community. These tumors are characterized by a triple negative (TN) phenotype, lacking the expression of hormone receptors (HR) [estrogen and progesterone receptors (ER and PR, respectively)] and HER2. Basal-like breast carcinomas (BLBC) are associated with

aggressive tumor behavior and shorter overall survival when compared to the luminal and HER2-overexpressing subtypes and there is an enthusiastic search for molecular markers expressed in BLBC that could be used as targets to therapy (Nielsen et al., 2004). Histologically, they are poorly differentiated carcinomas, present high nuclear and histological grade and frequently show medullary and metaplastic features (Tsuda et al., 2000; Fulford et al., 2006; Livasy et al., 2006; Rakha et al., 2006a,b). A distinct pattern of metastasis to brain and lungs, known to be associated with poor prognosis, and less significant involvement of axillary lymph nodes, has also been described in BLBC (Tsuda et al., 2000; Banerjee et al., 2006; Fulford et al., 2007). Nowadays, gene expression profiles (GEP) or cDNA microarrays studies are currently considered the "gold standard" methods for the identification of breast carcinomas with basal phenotype, since these technologies were the first to identify BLBC as a distinct subgroup with a specific molecular signature (Perou et al., 2000) and clinical identity (Sorlie et al., 2001, 2003; van't Veer et al., 2002). However, GEP are expensive, not easily applicable as a routine laboratory diagnostic tool in large scale clinical-pathological analysis and have limited value in retrospective studies using formalin-fixed paraffin-embedded (FFPE) tissues (Cheang et al., 2008; Reis-Filho and Tutt, 2008). Thus, the idea of developing an immunohistochemical (IHC)-based assay for the identification of BLBC is appealing. The variation in the transcriptional and translational programs of cells that accounts for the different molecular identities of breast carcinomas also reinforces the interest in creating an IHC-based assay for BLBC definition. The characteristic protein expression of tumors would be a useful surrogate of GEP, and the IHC profile would help to standardize investigations and uniformly identify a group of tumors with a basal-like transcriptional program (Reis-Filho and Tutt, 2008).

However, the most appropriate panel of antibodies to be used, in order to identify breast carcinomas with basal phenotype, has not reached a consensus yet. In 2008, Tang et al. (2008) compared the different IHC classifications that have been used to define basal-like and non basal-like breast carcinomas; interestingly, they showed that in high grade breast carcinomas, which is a common feature of basal phenotype, the rates of BLBC ranged between 19% and 76%, indicating the need for a more consensual strategy between laboratories.

The TN phenotype criterion is used by some authors who assume that Triple Negative tumors and BLBC are synonymous (Kreike et al., 2007; Spitale et al., 2008). In fact, this criterion is quite convenient, since it includes standard biomarkers already used in the clinical management of breast cancer. However, relying on negative results to perform a diagnostic interpretation may be risky due to technical failures leading to a decrease in specificity. Other authors use high molecular weight cytokeratins alone (CK5/6, CK14 or CK17) to identify BLBC, claiming that BLBC and triple negative tumors are different identities (van de Rijn et al., 2002; Abd El-Rehim et al., 2004; Fulford et al., 2007; Rakha et al., 2007b). In addition, since basal-like breast carcinomas express proteins that are characteristic from the basal/myoepihelial outer layer of the mammary gland, such as EGFR, p63, P-cadherin, calponin, CD10, S100 and α-smooth-muscle actin (α-SMA) (Jones et al., 2001; Reis-Filho et al., 2003; Nielsen et al., 2004; Livasy et al., 2006), some definitions of BLBC associate the lack of expression of ER, PR and HER2 with the immunoreactivity for some of these basal markers that were already correlated with basal phenotype and poor prognosis (Nielsen et al., 2004; Matos et al., 2005; Laakso et al., 2006). Our group has previously demonstrated that using a panel of antibodies for ER, PR, HER2, CK5/6 and/or EGFR and/or P-cadherin and/or p63 it is possible to distinguish invasive (Matos et al., 2005) and i*n situ* (Paredes et al., 2007b) BLBC. However, Nielsen et al. (2004) found that expression of CK5/6 and EGFR together with negativity for ER and HER2 would be the immunoprofile that identifies the same basal-like carcinomas found by cDNA microarrays, with a sensitivity of 76% and a specificity of 100%. This criterion is, therefore, considered the "gold standard" immunoprofile to classify BLBC.

In this study, we aim to refine the immunohisto-chemical criterion to identify BLBC by analyzing the sensitivity and the specificity of the main basal markers that have been described, namely CK5, EGFR, P-cadherin, CK14, Vimentin and p63 and suggest possible additional markers for BLBC identification, especially in CK5 and EGFR negative breast carcinomas.

Materials and methods

Breast tumour samples

Formalin-fixed, paraffin-embedded tissues of 462 invasive breast carcinomas were consecutively retrieved from the histopathology files of three Departments of Pathology: University Hospital of the Federal University of Santa Catarina (Florianópolis, Brazil), Hospital Divino Espírito Santo (HDES), (Ponta Delgada, São Miguel, Portugal), and a private Laboratory of Pathology in Araçatuba, Brazil. All cases were reviewed by three pathologists (FM, FS and LV) on haematoxylin and eosin-stained (H&E) sections.

TMA construction

Representative areas of the invasive breast carcinomas were carefully selected on the H&E-stained sections and marked on individual paraffin blocks. Two tissue cores (2 mm in diameter) were obtained from each specimen and precisely deposited into a recipient paraffin block using a TMA workstation (TMA builder 20010.02, Histopatholoy Ltd, Hungary). Forty seven TMA blocks were constructed, each one containing 24 tissue cores, arranged in a 4x6 sector. In each TMA

block, normal breast and testicular tissue were included as controls. After construction, 2 μm tissue sections were cut and adhered to glass slides (Polysine*TM*, Menzel-Glasser, Germany) for the immunohistochemical studies and a H&E-stained section from each TMA block was reviewed in order to confirm the presence of morphological representative areas of the original lesions.

Immunohistochemistry

All the immunohistochemical assays were performed with specific monoclonal antibodies. Details about primary antibodies, antigen retrieval and IHC detection systems are described in Table1. Except for EGFR, in which epitope retrieval was performed by proteolytic enzyme digestion for 20 minutes (pepsin A, 4 g/l; Sigma-Aldrich, USA) at 37°C, all epitope retrieval was heat-induced at 98ºC in a water-bath during 30 minutes, using a commercially available citrate buffer solution (Vector Laboratories, USA), 1:100, pH=6.0, or an ethylenediaminetetraacetic (EDTA) solution (Novocastra, UK), 1:10, pH=9.0, as antigen unmasking solutions. After the respective antigen retrieval and washes in a phosphate buffer solution (PBS), endogenous peroxidase activity was blocked with a 3% hydrogen peroxide solution (Panreac, Spain) in methanol (Sigma-Aldrich, USA) for 10 minutes. The slides were incubated in a blocking serum (LabVision, USA) for 15 min and then incubated with the respective primary monoclonal antibodies. Immunoassays were performed using the streptavidin-biotin-peroxidase technique (SABC), (LabVision Corporation, Fremont, CA, USA) or the HRP labeled polymer (DakoCytomation, USA) detection system, according to manufacturer's instructions. All reactions were revealed with diamino-benzidine (DAB) chromogen (DakoCytomation). Tissues were then counterstained with Mayer's haematoxylin, dehydrated and coverslipped using a permanent mounting solution (Mounting Medium, Richard Allan Scientific, USA). Positive and negative controls were included in every set of reactions for each antibody used. Normal breast ducts and lobules present in many of the selected areas were also used as internal controls, as well as the non-neoplastic breast tissue cores included in each array. The evaluation of immunohistochemistry results was performed by three pathologists as follows: ER, PR and p63 were considered positive whenever more than 10% of the neoplastic cells showed nuclear staining; similarly, the same cutoff was used for CK5, CK14 and Vimentin cytoplasmic staining, as well as for P-cadherin membrane staining. Membrane expression for HER2 and EGFR was evaluated according to the DakoCytomation HercepTest® scoring system (Reis-Filho et al., 2005). Breast carcinomas were considered HER2-overexpressing whenever the immunohistochemical reaction was classified as 3+ or when gene amplification was confirmed by Chromogenic *In Situ* hybridization (CISH) in the 2+ cases, as described in other works (Ricardo et al., 2007). For EGFR, the cases were considered positive whenever the immunostaining was 2+ or 3+.

Hormone receptor (ER and PR) positive tumors were considered luminal A and B whether or not they overexpressed HER2, respectively (Sotiriou et al., 2003; Matos et al., 2005; Paredes et al., 2007b; Spitale et al., 2008; Tamimi et al., 2008). Cases lacking ER/PR with overexpression of HER2 were classified as HER2 overexpressing tumors. ER-/PR-/HER2- cases with immunoreactivity for EGFR and/or CK5 were considered BLBC according to the gold standard Nielsen's criterion and cases without expression of the five biomarkers were considered unclassified. When the immunoreactivity for the additional basal markers, namely P-cadherin, CK14 and Vimentin are used, the positive cases for at least one of these markers were considered as BLBC (P-cad and/or CK14 and/or Vim). Since for some markers the immunohistochemical result was not interpretable, the statistical analyses were performed using only 387 breast tumors cases which were classified for all the biomarkers tested.

Table 1. Conditions of the immunohistochemical reactions performed in this study.

Antigen	Primary antibodies				Antigen retrieval buffer	Detection method
	Clone	Origin	Incubation time (min)	Dilution		
ER	SP1	Neomarkers, USA	30	1:150	Citrate	SABC*
PR	SP2	Neomarkers, USA	30	1:300	Citrate	HRP-Polymer **
HER2	SP3	Neomarkers, USA	30	1:80	Citrate	SABC*
CK5	XM26	Neomarkers, USA	60	1:50	Tris-EDTA	SABC*
EGFR	31G7	Zymed	60	1:100	Pepsin	HRP-Polymer **
P-cadherin	56	BD Transduction	60	1:50	Tris-EDTA	HRP-Polymer **
CK14	LL002	Novocastra, UK	60	1:400	Tris-EDTA	HRP-Polymer **
Vimentin	V9	Dako, USA	30	1:150	Citrate	SABC*
p63	4A4	Neomarkers, USA	60	1:150	Citrate	SABC*

* SABC: streptavidin-avidin-biotin-complex; **: HRP-Polymer (horseradish peroxidase - polymer).

Statistical analysis

Statistical analysis was performed by SPSS statistics 17.0 (SPSS Inc., Chicago, IL, USA) software program. χ^2 contingency test was used to determine associations between groups and the results were considered statistically significant if the p value was lower than 0.05. In order to determine which were the most sensitive and specific biomarkers to identify BLBC, the sensitivity and the specificity of the antibodies used were calculated. Sensitivity measurement was defined by the quotient between the true positive (TrueP) cases and the sum of the true positive and the false negative (FalseN) cases [sensitivity = TrueP/(TrueP+FalseN)]. Specificity was measured in a similar way, by the quotient between the true negative (TrueN) cases with the sum of the true negatives and the false positives (FalseP) [specificity = TrueN/(TrueN+FalseP)]. PPV (Positive Predictive Value) and NPV (Negative Predictive Value) were calculated as follows: PPV = TrueP/(TrueP+FalseP) and PNV = TrueN/(TrueN+FalseN). As described before, ER/PR/HER2 negative tumors that express CK5/6 and/or EGFR were considered BLBC. Consequently, TrueP and TrueN cases were the BLBC tumors that were positive or negative, respectively, to the marker or pair of markers in analysis. Inversely, FalseP and FalseN were non BLBC positive or negative to the basal markers in study.

Follow-up information was available for 282 of the 387 cases and a maximum cutoff of 77 months was considered. Survival curves were estimated by the Kaplan-Meier method using log-rank test to assess significant differences for overall survival.

Results

In this series of 387 breast carcinomas, 223/387 (57.6%) and 144/387 (37.2%) cases were ER and PR positive, respectively, and 65/387(16.8%) overexpressed HER2. Using the ER/PR/HER2- (TN) criterion, this series comprises 109 (28.2%) triple negative and 278 (71.8%) non-Triple Negative tumors. Considering the molecular subtypes of breast cancer, 213 (55%) cases were luminal A, 13 (3.4%) luminal B and 52 (13.4%) HER2-overexpressing tumors. According to Nielsen's criterion, 37 (9.6%) cases presented a basal-like phenotype and 72 (18.6%) were considered "unclassified" by this criterion. We analyzed the associations between CK5, EGFR, P-cadherin, CK14, p63 and Vimentin and the BLBC versus non BLBC (Table 2). As expected, the markers were significantly associated with the basal phenotype (p<0.0001), with the exception for p63 (p=0.5403). Fig. 1 shows the immunohistochemical staining for CK5, EGFR, P-cadherin, Vimentin and CK14 in BLBC.

Afterwards, the sensitivity, specificity, PPV and NPV of each biomarker for the identification of BLBC were calculated (Table 3), except for p63 which was not even related with basal phenotype. CK5 was the most sensitive biomarker (91.9%), followed by P-cadherin (67.6%). CK14 and EGFR were the most specific markers, presenting 98.6% and 97.1% of specificity, respectively, and vimentin was also shown to be very specific (86.9%).
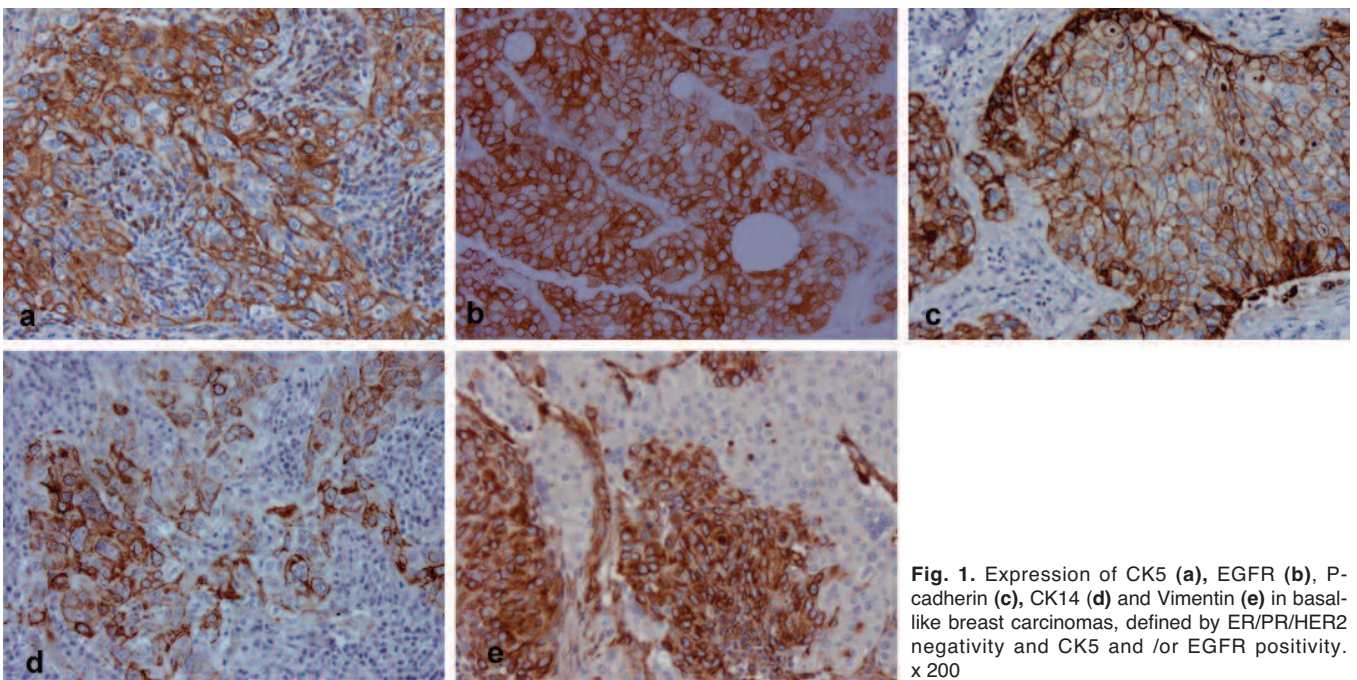

Fig. 1. Expression of CK5 **(a)**, EGFR **(b)**, P-cadherin **(c)**, CK14 **(d)** and Vimentin **(e)** in basal-like breast carcinomas, defined by ER/PR/HER2 negativity and CK5 and /or EGFR positivity. x 200

In order to find the best combination of basal markers with the ability to identify BLBC, we evaluated the most sensitive and the most specific markers in pairs (CK5, P-cadherin with CK14, EGFR or Vimentin). Since P-cadherin presented good sensitivity and specificity values, we also evaluated its association with CK5 (Table 4). The statistical associations considered cases that were positive for both markers (+/+), positive for at least one marker (+/- or -/+) or negative for both (-/-). Table 5 shows the percentages of sensitivity, specificity, PPV and NPV for the several pairs of markers. In these analyses, we considered as true positive the cases that were +/+ and positive for at least one of the markers in the subgroup of BLBC previously distinguished by Nielsen's criterion, and as false positive the cases that

Table 2. Association between the expression of CK5, EGFR, P-cadherin, CK14, p63 and vimentin with basal-like and non basal-like breast carcinomas.

	n	Basal n (%)	Non basal n(%)	P
	387	37(9.6%)	350(90.4%)	
CK5				<0.0001
+	89	34(91.9%)	55(15.7%)	
-	298	3(8.1%)	295(84.3%)	
EGFR				<0.0001
+	21	11(29.7%)	10(2.9%)	
-	366	26(70.3%)	340(97.1%)	
P-cadherin				<0.0001
+	123	25(67.6%)	98(28%)	
-	264	12(32.4%)	252(72%)	
CK14				<0.0001
+	17	12(32.4%)	5(1.4%)	
-	370	25(67.6%)	345(98.6%)	
p63				0.5403
+	14	2(5.4%)	12(3.4%)	
-	373	35(94.6%)	338(96.6%)	
Vimentin				<0.0001
+	63	17(45.9%)	46(13.1%)	
-	324	20(54.1%)	304(86.9%)	

Table 3. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the IHC method for the basal-markers studied to discriminate a basal-like carcinoma.

	Sensitivity (%)	Specificity (%)	PPV (%)	PNV (%)
CK5	91.9	84.3	38.2	99.0
EGFR	29.7	97.1	52.4	92.9
P-cadherin	67.6	72.0	20.3	95.5
CK14	32.4	98.6	70.6	93.2
Vimentin	45.9	86.9	27.0	93.8
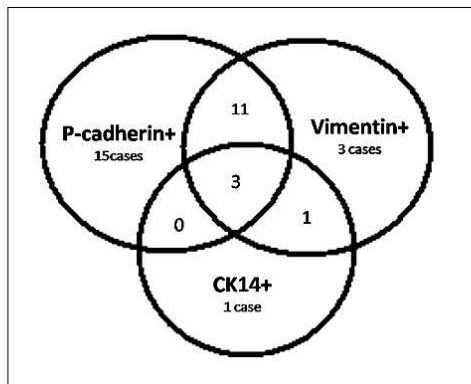
Fig. 2. Distribution of P-cadherin, vimentin and CK14 expression in triple negative tumors that were negative for CK5 and EGFR.

Table 4. Association between the expression of pairs of basal markers with basal-like and non basal-like breast carcinomas.

	n	Basal n (%)	Non basal n(%)	p
CK5/EGFR				<0.0001
+/+	11	8(21.6%)	3(0.8%)	
At least one +	88	29(78.4%)	59(16.9%)	
-/-	288	0(0%)	288(82.3%)	
CK5/CK14				<0.0001
+/+	11	11(29.7%)	0(0%)	
At least one +	83	23(62.2%)	60(17.1%)	
-/-	293	3(8.1%)	290(82.9%)	
CK5/Vim				<0.0001
+/+	24	16(43.2%)	8(2.3%)	
At least one +	104	19(51.4%)	85(24.3%)	
-/-	259	2(5.4%)	257(73.4%)	
P-cadherin/EGFR				<0.0001
+/+	13	8(21.6%)	5(1.4%)	
At least one +	118	20(54.1%)	98(28%)	
-/-	256	9(24.3%)	247(70.6%)	
P-cadherin/CK14				<0.0001
+/+	12	9(24.3%)	3(0.9%)	
At least one +	116	19(51.4%)	97(27.7%)	
-/-	259	9(24.3%)	250(71.4%)	
P-cadherin/Vim				<0.0001
+/+	41	11(29.7%)	30(8.6%)	
At least one +	104	20(54.1%)	84(24%)	
-/-	242	6(16.2%)	236(67.4%)	
P-cadherin/CK5				<0.0001
+/+	38	23(62.2%)	15(4.3%)	
At least one +	136	13(35.1%)	123(35.1%)	
-/-	213	1(2.7%)	212(60.6%)	

Table 5. Sensitivity, specificity, PPV and NPV of the IHC method for the pairs of basal-markers antibodies studied to discriminate a basal-like carcinoma.

	Sensitivity (%)	Specificity (%)	PPV (%)	PNV (%)
CK5/EGFR	100	82.3	11.4	100
CK5/CK14	91.9	82.9	10.5	99
CK5/Vim	94.6	73.4	12.0	99.2
P-cadherin/EGFR	75.7	70.6	10.2	96.5
P-cadherin/CK14	75.7	71.4	10.1	96.5
P-cadherin/Vim	83.8	67.4	11.6	97.5
P-cadherin/CK5	97.3	60.6	14.5	99.5

were positive for the two markers and the ones expressing at least one marker in non basal-like tumors. True negative and false negative were the -/- cases in non basal-like and in BLBC, respectively. All the associations were statistically significant (p‹0.0001). The pair CK5/EGFR presented, as expected, the highest values of sensitivity and specificity, 100% and 82.3%, respectively. However, concerning sensitivity, the pairs

CK5/CK14, P-cadherin/CK5 and CK5/Vimentin showed similar values to the "gold standard" CK5/EGFR pair, with 91.9%, 97.3% and 94.6% of sensitivity, respectively. The specificity of CK5/CK14 combination (82.9%) was approximately equal to the one presented by CK5/EGFR (82.3%).

In the BLBC group, when analyzing the number of cases that were +/+ and positive for at least one of the markers of the pair, against the -/- cases (Table 6), it is possible to observe that only one basal-like breast carcinoma was negative for both markers in P-cadherin/CK5 pair. The CK5/Vimentin pair missed the expression in 2 cases, while CK5/CK14 did not stain three BLBC. All the other pairs were positive in BLBC for the two markers, or for at least one of them, in at least 75.7% of breast carcinomas with basal phenotype.

More importantly, given the sensitivity of P-cadherin and the specificity of CK14 and Vimentin, we also analyzed their expression among the TN/CK5 and EGFR

Table 6. Analyzes of the distribution of expression of the pairs of markers in BLBC.

		Basal n (%)
CK5/EGFR	+/+ and at least one +	37(100%)
	-/-	0(0%)
CK5/CK14	+/+ and at least one +	34(91.9%)
	-/-	3(9.1%)
CK5/Vim	+/+ and at least one +	35(94.6%)
	-/-	2(5.4%)
P-cadherin/EGFR	+/+ and at least one +	28(75.7%)
	-/-	9(24.3%)
P-cadherin/CK14	+/+ and at least one +	28(75.7%)
	-/-	9(24.3%)
P-cadherin/Vim	+/+ and at least one +	31(83.8%)
	-/-	6(16.2%)
P-cadherin/CK5	+/+ and at least one +	36(97.3%)
	-/-	1(2.7%)

Table 7. Expression of P-cadherin, vimentin and CK14 in the 72 TN tumors also negative for CK5 and EGFR.

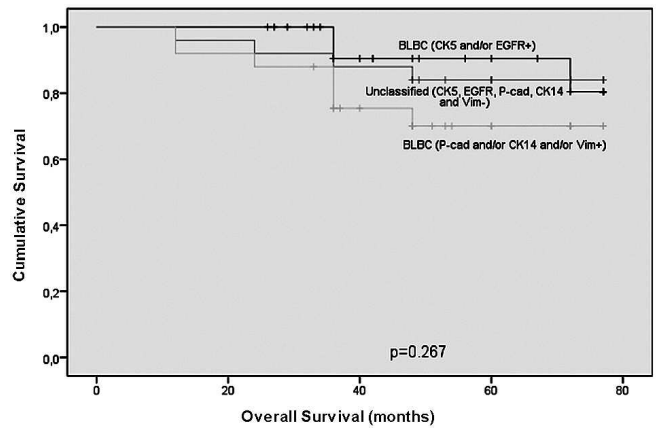|		TN/CK5 and EGFR- n=72
P-cadherin	+	29(40.3%)
	-	43(59.7%)
Vimentin	+	18(25%)
	-	54(75%)
CK14	+	5(6.9%)
	-	67(93.1%)

Fig. 3. Kaplan-Meier survival curves for overall survival (OS) of triple negative breast carcinoma patient's cohort, with a 77 months cut-off. BLBC defined by TN/CK5 and/or EGFR+ [BLBC (CK5 and/or EGFR+)], BLBC defined as ER/PR/HER2-, CK5/EGFR- and immunoreactivity for P-cadherin and/or CK14 and/or Vimentin [BLBC (P-cad and/or CK14 and/or Vim)] and tumors that were negative for all the basal markers in study were analyzed, p=0.267 (not statistically significant).

Table 8. Distribution of histological grade among triple negative breast carcinomas of the studied series.

	Histological grade		
Triple negative tumors (n=103*)	I	II	III
BLBC (CK5 and/or EGFR+) (n=34)	3 (9%)	12 (35%)	19 (56%)
BLBC (P-cadherin and/or CK14 and/or Vimentin+) (n=32)	2 (6%)	15 (47%)	15 (47%)
Unclassified (TN,CK5, EGFR, P-cad, CK14 and Vim-) (n=37)	17 (46%)	15 (40%)	5 (14%)

BLBC (CK5 and/or EGFR+) are the TN tumors that were positive for CK5 and/or EGFR and BLBC (P-cadherin and/or CK14 and/or Vimentin+) are the TN/CK5 and EGFR- tumors immunoreactive for one of the additional markers in study: P-cadherin, CK14 and vimentin. *: Histological grade of some cases could not be assessed because the patients were submitted to preoperative chemotherapy.

negative tumors ("unclassified" by Nielsen's criterion). In 38/72 (52.8%) cases, none of the biomarkers were expressed; however, in the other 34/72 cases (47.2%), there was the expression of, at least, one of the biomarkers. P-cadherin was present in 29 (40.3%), Vimentin in 18 (25%) and CK14 in 5 (6.9%) of these tumors (Table 7). In a more detailed analysis, 15 cases were positive only for P-cadherin, while only one and three cases were positive for CK14 and for Vimentin alone, respectively (Fig. 2).

Interestingly, if we consider as BLBC these TN/CK5 and EGFR- "unclassified" cases that presented immunoreactivity for P-cadherin, CK14 and/or Vimentin [BLBC (Pcad and/or CK14 and/or Vimentin+)], this series presents 71/387 (18%) of BLBC. BLBC defined by TN/CK5 and/or EGFR+ and BLBC defined as ER/PR/HER2-, CK5/EGFR- and immunoreactivity for P-cadherin and/or CK14 and/or Vimentin were analyzed separately. These two differently defined BLBC presented a similar percentage of high histological grade tumors [56% and 47% in BLBC (CK5 and/or EGFR+) and in BLBC (Pcad and/or CK14 and/or Vimentin+), respectively], (Table 8). The overall survival was similar for the two groups as we can see in Figure 3.

Discussion

The need for a more precise diagnosis of breast cancer that converges with the clinical outcome and the choice of the most appropriate therapy has motivated studies in different areas of breast cancer research. The cDNA microarray technology is a "gold standard" method for the recognition of the basal phenotype, but from a practical point of view, we need to translate these results to an accessible method. It is undeniable that the BLBC immunohistochemistry definition requires cDNA microarray validation, since these tumors were first identified by this technique (Perou et al., 2000; Livasy et al., 2006). However, from the pathologists and oncologists point of view, the lack of molecular targets for therapy in this subgroup of patients indicates the urgent need for an easier and less expensive way to identify BLBC patients. Based on this, there is an attempt to establish an immunohistochemical surrogate panel, easily applied on FFPE samples, which identifies a pool of breast cancer patients who may require more aggressive systemic therapy and that would be the most appropriate subjects for clinical trials, specifically targeting this molecular subgroup of breast cancer. However, there is still no consensual definition about the ideal IHC panel of biomarkers to distinguish the basal phenotype. In fact, many different panels have been used, in which CK5, EGFR, P-cadherin, CK14 and Vimentin are included. Due to this diversity of criteria, a wide range of percentages of BLBC are described in the several studied series (van de Rijn et al., 2002; Foulkes et al., 2004; Jones et al., 2004; Abd El-Rehim et al., 2005; Arnes et al., 2005; Collett et al., 2005; Kusinska et al., 2005; Laakso et al., 2005; Potemski et al., 2005;

Banerjee et al., 2006; Fulford et al., 2006, 2007; Kim et al., 2006; Rakha et al., 2006a,b, 2007a,b,c; Rodriguez-Pinilla et al., 2006, 2007; Siziopikou and Cobleigh, 2007). Nielsen et al. (2004) demonstrated that CK5 and EGFR could reliably discriminate BLBC that were identified by GEP, considering these two basal markers the "gold standard" immunohistochemical panel of antibodies to the BLBC identification, together with ER and HER2 lack of expression. Recently, Cheang et al. (2008) compared two BLBC immuno-panels and concluded that the ER-/PR-/HER2- and expression of CK5 and/or EGFR provides the more accurate definition of BLBC and can better predict breast cancer patient's survival.

However, we cannot assure which are the best antibodies to be included in a daily practice panel for the recognition of the basal phenotype in breast carcinomas: should we look for the most sensitive or the most specific ones? None of these markers are actually pathognomonic of a basal phenotype, since they are variably expressed in the other subgroups of breast carcinomas, which support the search for "ideal" biomarkers to be used in the anatomic pathology workup and with clinical relevance.

We demonstrate herein that P-cadherin, Vimentin or CK14 may possibly be useful biomarkers to include in IHC panels for distinguishing BLBC. P-cadherin reveals consistent values of sensitivity and specificity, while Vimentin and CK14 presented high specificity values. The three markers were able to reliably recognize the basal phenotype, especially when associated to CK5.

The presence of P-cadherin, an adhesion molecule expressed in myoepithelial cells of the normal mammary gland, was already described in invasive and in *in situ* breast carcinomas with worst prognosis, namely in those with high histological grade and basal phenotype (Peralta Soler et al., 1999; Gamallo et al., 2001; Kovacs and Walker, 2003; Paredes et al., 2005, 2007b). The role of P-cadherin in breast carcinogenesis has been one of the main fields of our research group's interest and we have observed that this molecule presents an inverse correlation with HR (Peralta Soler et al., 1999; Gamallo et al., 2001; Kovacs and Walker, 2003; Paredes et al., 2005) and a direct correlation with EGFR (Kovacs and Walker, 2003), HER2 and high proliferation rates, strengthening the value of P-cadherin as a poor prognostic indicator in breast cancer (Palacios et al., 1995; Peralta Soler et al., 1999; Gamallo et al., 2001; Paredes et al., 2005). The expression of P-cadherin in neoplastic cells has already been related to a histogenetic origin in cap cells or to the acquisition of a stem cell-like phenotype, suggesting that P-cadherin-expressing tumors could be associated to a stem cell origin (Peralta Soler et al., 1999, Gamallo et al., 2001, Paredes et al., 2007). Recently, it has been suggested that basal-like breast carcinomas may be genuine stem/early progenitor cell tumors of the mammary gland, relating their origin to a more undifferentiated type of precursor cells (Honeth et al., 2008). Also, Rakha et al. (2009)

demonstrated more evidence of the features of dual-lineage differentiation/stem cell phenotype of BLBC by showing a higher frequency of CK19 expression in this type of tumor.

CK14 does not show a differential presence in breast carcinomas with basal phenotype identified by cDNA microarray technology, but this cytokeratin is frequently associated with poor prognosis (Jones et al., 2004) and with the morphological features observed in BLBC (Tsuda et al., 2000). For this reason, CK14 has been included in the immunopanel used to identify BLBC by several other authors (Laakso et al., 2005, 2006; Rakha et al., 2006a,b; Reis-Filho et al., 2006).

Vimentin is an intermediate filament protein whose expression in normal mammary gland is also restricted to myoepithelial/ basal layer. Its expression has been associated with high histological grade, lack of ER, p53 mutations, high proliferation rates (Raymond and Leong, 1989; Domagala et al., 1990a,b; Koutselini et al., 1995; Santini et al., 1996; Thomas et al., 1999) and expression of CK5/6 and EGFR (Korsching et al., 2005; Reis-Filho, 2005). Vimentin-expressing carcinomas have been observed in association with sporadic and familial BLBC and with a specific pattern of metastasis similar to BLBC (Rodriguez-Pinilla et al., 2007). Like P-cadherin, Vimentin was also described to be differentially expressed by BLBC identified by GEP, being proposed to integrate the panel of antibodies for the identification of BLBC (Livasy et al., 2006).

Our results show that P-cadherin, CK14 and Vimentin, together with CK5, can identify almost all BLBC that were classified as such using the most widely accepted IHC panel to classify BLBC: ER/PR/HER2- and CK5 and/or EGFR+.

Triple negative phenotype by IHC is one of the characteristic features of BLBC and several authors claim that basal tumors are almost all TN tumors (Diaz et al., 2007; Kreike et al., 2007). Kreike et al. (2007), in a series of 97 TN cases, observed that 90% of these tumors have a basal phenotype by cDNA microarray analysis. However, the lack of expression of ER, PR and HER2 as the sole criterion to identify these tumors is risky (Rakha et al., 2008) because there are technique limitations when dealing with FFPE tissue samples, which reinforces the need for a more suitable panel.

There is a significant overlapping of features shared by triple negative and BLBC in what concerns, for example, the prevalence of these types of cancer in younger patients, in African-American women (Morris et al., 2007), their presentation as interval cancers, a similar pattern of recurrence (Dent et al., 2007; Tischkowitz et al., 2007), the more aggressive behavior comparing with other types of breast cancer (Reis-Filho and Tutt, 2008) and the biological and clinical similarity between sporadic TN and BLBC with breast carcinomas arising from BRCA1 mutation carriers (Reis-Filho and Tutt, 2008). However, several studies claim that this overlap is not complete (Bertucci et al., 2008, Rakha and Ellis, 2009). It is known that TN carcinomas with basal

phenotype have a significant shorter disease-free survival than TN without expression of basal markers (Rakha et al., 2007a; Tischkowitz et al., 2007) and that germline BRCA1 mutation carriers are more probably found in TN tumors expressing CK5/6 and /or EGFR than in TN with no expression of these basal markers (Turner et al., 2007; Rakha et al., 2009). It has also been observed in GEP that triple negative group is composed by other subgroups of tumors with different outcomes, namely the normal breast-like tumors (Perou et al., 2000; Sorlie et al., 2001, 2003; Sotiriou et al., 2003; Fan et al., 2006; Hu et al., 2006; Hennessy et al., 2009) and a recently described subgroup of claudin-low tumors (Herschkowitz et al., 2007; Hennessy et al., 2009). The existence of TN tumors that do not react immunohistochemically with any of the basal markers routinely used has been described, and variably designated as non basal triple negative, unclassified, undetermined, null phenotype (Liu et al., 2008) or TN3BKE- (Triple Negative 3 Basal Keratins and EGFR-) (Rakha et al., 2009). It seems extremely important to distinguish BLBC from the whole triple negative group, reducing the TN heterogeneity, since their biological behavior appears to be different. The lightening of this heterogeneity would enable patients to benefit from their differential recognition (Rakha et al., 2007a, 2008, 2009; Liu et al., 2008; Reis-Filho and Tutt, 2008; Tan et al., 2008; Rakha and Ellis, 2009). This distinction is also important because TN tumors defined by IHC tend to be clinically considered as BLBC and selected for clinical trials (Bertucci et al., 2008), probably misleading the effect of the drugs in the clinical trials.

It is interesting to emphasize that among the analyzed TN/CK5 and EGFR- tumors that were also negative for P-cadherin, CK14 and Vimentin, approximately 50% of these cases presented low histological grade (Table 8). P-cadherin was expressed alone in a higher number (15 cases) of TN/CK5 and EGFR negative tumors, compared with CK14 (1 case) and Vimentin (3 cases). When P-cadherin, CK14 and Vimentin expression are considered along with CK5 and EGFR for the BLBC identification, 34 cases are added to the 37 already identified BLBC (CK5 and/or EGFR+) and the percentage of basal-like tumors in the pool of TN cases of our series rounds the 65% (71/109). This rate is similar to the one identified by Bertucci (Bertucci et al., 2008), where 70% of IHQ TN tumors presented a basal phenotype by GEP. It is worth noticing that using P-cadherin, CK14 and Vimentin to recruit BLBC from the pool of tumors that could not be classified using only CK5 and EGFR as basal makers, these newly identified BLBC are clinically similar to basal-like tumors identified by Nielsen's criterion, since the majority of the cases presented high histological grade and there are no significant differences in what concerns overall survival of the patients.

Although CK5 and EGFR have been consistently used to recognize BLBC, P-cadherin, CK14 and

Vimentin could also be recruited for an immunohistochemical recognition of BLBC (Paredes et al., 2002, 2007a,b; Matos et al., 2005; Livasy et al., 2006; Rodriguez-Pinilla et al., 2007). Our results showed that these three markers can reliably identify the basal phenotype, especially when associated to CK5, and can be alternative options in this setting. We also demonstrate that P-cadherin, due to its high sensitivity, can recognize possible BLBC among the IHC TN tumors, probably identifying patients with poor prognosis that can benefit from this differential recognition. Pathologists have faced continuous changes in the diagnostic approach of breast cancer and, regarding its classification, it is still controversial whether or not the histological classification should be replaced by the "molecular" taxonomy. Therefore, it is essential to move towards a standardized methodology to establish an IHC panel of biomarkers to the most appropriate recognition of basal-like breast carcinomas.

Conflict of interest. The authors declare that they have no conflict of interest.

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Alterations in Vitamin D signalling and metabolic pathways in breast cancer progression: a study of VDR, CYP27B1 and CYP24A1 expression in benign and malignant breast lesions

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Alterations in Vitamin D signalling and metabolic pathways in breast cancer progression: a study of VDR, CYP27B1 and CYP24A1 expression in benign and malignant breast lesions

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Abstract

Background: Breast cancer is a heterogeneous disease associated with different patient prognosis and responses to therapy. Vitamin D has been emerging as a potential treatment for cancer, as it has been demonstrated that it modulates proliferation, apoptosis, invasion and metastasis, among others. It acts mostly through the Vitamin D receptor (VDR) and the synthesis and degradation of this hormone are regulated by the enzymes CYP27B1 and CYP24A1, respectively. We aimed to study the expression of these three proteins by immunohistochemistry in a series of breast lesions.

Methods: We have used a cohort comprising normal breast, benign mammary lesions, carcinomas *in situ* and invasive carcinomas and assessed the expression of the VDR, CYP27B1 and CYP24A1 by immunohistochemistry.

Results: The results that we have obtained show that all proteins are expressed in the various breast tissues, although at different amounts. The VDR was frequently expressed in benign lesions (93.5%) and its levels of expression were diminished in invasive tumours (56.2%). Additionally, the VDR was strongly associated with the oestrogen receptor positivity in breast carcinomas. CYP27B1 expression is slightly lower in invasive carcinomas (44.6%) than in benign lesions (55.8%). In contrast, CYP24A1 expression was augmented in carcinomas (56.0% in *in situ* and 53.7% in invasive carcinomas) when compared with that in benign lesions (19.0%).

Conclusions: From this study, we conclude that there is a deregulation of the Vitamin D signalling and metabolic pathways in breast cancer, favouring tumour progression. Thus, during mammary malignant transformation, tumour cells lose their ability to synthesize the active form of Vitamin D and respond to VDR-mediated Vitamin D effects, while increasing their ability to degrade this hormone.

Background

Breast cancer is one of the major causes of death by cancer in women worldwide [1]. Nowadays, breast cancer is no longer considered to be a single disease, but is rather comprised of distinct tumour subtypes displaying different clinical outcomes [2]. Over the lifetime of the individual, in order to a tumour to develop it needs a combination of low-penetrance genetic factors and environmental aspects. Ultimately, cancer results from alterations in the control of the complex balance of proliferation, differentiation and programmed cell death [3] and these processes appear to be regulated by intrinsic and extrinsic factors, like niche signals, hormonal and dietary aspects, among others [4], [5].

Vitamin D is a lipid soluble substance that belongs to the family of secosteroid hormones. Its physiological role has been classically associated with calcium regulation and phosphate transport in bone metabolism. Apart from this endocrine role, subsequent studies have widened the range of functions for Vitamin D and this

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has been particularly important in the field of cancer research. Several authors have demonstrated, in various models of cancer (including the breast), the ability of Vitamin D to perform autocrine and paracrine functions. Specifically, it has been demonstrated the capacity to modulate cancer features, namely proliferation and differentiation [6], apoptosis [7], angiogenesis [8], invasion and metastasis [9].

Vitamin D exerts most of its biological activities by binding to a specific high-affinity receptor, the Vitamin D Receptor (VDR), that was first identified in a breast cancer cell line in 1979 [10]. The VDR belongs to the superfamily of nuclear receptors for steroid hormones and regulates gene expression by acting as a ligand-activated transcription factor [11]. Several studies have demonstrated that the VDR knockout mice display a higher incidence rate of carcinogen-induced preneoplastic breast lesions when compared with their littermates [12], [13]. These reports highlight the importance of the VDR deficiency in sensitizing the mammary gland to transformation in response to a carcinogenic agent. Immunohistochemical studies have confirmed that the VDR is expressed in samples from normal breast tissues [14] and also in breast cancer biopsy specimens [15]. Because the VDR is expressed in the mammary gland and Vitamin D has been shown to display anticarcinogenic properties, this hormone has emerged as a promising targeted therapy. But in order to keep the homeostasis of the organism the amount of circulating Vitamin D has to be tightly regulated. This is a very complex process, in which the main components are the enzymes 1α-hydroxylase/CYP27B1 (encoded by the gene *CYP27B1*) and 24-hydroxylase/CYP24A1 (encoded by the gene *CYP24A1*). CYP27B1 is responsible for the synthesis of the biologically active form of Vitamin D (1,25-dihydroxyvitamin D), whereas CYP24A1 mediates the catabolism of Vitamin D [16]. Several studies have focused their attention in the comparison of the levels of these enzymes in normal and tumour tissue. It has been observed that both CYP27B1 and CYP24A1 are up-regulated in breast tumours when compared with normal tissue. However, deregulated expression of CYP24A1 seems to abrogate the effects of CYP27B1, resulting in the degradation of Vitamin D to less active metabolites [17]. In contrast, a recent paper has demonstrated that CYP27B1 mRNA in breast tumours is decreased in comparison with normal mammary tissue [18]. Despite these findings, no reports regarding the expression by immunohistochemistry of the VDR, CYP27B1 and CYP24A1 in the mammary gland have been described. The main purpose of this work was to perform an immunohistochemical study of the expression of the VDR, CYP27B1 and CYP24A1 in a comprehensive series of human breast tissues comprised of normal breast, benign mammary lesions, carcinomas *in situ* and invasive breast carcinomas.

Methods

Patient's selection and Tissue Microarray construction

We have studied a cohort of 379 benign lesion samples and 189 cases of carcinomas *in situ*, collected from the archives of the Pathology Department of General Hospital of UNIMED in Araçatuba, Brazil. Three hundred and fifty cases of invasive breast carcinomas were retrieved from the archives of the Pathology Department of the Federal University of Santa Catarina, Florianópolis, Brazil (161 cases) and from the Pathology Department of General Hospital of UNIMED in Araçatuba, Brazil (189 tumour samples). This last series of 189 invasive carcinomas contains, in the same block, the aforementioned carcinomas *in situ*. Additionally, 29 cases of normal breast tissue were included in the study. The normal breast tissue, carcinomas *in situ* and invasive tumour samples were collected between 1994 and 2004. The series of benign lesions was collected between 2002 and 2006.

Representative areas of the different lesions were carefully selected on the H&E-stained sections, by 2 pathologists (DV and LAV) and marked on individual paraffin blocks. Two tissue cores (2 mm in diameter) were obtained from each selected specimen and precisely deposited into a recipient paraffin block using a TMA (Tissue Microarray) workstation (TMA builder, LabVision Corporation, USA). Several TMA blocks were constructed (40 for the invasive breast carcinomas, 22 for the carcinomas *in situ* and 17 for the benign lesions), each containing 24 tissue cores, arranged in a 4×6 sector. In each TMA block, at least 3 nonneoplastic breast tissue cores were also included as controls and 1 core of a non-breast sample (we have used testicular and liver tissues). To homogenize the paraffin of the receptor block and the paraffin of the cores extracted from the donor blocks, the TMAs were kept at 37°C for 3 hours. After construction, 2-µm tissue sections were cut and adhered to Superfrost Plus glass slides. An H&E-stained section from each block was reviewed to confirm the presence of morphological representative areas of the original lesions.

The present study has been conducted under the national regulative law for the usage of biological specimens from tumour banks, where the samples are exclusively available for research purposes in the case of retrospective studies.

Immunohistochemistry

Immunohistochemical staining for Oestrogen Receptor (ER), HER2 and CK5 (Cytokeratin 5) was performed

using the streptavidin-biotin-peroxidase technique (Lab-Vision Corporation) in each set of glass slides comprising the TMAs, whereas P-cadherin (P-cad), EGFR (Epidermal Growth Factor Receptor) and Progesterone Receptor (PgR) used the HRP labelled polymer (Dako-Cytomation, USA) as described elsewhere [19]. Antigen unmasking for VDR was performed using a solution of pepsin A (4 g/L; Sigma-Aldrich) for 30 minutes at 37°C. Epitope retrieval for CYP27B1 and CYP24A1 was performed using a dilution of 1:100 of citrate buffer, pH = 6.0 (Vector Laboratories, Burlingame, CA, USA) at 98°C for 30 minutes. The antigen retrieval times, antibodies, dilutions and suppliers are listed in Table 1. Primary antibody incubation was performed overnight at 4°C for VDR and CYP24A1 and for 1 h at room temperature for CYP27B1. After washes, the slides were incubated with secondary antibody associated with HRP labelled polymer (ImmunoLogic, The Netherlands) for VDR or incubated with biotinylated secondary antibody (Santa Cruz, USA) followed by streptavidin-conjugated peroxidase (Labvision) during 15 min for CYP24A1 and CYP27B1, and immediately revealed with DAB (Dako-Cytomation). Tissues were then counterstained with Mayer's haematoxylin, dehydrated and cover-slipped using a permanent mounting solution (Zymed, USA). Positive and negative controls were included in each run in order to guarantee the reliability of the assays. Paraffin sections of a basal cell carcinoma of the skin, normal colon and normal liver were used as positive controls for VDR, CYP27B1 and CYP24A1 expression, respectively.

Scoring and statistical analysis

The evaluation of the immunohistochemical results was performed by three pathologists (FS, FM and LAV). VDR nuclear expression was evaluated using the H-score method: intensity ranked from 1 to 3 (1 - weak, 2 - moderate, 3 - strong), and extension ranked from 1 to 10 (1 - 0-10% cells, 2 - 11-20% cells and so on, until a maximum score of 10) [20]. The scores for intensity and extension were multiplied and the following criterion was applied: the cases were considered negative when ranging from 1 to 4; samples ranking from 5 to 30 were considered to be positive. Considering the lack of previous reports for the immunohistochemical evaluation of the CYP27B1 and CYP24A1, we considered the cases to be positive only when cytoplasmic staining was observed. The other markers were scored as described in previous studies from our group [19], [21].

The Statview 5.0 software package (SAS Institute, USA) was used for all statistical analysis. Correlations between discrete variables were performed using the chi-square test and analysis of variance was employed to search for associations between continuous and discrete variables. In all analyses, a p value < 0.05 was considered to be statistically significant.

Cell culture and Western blotting

MDA-MB-231 breast cancer cells were grown in complete Dulbecco's Modified Eagle Medium (DMEM) in the presence of 10% foetal bovine serum (Invitrogen, USA). Treatments with Vitamin D 100 nM (Cayman Chemical, USA) and ethanol (vehicle) were performed for 72 h, while the treatment with PTH (Parathyroid Hormone) (Sigma-Aldrich, Germany) 100 nM and water (vehicle) were performed for 4 h. Total cell lysates were obtained and the samples were separated in an SDS-polyacrylamide gel. After blotting into a nitrocellulose membrane (GE Healthcare Life Sciences, UK), staining for CYP27B1 and CYP24A1 was performed using the antibodies (Santa Cruz, USA) presented on Table 1 overnight at a dilution of 1:200. After washes, the membranes were incubated with a mouse anti-goat HRP secondary antibody (Santa Cruz) and were revealed with ECL (GE Healthcare Life Sciences).

RNA extraction and Real-time PCR

RNA was extracted from formalin-fixed paraffin-embedded breast lesions using the RecoverAll Total Nucleic Acid Isolation Kit (Ambion, USA), according to the manufacturer's protocol. After extraction, RNA was quantified using NanoDrop spectrophotometer (Thermo Scientific, USA). cDNA was synthesized using the Omniscript Reverse Transcription kit (Qiagen, Germany) following the manufacturer's instructions. Finally, real-time PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems, USA), using 2 mL of cDNA and in accordance to the manufacturer's protocol. The TaqMan Gene Expression Assays used were Hs00172113_m1 (VDR), Hs00168017_m1 (CYP27B1) and Hs00167999_m1 (CYP24A1). Reactions were performed using standard cycle parameters on an ABI PRISM Sequence 7000 Detection System (Applied

Table 1 Sources and dilutions of primary antibodies related to the Vitamin D metabolism used in this study for immunohistochemistry

Antibody	Clone	Manufacturer	Time of incubation (min)	Dilution	Antigen retrieval (min)
VDR	9A7γE10.4	Calbiochem, Germany	overnight	1:50	30
CYP27B1	C12	Santa Cruz, USA	60	1:200	30
CYP24A1	C18	Santa Cruz, USA	overnight	1:75	30

Biosystems). Relative transcript levels were determined using Human GAPDH Endogenous Control (Applied Biosystems) as an internal reference. Differences between the breast tissue samples were determined using comparative delta C_T method [22]. All reactions were done in triplicate and expressed as mean of the values from three separate experiments.

Results

VDR, CYP27B1 and CYP24A1 immunohistochemical staining

The expression patterns of the VDR, CYP27B1 and CYP24A1 have been evaluated by immunohistochemistry in 947 breast tissue samples arranged in 79 TMAs. From this set of cases, some samples could not be assessed due to the fact that either the core had fallen out or it did not have enough biological material to study. In all TMAs, positive and negative cases were obtained for each protein. The immunostainings for these markers had been previously validated in whole tissue sections with an overall agreement of 90%. A panel with representative immunostainings for each protein in different breast tissues is shown in Figure 1. We have observed that the VDR displays nuclear staining, as would be expected from a nuclear receptor which acts as a transcription factor. Considering CYP27B1 and CYP24A1 expression, nothing has ever been described on their expression status in the mammary gland, as far as we know. This is the first report showing the expression of these two enzymes in breast lesions. These proteins present cytoplasmic and granular staining, which could reflect their mitochondrial localisation. All proteins (VDR, CYP27B1 and CYP24A1) have been found to be expressed in all lesions studied and also in the normal breast tissue, although at different levels.

The differential expression of CYP27B1 and CYP24A1 was technically validated. MDA-MB-231 breast cancer cells have been treated with PTH 100 nM and Vitamin D 100 nM and total cell lysates have been extracted. Western blotting analysis has confirmed the expression of CYP27B1 and CYP24A1 upon treatment with the aforementioned hormones (Additional file 1: Figure S1). Additionally, using a group of randomly selected tissue samples, RNA was isolated and used in real-time PCR to confirm the immunohistochemical results (Additional file 2: Table S1). Our results have shown that positive cases in the TMAs displayed cDNA amplification in the real-time PCR and the opposite situation was observed for cases where no staining was present in the TMAs.

Expression of the VDR, CYP27B1 and CYP24A1 in benign lesions of the mammary gland

In order to study the VDR, CYP27B1 and CYP24A1 expression in benign lesions of the mammary gland, we have evaluated 379 cases arranged in 17 TMAs. The series consisted of a variety of breast lesions, namely usual and atypical ductal hyperplasias (UDH represent 20.1%, corresponding to 76 samples; while ADH represent 5.4%, corresponding to 21 samples), columnar cell lesions (CCL - 25.6% of cases, corresponding to 97 samples), papillomatosis (16.9% of cases, corresponding to 64 samples) and adenosis (17.2% of cases, corresponding to 65 samples). The percentage of immunoreactive cases for the VDR was very high (93.5%, corresponding to 259 cases out of 277). Regarding the expression of CYP27B1, we have observed 55.8% of positive cases, corresponding to 173 lesions out of 310. Concerning CYP24A1 expression, we have detected 62 positive cases out of 327 samples (19.0%). Amongst all lesions, ADH cases were overall less immunoreactive to the three proteins.

We have correlated the histological classification of the benign lesions with the VDR, CYP27B1 and CYP24A1 expression, but no significant associations have been found (see Table 2 for further details).

Expression of the VDR, CYP27B1 and CYP24A1 in breast carcinomas *in situ*

A fully characterized series of 189 breast carcinomas *in situ* arranged in 22 TMAs was assessed for the expression patterns of VDR, CYP27B1 and CYP24A1. For the VDR, we have observed that 62 cases out of 131 cases (47.3%) displayed staining for this protein. Concerning CYP27B1 expression, we have encountered positive staining in 66.4% of the cases (91 out of 137 samples); whereas CYP24A1 expression was observed in 56.0% of the tumours (70 out of 125 cases).

We have also assessed the expression of other breast cancer biomarkers in our cohort (ER, HER2 and PgR and basal markers as defined by our group [19] and others [23]) and looked for the existence of correlations between the expression of the Vitamin D partners and these molecular markers (Table 3). ER expression has been observed in 117 cases (61.9%), HER2 protein was present in 37 cases (15.6%) and PgR expression was detected in 90 cases (47.6%). We have also tested our series for basal markers and have obtained the following results: EGFR expression is present in 10 cases (5.3%), CK5 is positive in 15 cases (7.9%) and P-cadherin was observed in 36 samples (19.0%). Expression of the VDR correlated positively with ER status (p = 0.0227), with a higher percentage of VDR-positive cases among the ER-positive tumours - 74.2% (46 out of 62 cases). Additionally, we have seen that there is an inverse correlation between the expression of the VDR and P-cadherin (p = 0.0078). CYP27B1 expression only presented an inverse correlation (p = 0.0295) with EGFR expression, but the number of cases positive for EGFR was very low. No statistically significant associations have been observed between CYP24A1 expression and the markers studied.
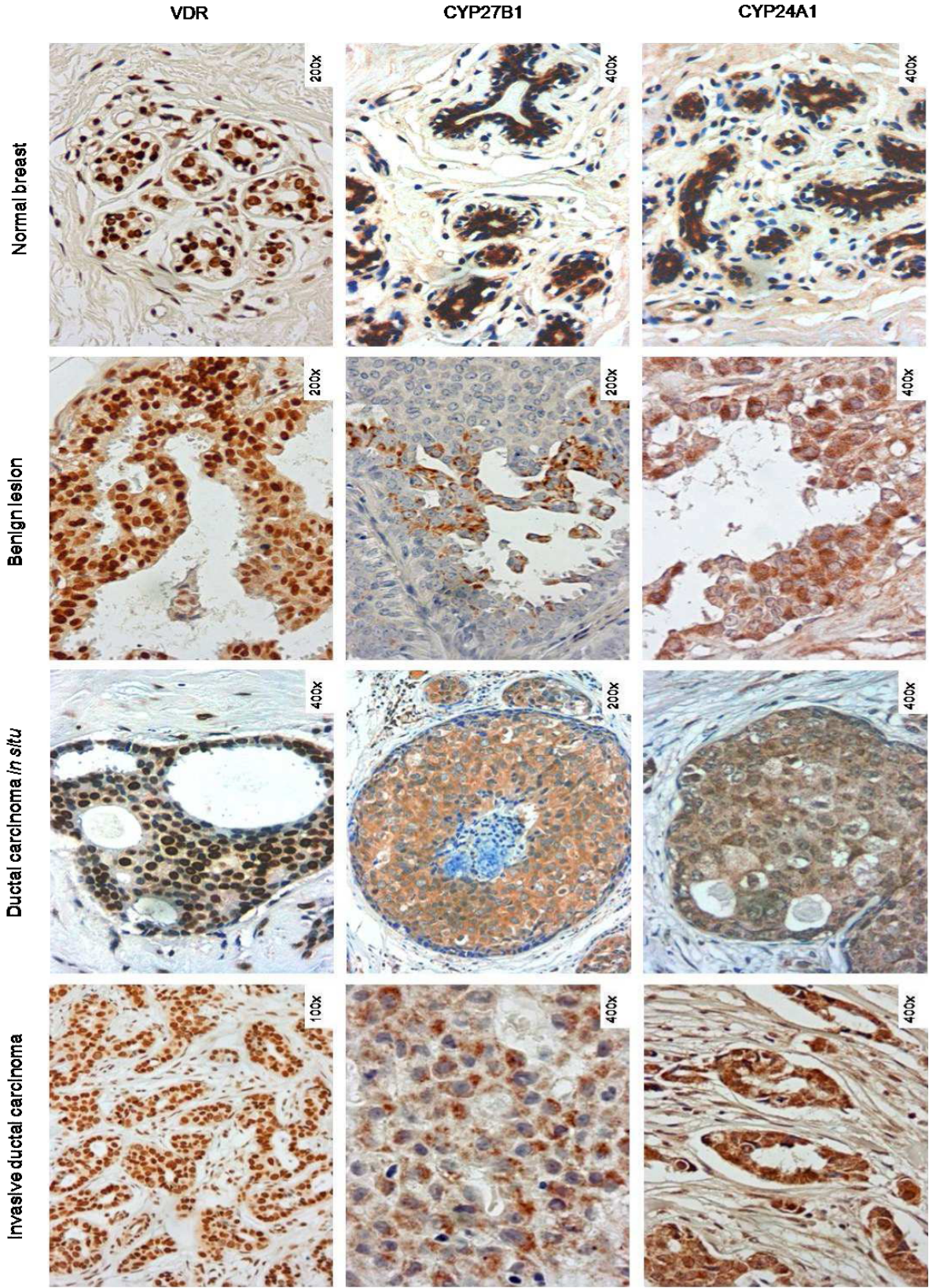
Figure 1 Immunohistochemical staining for the VDR, CYP27B1 and CYP24A1 in the different types of breast tissue

Table 2 VDR, CYP27B1 and CYP24A1 expression in the various types of benign breast lesions

	VDR		CYP27B1		CYP24A1	
	+ (%)	- (%)	+ (%)	- (%)	+ (%)	- (%)
Usual ductal hyperplasia	84 (92.3)	7 (7.7)	57 (55.9)	45 (44.1)	23 (20.5)	89 (79.5)
Atypical ductal hyperplasia	9 (81.8)	2 (18.2)	4 (36.4)	7 (63.6)	1 (7.1)	13 (92.9)
Columnar cell lesions	63 (95.5)	3 (4.5)	43 (55.8)	34 (44.2)	13 (16.5)	66 (83.5)
Papillomatosis	45 (95.7)	2 (4.3)	30 (56.6)	23 (43.4)	9 (17.0)	44 (83.0)
Adenosis	49 (92.5)	4 (7.5)	32 (55.2)	26 (44.8)	13 (22.0)	46 (78)
p value	**0.4847**		**0.7994**		**0.6842**	

Expression of the VDR, CYP27B1 and CYP24A1 in invasive mammary carcinomas

We have evaluated 350 cases of invasive breast carcinomas arranged in 40 TMAs. The cohort corresponds to 189 cases of the series for which there was an *in situ* component in the adjacent area of the invasive tumour and an additional series of 161 cases of invasive breast carcinomas. Positive staining for the VDR has been observed in 56.2% of the cases (172 out of 306 cases). Regarding CYP27B1 expression, 44.6% of cases were positive (123 out of 276 samples), whereas 53.7% of cases (151 out of 281 tumours) presented positivity for CYP24A1.

Next, we searched for associations between the expression of Vitamin D partners and the expression of the molecular markers mentioned in the previous section (Table 4). We have obtained 197 cases (56.3%) positive for ER, 70 cases (20%) for HER2 and 143 cases (40.9%) for PgR. As for basal markers, we have observed

that 13 cases (3.7%) were positive for EGFR expression, 48 cases (13.7%) presented positivity for CK5 and 93 cases (26.6%) stained for P-cadherin.

A statistically significant association was observed between the VDR-positive cases and ER-positive cases (p = 0.0002). Additionally, VDR-positive cases have also been significantly correlated with HER2-negative cases (p = 0.0238), but this is probably due to the low number of positive cases for HER2 in our series of mammary carcinomas. CYP27B1 expression presented no significant associations with any of the markers analyzed. PgR was the only marker that displayed an inverse correlation with CYP24A1: specifically, cases positive for PgR were mostly negative for CYP24A1 (p = 0.0485).

The series of 189 tumours with both components (carcinomas *in situ* and the corresponding invasive tumour) allowed the evaluation of the expression of the VDR, CYP27B1 and CYP24A1 simultaneously in the

Table 3 VDR, CYP27B1 and CYP24A1 and other breast cancer biomarkers expression in carcinomas *in situ*

		VDR		CYP27B1		CYP24A1	
		+ (%)	- (%)	+ (%)	- (%)	+ (%)	- (%)
ER	+ (%)	46 (35.1)	38 (29.0)	58 (42.3)	29 (21.2)	41 (32.8)	36 (28.8)
	- (%)	16 (12.2)	31 (23.7)	33 (24.1)	17 (12.4)	29 (23.2)	19 (15.2)
	p value	**0.0227**		ns		ns	
HER2	+ (%)	9 (6.9)	14 (10.7)	18 (13.1)	7 (5.1)	9 (7.2)	12 (9.6)
	- (%)	53 (40.5)	55 (42.0)	73 (53.3)	39 (28.5)	61 (48.8)	43 (34.4)
	p value	ns		ns		ns	
PgR	+ (%)	35 (26.7)	30 (22.9)	49 (35.8)	18 (13.1)	38 (30.4)	22 (17.6)
	- (%)	27 (20.6)	39 (29.8)	42 (30.7)	28 (20.4)	32 (25.6)	33 (26.4)
	p value	ns		ns		ns	
CK5	+ (%)	3 (2.3)	8 (6.1)	7 (5.1)	4 (2.9)	8 (6.4)	4 (3.2)
	- (%)	59 (45.0)	61 (46.6)	84 (61.3)	42 (30.7)	62 (49.6)	51 (40.8)
	p value	ns		ns		ns	
EGFR	+ (%)	1 (0.8)	5 (3.8)	2 (1.5)	5 (3.7)	5 (4.0)	3 (2.4)
	- (%)	61 (46.6)	64 (48.9)	89 (65.0)	41 (29.9)	65 (52.0)	52 (41.6)
	p value	ns		**0.0295**		ns	
P-cad	+ (%)	4 (3.1)	16 (12.2)	14 (10.2)	12 (8.8)	16 (12.8)	7 (5.6)
	- (%)	58 (44.3)	53 (40.5)	77 (56.2)	34 (24.8)	54 (43.2)	48 (38.4)
	p value	**0.0078**		ns		ns	

ns: not significant.

Table 4 VDR, CYP27B1 and CYP24A1 and other breast cancer biomarkers expression in invasive breast tumours

		VDR		CYP27B1		CYP24A1	
		+ (%)	- (%)	+ (%)	- (%)	+ (%)	- (%)
ER	+ (%)	114 (37.3)	60 (19.6)	70 (25.4)	86 (31.2)	93 (33.1)	66 (23.5)
	- (%)	58 (19.0)	74 (24.2)	53 (19.2)	67 (24.3)	58 (20.6)	64 (22.8)
	p value	0.0002		ns		ns	
HER2	+ (%)	26 (8.6)	34 (11.3)	31 (11.4)	25 (9.2)	29 (10.4)	30 (10.8)
	- (%)	144 (47.7)	98 (32.5)	90 (33.1)	126 (46.3)	121 (43.5)	98 (35.3)
	p value	0.0238		ns		ns	
PgR	+ (%)	71 (23.3)	59 (19.3)	52 (18.8)	64 (23.2)	71 (25.3)	46 (16.4)
	- (%)	100 (32.8)	75 (24.6)	71 (25.7)	89 (32.2)	80 (28.5)	84 (29.9)
	p value	ns		ns		0.0485	
CK5	+ (%)	27 (8.8)	19 (6.2)	15 (5.4)	24 (8.7)	27 (9.6)	16 (5.7)
	- (%)	145 (47.4)	115 (37.6)	108 (39.1)	129 (46.7)	124 (44.1)	114 (40.6)
	p value	ns		ns		ns	
EGFR	+ (%)	4 (1.3)	7 (2.3)	4 (1.5)	6 (2.2)	6 (2.1)	3 (1.1)
	- (%)	166 (54.8)	126 (41.6)	118 (43.1)	146 (53.3)	145 (51.8)	126 (45.0)
	p value	ns		ns		ns	
P-cad	+ (%)	42 (13.8)	40 (13.1)	30 (10.9)	42 (15.2)	40 (14.3)	37 (13.2)
	- (%)	129 (42.3)	94 (30.8)	93 (33.7)	111 (40.2)	110 (39.3)	93 (33.2)
	p value	ns		ns		ns	

ns: not significant.

two types of tumours (Additional file 2: Table S2). The results obtained show that the three proteins (VDR, CYP27B1 and CYP24A1) display a statistically significant correlation of expression between the two sections (carcinomas *in situ* and the matching invasive tumour). Thus, positive cases in the *in situ* component are also positive in the invasive component and the same is observed for the negative cases.

Expression of the VDR, CYP27B1 and CYP24A1 according to the type of breast lesion

The frequencies of protein expression of the VDR, CYP27B1 and CYP24A1 in the different mammary tissues are shown in Figure 2. The normal mammary gland (29 cases), as expected, is positive for the expression of the VDR in all the cases studied (100%). The majority of the samples also displays immunostaining for CYP27B1 (63.6%) and, in contrast, the levels of expression of CYP24A1 are low (29.6%). The VDR is also highly expressed in benign lesions (93.5%) with a reduction in the percentage of positive cases in carcinomas *in situ* (47.3%) and in invasive carcinomas (56.2%). CYP27B1 expression does not vary greatly between the different breast lesions. However, between *in situ* and invasive carcinomas, a statistically significant decrease in the percentage of positive cases was observed (from 66.4% in carcinomas *in situ* to 44.6% in invasive carcinomas). In contrast, the expression of CYP24A1 is increased in carcinomas (56.0% in carcinomas *in situ*

and 53.7% in invasive carcinomas) compared with the benign lesions (19.0%), which are mostly negative.

Discussion

Vitamin D mediates anti-proliferative and pro-differentiation signalling in various epithelial tissues, including
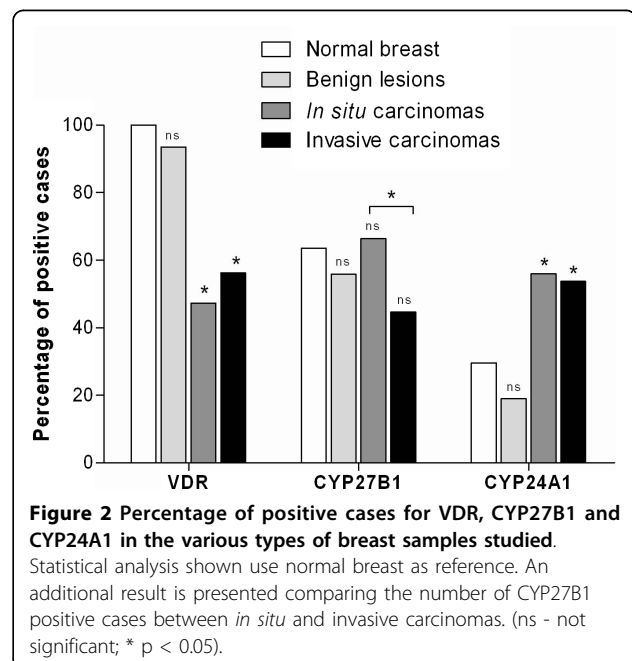

Figure 2 Percentage of positive cases for VDR, CYP27B1 and CYP24A1 in the various types of breast samples studied. Statistical analysis shown use normal breast as reference. An additional result is presented comparing the number of CYP27B1 positive cases between *in situ* and invasive carcinomas. (ns - not significant; * p < 0.05).

the mammary gland [6]. Therefore, it is reasonable to assume that disruption of the Vitamin D signalling and metabolic pathways may occur during tumour development. To explore this hypothesis, we have evaluated a cohort of 947 samples of human breast tissues for the presence of VDR, CYP27B1 and CYP24A1. Specifically, our series consisted of normal breast tissue (29 cases), preneoplastic benign mammary lesions (379 cases), carcinomas *in situ* (189 cases) and invasive breast carcinomas (350 cases). To the best of our knowledge, this is the first time that the expression of the VDR, CYP27B1 and CYP24A1 has been evaluated in histological sections of mammary lesions.

The three proteins have been found to be expressed in all breast tissues, although at different levels. VDR presented a nuclear localisation, as it would be expected for a nuclear receptor, while CYP27B1 and CYP24A1 enzymes displayed cytoplasmic staining with a granular pattern, which is consistent with their mitochondrial localisation. The immunohistochemical results were further validated and confirmed using quantitative real-time PCR and Western blotting.

Some studies have demonstrated that the VDR protein is expressed in samples from normal breast tissues and also in breast cancer biopsy specimens [14,15,24,25]. Our results have shown that the VDR is expressed in carcinomas. However, the percentage of positive cases that we have obtained (47.3% in carcinomas *in situ* and 56.2% in invasive carcinomas) is lower than the 80% to 90% that had been previously described in the literature [26,27]. This discrepancy can be explained by the development of new detection techniques and the use of different scoring methods. In this study, we have used the H-Score, the current method employed for other nuclear receptors, like ER [20], whereas in previous studies the presence of any staining was marked as positive. As far as we know, our study is the first to investigate the immunohistochemical expression of the VDR in a range of benign lesions and carcinomas *in situ* of the mammary gland. The percentage of positive cases for the VDR is higher in benign lesions than in invasive tumours (93.5% and 56.2%, respectively), while the carcinomas *in situ* display the lowest value of all (47.3%). There are some studies showing higher levels of VDR in tumour tissues [18,28], but this discrepancy can be attributed to the use of different evaluation techniques.

An interesting finding is the correlation between the expression of the VDR and the ER in both *in situ* and invasive carcinomas. In fact, the VDR is expressed in most ER-positive cases (54.7% in *in situ* carcinomas and 65.5% in invasive tumours). It is thought that one of the VDR functions is to counteract oestrogen-mediated proliferation and maintain differentiation [12]. Indeed, data support the concept that the anti-tumour effects of

Vitamin D and its analogues on ER-positive human breast cancer cells are mediated through the down regulation of the ER itself and the attenuation of oestrogen responses, such as breast cancer cell growth [29,30]. Thus, being the VDR mostly expressed in ER-positive carcinomas, Vitamin D or its analogues may become an alternative therapy for these tumours in cases of resistance to ER-targeted therapy.

The levels of protein expression of CYP27B1 and CYP24A1 have not been previously studied in breast cancer. In colon cancer, a study using immunohistochemistry has demonstrated that CYP27B1 is present at equally high levels in normal colonic epithelium and colorectal cancer [31]. For CYP24A1 it has been shown that increasing amounts of this enzyme are present in normal colon tissue and pre-malignant lesions. In cancer, the expression of CYP24A1 decreases as a function of tumour cell dedifferentiation [32]. In breast tissues, McCarthy *et al.*[18] have demonstrated that CYP27B1 mRNA expression was significantly down regulated in adjacent non-cancerous tissue from women with breast cancer in comparison with individuals without cancer. Additionally, it has been shown that the expression of mRNA for CYP27B1 and the VDR was higher in carcinomas versus non-neoplastic tissue [17]. Considering differences in expression in benign and malignant breast tissues, we have observed an increased expression of CYP24A1 and a decreased expression of CYP27B1 with malignant progression. In fact, CYP27B1 was expressed in 55.8% of the preneoplastic lesions and this percentage is decreased in invasive tumours (44.6%), while carcinomas *in situ* display the highest value (66.4%) and these differences are statistically significant. In contrast, CYP24A1 is augmented more than 2.5 fold in invasive tumours (53.7%), compared with benign breast lesions (19.0%) and this difference is also significant (p < 0.0001). The *in situ* carcinomas exhibit the highest percentage of positive cases (56.0%). These observations are consistent with the results of Townsend and colleagues [17], which have demonstrated that there was an up regulation of CYP24A1 mRNA in breast tumour tissue, in comparison with normal breast. It has also been described that the *CYP24A1* gene is amplified in breast cancer [33]. In contrast, another study has found no differences in the expression of the VDR, CYP27B1 and CYP24A1 mRNA in breast cancer and non-neoplastic mammary tissue [34]. These contradictory results may be explained by recent reports where it is described that VDR and CYP24A1 are under the post-transcriptional control of miRNAs [35,36].

Breast cancer is a process that evolves through the accumulation of (epi)genetic events that drive uncontrolled proliferation and resistance to apoptosis. The active form of Vitamin D is known for its capacity to modulate proliferation and induce apoptosis [6]. Consequently, malignant cells would need to develop

mechanisms to deregulate Vitamin D metabolic and signalling pathways in order to allow tumour development [37]. Furthermore, it has been suggested that the Vitamin D produced in non-renal tissues is not released into the blood stream, but instead acts locally [38]. Therefore, the amount of Vitamin D available in the tissue depends on the relative amounts of CYP27B1 (synthesis) and CYP24A1 (catabolism). Accordingly, our results show a deregulation of these two enzymes in the different stages of breast carcinogenesis. The crucial step of transformation introduces a clear unbalance in the Vitamin D signalling and metabolic pathways. A reduction in the expression of the VDR in carcinomas indicates lower sensitivity of the tissue to Vitamin D control. Furthermore, a strong increase in CYP24A1 positive cases points to an enhanced ability of the cells to degrade this hormone. In contrast, the stable levels of CYP27B1 throughout the transformation process, with only a small decrease in invasive carcinomas, may reflect a lower capacity to metabolize Vitamin D into its active form.

Conclusions

In summary, this is the first study to report the expression of the VDR, CYP27B1 and CYP24A1 in a series of normal breast, preneoplastic mammary lesions, breast carcinomas *in situ* and invasive tumours. We have correlated the expression of these Vitamin D partners with the expression of a panel of tumour biomarkers. Furthermore, we have confirmed these results by real-time RT-PCR. Overall, our results on the expression of the VDR, CYP27B1 and CYP24A1 suggest that there is a deregulation of the Vitamin D metabolic and signalling pathways in breast cancer, in order to favour tumour progression. Thus, during breast malignant transformation, tumour cells lose their ability to synthesize the active form of Vitamin D and to respond to Vitamin D effects, while increasing their ability to degrade this hormone.

Additional material

Additional file 1: Figure S1: In MDA-MB-231 breast cancer cells CYP27B1 expression is induced by the treatment with PTH 100 nM for 4 h and CYP24A1 expression is induced by the treatment with Vitamin D $(1,25(OH)_2D_3)$ 100 nM for 72 h. α-tubulin was used as a loading control

Additional file 2: Table S2: VDR, CYP27B1 and CYP24A1 expression in tumours that display both the *in situ* and the invasive component in the same histological section.

List of abbreviations

VDR: Vitamin D Receptor; TMA: Tissue Microarray; ER: oestrogen receptor; CK: Cytokeratin; EGFR: Epidermal Growth Factor Receptor; UDH: Usual Ductal Hyperplasia; ADH: Atypical Ductal Hyperplasia; CCL: Columnar Cell Lesions

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1alpha,25-dihydroxyvitamin D_3 induces *de novo* E-cadherin expression in triple-negative breast cancer cells by *CDH1* promoter demethylation

1Alpha,25-dihydroxyvitamin D$_3$ Induces *de novo* E-cadherin Expression in Triple-negative Breast Cancer Cells by *CDH1*-promoter Demethylation

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Abstract. *Background: The triple-negative subgroup of breast cancer includes a cluster of tumors exhibiting low E-cadherin expression (metaplastic carcinomas). In several cancer models, 1alpha,25-dihydroxyvitamin D$_3$ (1α,25(OH)$_2$D$_3$) induces differentiation by increasing E-cadherin expression. The Vitamin D receptor (VDR) was evaluated as a possible therapeutic target for metaplastic carcinomas and 1α,25(OH)$_2$D$_3$ effects as a differentiating agent in triple-negative breast cancer cells were assessed. Materials and Methods: Metaplastic carcinomas were assessed for VDR expression by immunohistochemistry; differences in E-cadherin expression in triple-negative breast cancer cells were evaluated by real-time PCR, western blotting and Cadherin 1 (CDH1) methylation status. Results: Most of the metaplastic carcinomas were positive for VDR expression. Furthermore, 1α,25(OH)$_2$D$_3$ promoted differentiation of MDA-MB-231 cells by inducing de novo E-cadherin expression, an effect that was time- and dose-dependent. Also, E-cadherin expression was due to promoter demethylation. Conclusion: Metaplastic carcinomas may respond to 1α,25(OH)$_2$D$_3$, since they express VDR and 1α,25(OH)$_2$D$_3$ induces de novo E-cadherin expression in breast cancer cells by promoter demethylation.*

Breast cancer is a heterogeneous disease, comprised of diverse molecular subtypes associated with different biological behaviours and clinical outcomes (1, 2). Among all breast cancer subgroups, the triple-negative basal-like type is the most aggressive, presents poor patient outcome

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(2) and comprises a rare cluster of carcinomas entitled metaplastic tumors (3-5). Our group and others have demonstrated that metaplastic carcinomas are distinguished by high levels of expression of classical basal-like markers, such as cytokeratin (CK) 5/6, CK14, epidermal growth factor receptor (EGFR), vimentin and P-cadherin, as well as E-cadherin down-regulation (5-7). Furthermore, patients harbouring metaplastic tumors display a worse prognosis, exhibiting lower rates of disease-free survival than those with invasive ductal carcinomas (8, 9). Due to their triple-negative phenotype, metaplastic carcinomas do not have a directed therapy. Since radiation and chemotherapy remain the only options to treat these carcinomas, intensive research on alternative therapeutic strategies is mandatory.

1Alpha,25-dihydroxyvitamin D$_3$ (1α,25(OH)$_2$D$_3$), the biologically active form of vitamin D, is a steroid hormone that exerts most of its biological activities by binding to a specific high-affinity receptor, the vitamin D receptor (VDR) (10). We previously reported that 56% of invasive breast carcinomas express the VDR and, among these, 56% of the cases classified as triple-negative basal-like tumors are positive for VDR expression (11), suggesting that they may be responsive to the anti-carcinogenic properties of 1α,25(OH)$_2$D$_3$. In several cancer models, 1α,25(OH)$_2$D$_3$ participates in cell growth regulation and cell differentiation (12). In breast cancer cells, it was demonstrated that 1α,25(OH)$_2$D$_3$ is able to induce cells to be more adhesive to each other, as well as to some substrates, through an increase in the expression of endogenous E-cadherin and other adhesion molecules (13). Additionally, 1α,25(OH)$_2$D$_3$ promotes the differentiation of colon cancer cells by inducing the expression of E-cadherin in VDR-expressing cells (14) and a similar result was obtained in prostate cancer with a 1α,25(OH)$_2$D$_3$ analogue (15).

These data provide good evidence for the ability of 1α,25(OH)$_2$D$_3$ to act as an epithelial differentiation-inducing agent. Therefore, the purpose of the current work was to study if the VDR could be a potential therapeutic target for

metaplastic triple-negative breast carcinomas. Additionally, the *in vitro* effects of $1\alpha,25(OH)_2D_3$ as a differentiating agent in triple-negative breast cancer cell lines were evaluated.

Materials and Methods

Immunohistochemistry. A series of 12 formalin-fixed paraffin-embedded metaplastic breast carcinomas were retrieved from the archives of the Federal University of São Paulo, Brazil and from the Federal University of Santa Catarina, Brazil. The cases were collected between 1994 and 2009. Immunohistochemical staining for the VDR was performed as described elsewhere (11).

Cell culture and treatments. All the breast cancer cells (MDA-MB-231, Hs578T and BT-549, commercially available from ATCC), representative of mesenchymal triple-negative breast cancer (16, 17) were grown in complete GIBCO, Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA, USA) in the presence of 10% foetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Treatments with $1\alpha,25(OH)_2D_3$ 100 nM (Cayman Chemical, Denver, CO, USA), 5-aza-2-deoxycytidine 5 μM (5-aza-dC, Sigma, Munich, Germany), DMSO (dimethyl sulfoxide, vehicle for 5-aza-dC and Trichostatin A [TSA]) and ethanol (vehicle for $1\alpha,25(OH)_2D_3$) were performed for 72 hours, while the treatment with TSA 100 nM (Sigma) was performed only for 16 hours. Every 24 hours, the culture medium was changed and a fresh new treatment agent was added.

Western blotting. Total protein lysates were prepared from the cultured cells and the protein concentration was determined using the Bradford assay (Bio-Rad protein quantification system, Berkeley, CA, USA). Equal protein samples were separated in an 8% SDS-PAGE and the proteins were transferred onto nitrocellulose membranes (GE Healthcare Life Sciences, Chalfont St Giles, UK). For immunostaining, the membranes were blocked for non-specific binding with 5% (w/v) non-fat dry milk, in PBS containing 0.5% (v/v) Tween-20. The membranes were incubated with the primary antibodies (α-tubulin, clone DM1A, Sigma, 1:10000 for 1 hour; β-actin, clone I19, Santa Cruz [Santa Cruz, CA, USA], 1:1000 for 1 hour; E-cadherin, clone 24E10, Cell Signaling [Beverly, MA, USA], 1:1000 for 1 hour; and VDR, clone 9A7γE10.4, Calbiochem [Darmstadt, Germany], 1:400 overnight), followed by four 5 min washes in PBS/Tween-20; then they were incubated with horseradish peroxidase-conjugated secondary antibodies (all 1:1000, Santa Cruz) for 60 min. The membranes were then washed six times more for 5 min and the proteins detected using the ECL detection system (GE Healthcare Life Sciences).

RNA extraction, cDNA synthesis and quantitative real-time PCR. The RNA was extracted from the breast cancer cells using TRIzol® reagent (Invitrogen) and cDNA was synthesised from 1 μg of RNA, using an Omniscript Reverse Transcription kit (Qiagen, Düsseldorf, Germany), following the manufacturer's instructions. Real-time PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad, CA, USA), using 1 μL of cDNA and in accordance with the manufacturer's protocol. The TaqMan Gene Expression Assays used were Hs01023895_m1 (for *CDH1* [Cadherin 1], Applied Biosystems) and TaqMan PreDeveloped Assay Reagents Human GAPDH (for *GAPDH* [Glyceraldehyde 3-

phosphate dehydrogenase], Applied Biosystems). The reactions were performed using standard cycle parameters and relative transcript levels were determined using human *GAPDH* as an internal reference. Differences between samples were determined using the Quantitation–Relative Standard Curve method.

DNA extraction and CDH1 promoter methylation analysis. The DNA was extracted from the breast cancer cell lines using an ULTRAPrep Genomic DNA Blood and Cell Culture Kit (AHN Biotechnologie, Nordhausen, Germany), according to the manufacturer's instructions. Bisulfite treatment was performed on 300 ng of DNA, using an EpiTect Bisulfite kit (Qiagen) following the manufacturer's guidelines. Unmethylated cytosines were converted to uracil, whereas methylated ones remained unmodified. The 12 CpG sites (cytosine-phosphate-guanine) within the 90 base pairs upstream of the *CDH1* translation start site (ATG) were analysed, as described elsewhere (18).

Immunofluorescence. The cells were seeded on coverslips and fixed with formaldehyde 4% (v/v) for 30 min The coverslips were washed three times with PBS for 5 min, followed by incubation with 50 mM NH4Cl in PBS for 10 min. Following another set of three 5 minute washes with PBS, the coverslips were incubated with Triton X-100 0.2% (v/v) for 5 min and washed with PBS three times for 5 min. Subsequently, they were blocked for non-specific binding with BSA 5% in PBS, containing 0.5% (v/v) Tween-20, for 30 min and incubated with the primary antibody for E-cadherin (Zymed, San Francisco, CA, USA, clone HECD1, 1:100) for 1 hour. After three 5 minute washes with PBS, the coverslips were incubated with a goat anti-mouse secondary antibody (Alexa Fluor 594, 1:500, Invitrogen), washed with PBS for 3 times 5 min and mounted using Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA).

Transfection with siRNA for VDR. MDA-MB-231 cells (2.5×10^5 cells) were cultured in 6-well plates for 24 hours. For each well, 150 nmol of siRNA against VDR (Hs_VDR_8 FlexiTube siRNA, Qiagen) or control siRNA (Qiagen) was used according to the manufacturer's instructions. After 5 hours of incubation, the cell medium was replaced and the cells were treated with $1\alpha,25(OH)_2D_3$ 100 nM and ethanol. The evaluation of siRNA efficiency occurred 48 hours after transfection.

Statistical analysis. Differences between groups were assessed using Student's *t*-test. Differences with *p*-values <0.05 were considered statistically significant. All the presented results are representative of at least three independent experiments, unless stated otherwise.

Results

VDR expression in metaplastic breast carcinomas. Out of the 12 metaplastic breast carcinomas, 8 cases (66.7%) were positive for the expression of VDR (Figure 1).

VDR expression in triple-negative breast cancer cell lines. By western blotting, it was shown that all the cell lines studied were positive for VDR expression. The MDA-MB-231 and BT-549 cells seem to be more sensitive to $1\alpha,25(OH)_2D_3$, as in these cells there was a clear increase in VDR expression upon hormonal treatment (Figure 2).

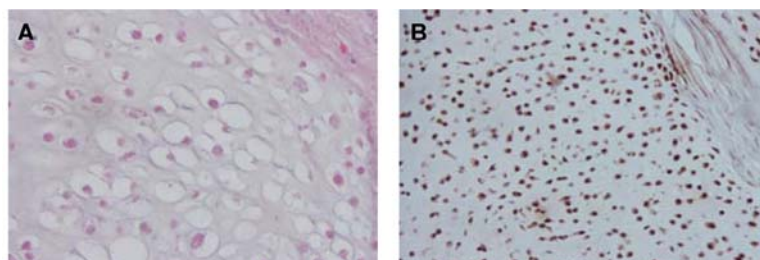Figure 1. *H&E, magnification ×630 (A) and VDR, magnification ×400 (B) staining in a case of metaplastic breast carcinoma.*
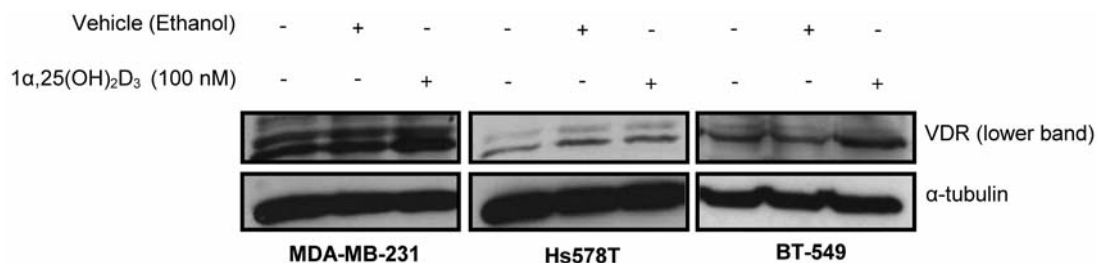

Figure 2. *Western blot of VDR expression in MDA-MB-231, Hs578T and BT-549 breast cancer cell lines.*

Effect of $1\alpha,25(OH)_2D_3$ on the expression of E-cadherin. A *de novo* expression of E-cadherin, by western blotting, was observed upon $1\alpha,25(OH)_2D_3$ treatment in the MDA-MB-231 cells (Figure 3A). As presented in Figure 3B, the expression of E-cadherin was dependent on the duration of treatment. The protein expression was first detected at 24 hours and increased with time. With 72 hours of treatment the E-cadherin expression level was dependent on the dose of $1\alpha,25(OH)_2D_3$ and was identified even with the very low dose of 1 nM (Figure 3C).

In the MDA-MB-231 cells, $1\alpha,25(OH)_2D_3$ was a potent inducer of *CDH1* mRNA expression, displaying more than 10-fold induction, compared with the control ($p < 0.01$) (Figure 4A). Furthermore, the level of expression induced by $1\alpha,25(OH)_2D_3$ was 2-fold higher than that produced by the demethylating agent 5-aza-dC alone and 3-fold higher than that induced by the histone deacetylation (HDAC) inhibitor agent TSA alone. However, both agents displayed an additive effect to $1\alpha,25(OH)_2D_3$, the highest levels of expression being induced when the three drugs were combined. These results were also confirmed by the protein expression (Figure 4A). In the BT-549 cells, $1\alpha,25(OH)_2D_3$ was unable to induce E-cadherin expression on its own. However, in the Hs578T cells CDH1 expression was significantly induced upon $1\alpha,25(OH)_2D_3$ treatment (Figure 4B). Furthermore, in both cell lines *CDH1* mRNA expression was induced upon treatment with 5-aza-dC. Interestingly, $1\alpha,25(OH)_2D_3$ seemed to display an additive effect when administered with both 5-aza-dC and TSA.
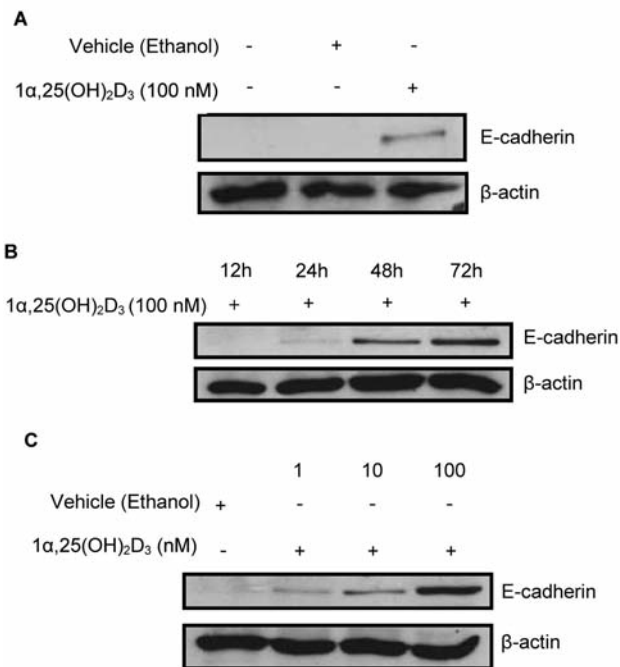

Figure 3. *Effect of $1\alpha,25(OH)_2D_3$ on E-cadherin expression in MDA-MB-231 breast cancer cells treated for 72 hours (A) or for various times (B) or at various dose rates (C), and assessed by western blotting.*

Again, the highest levels of CDH1 expression were achieved whenever all the agents were added together and, in this case, the BT-549 cells were more responsive than the
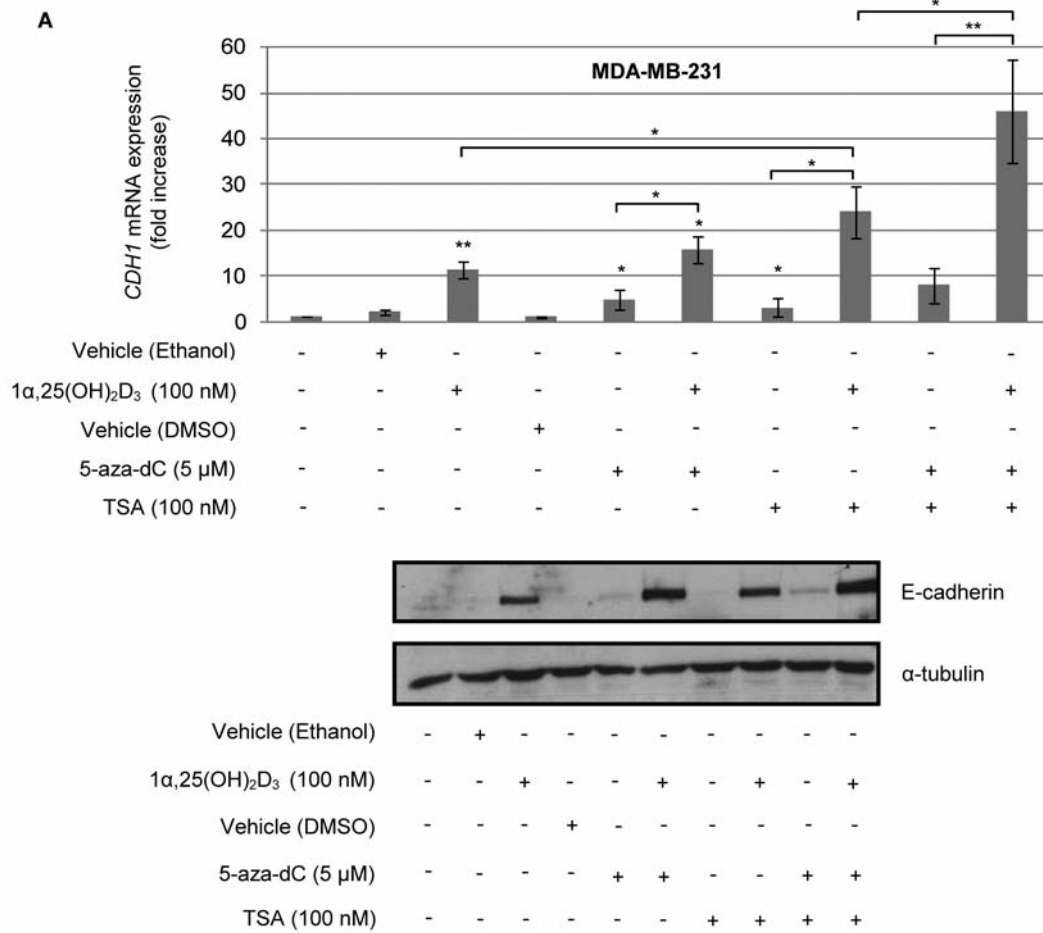
Figure 4. *Continued*

Hs578T cells, which corroborated the VDR expression results. Furthermore, these results were confirmed by the protein expression (Figure 4B and 4C).

As shown by immunofluorescence in Figure 5, upon treatment with $1\alpha,25(OH)_2D_3$, the MDA-MB-231 cells exhibited expression of E-cadherin at the plasma membrane. In contrast, the expression of E-cadherin induced by 5-aza-dC alone was granular and dispersed throughout the cytoplasm. However, when these cells were treated with both agents, the E-cadherin expression was located at the membrane.

Mediation of $1\alpha,25(OH)_2D_3$-induced expression of E-cadherin. Since $1\alpha,25(OH)_2D_3$ alone induced E-cadherin expression at the protein level only in the MDA-MB-231 cells, the experiments using *VDR* knockdown with siRNA were only conducted in this cell line. Upon silencing of the *VDR* in the MDA-MB-231 cells, the E-cadherin expression after hormonal treatment was abrogated (Figure 6).

Mechanism of E-cadherin expression. Upon $1\alpha,25(OH)_2D_3$ treatment, partial demethylation of the *CDH1* promoter in the MDA-MB-231 cells was observed (Figure 7). Demethylation was detected in 7 out of the 12 CpG sites analysed.

Discussion

The majority of the metaplastic breast carcinomas studied were positive for VDR expression, suggesting that they might be responsive to treatment with $1\alpha,25(OH)_2D_3$. In addition, 67% of the tumors had previously been characterised as negative for E-cadherin expression and 83.3% exhibited vimentin expression (unpublished results), showing that these tumors were indeed undifferentiated and could benefit from the differentiation-inducing properties of $1\alpha,25(OH)_2D_3$ treatment. In the *in vitro* model, $1\alpha,25(OH)_2D_3$ induced a *de novo* E-cadherin (epithelial differentiation marker) expression in the triple-negative MDA-MB-231 breast cancer cell line. This is an important finding, given the major role of E-
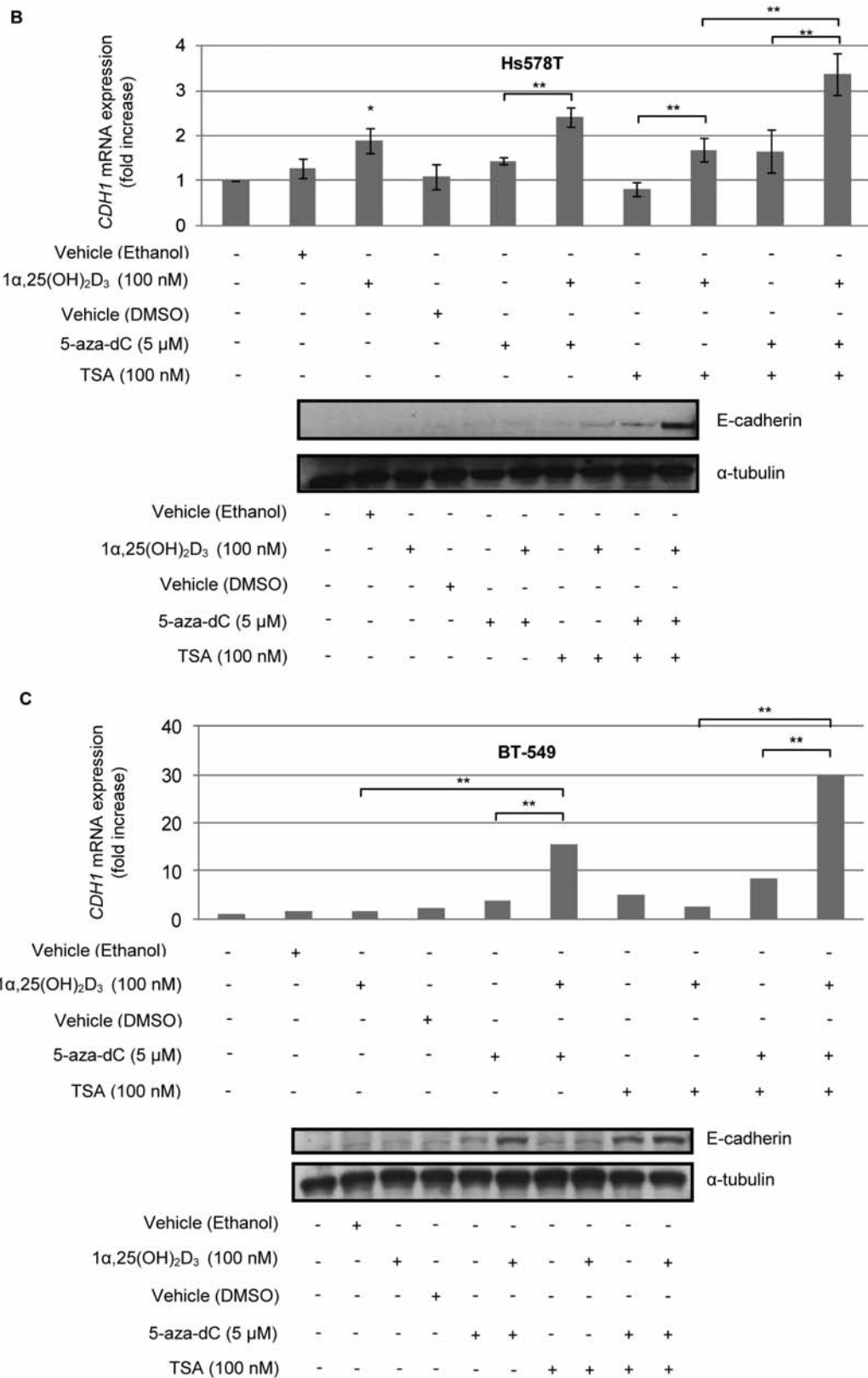
Figure 4. *Effect of 1α,25(OH)$_2$D$_3$, 5-aza-dC and TSA on CDH1 mRNA expression and E-cadherin expression in MDA-MB-231 (A), Hs578T (B) and BT-549 (C) breast cancer cells (*p<0.05, **p<0.01).*
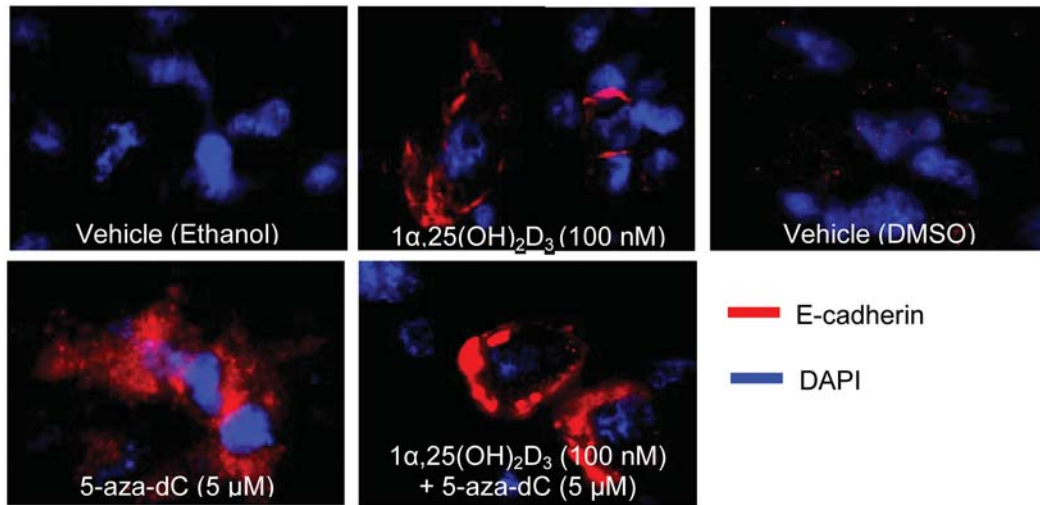
Figure 5. *Immunofluorescence of E-cadherin expression in MDA-MB-231 breast cancer cells (magnification ×400).*
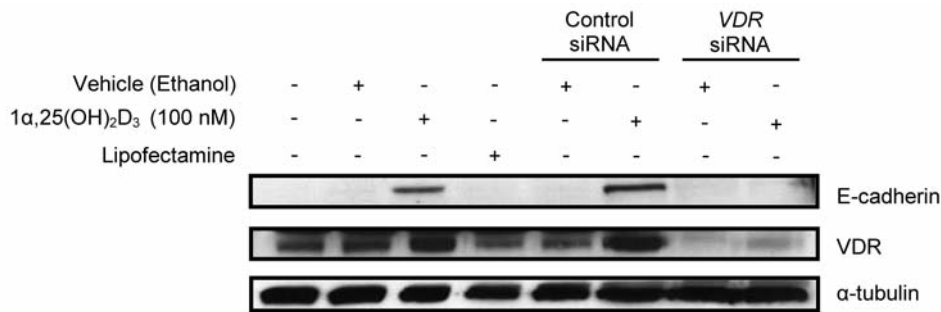

Figure 6. *Effect on E-cadherin expression induced by 1α,25(OH)$_2$D$_3$ of VDR knockdown by siRNA in MDA-MB-231 cells.*
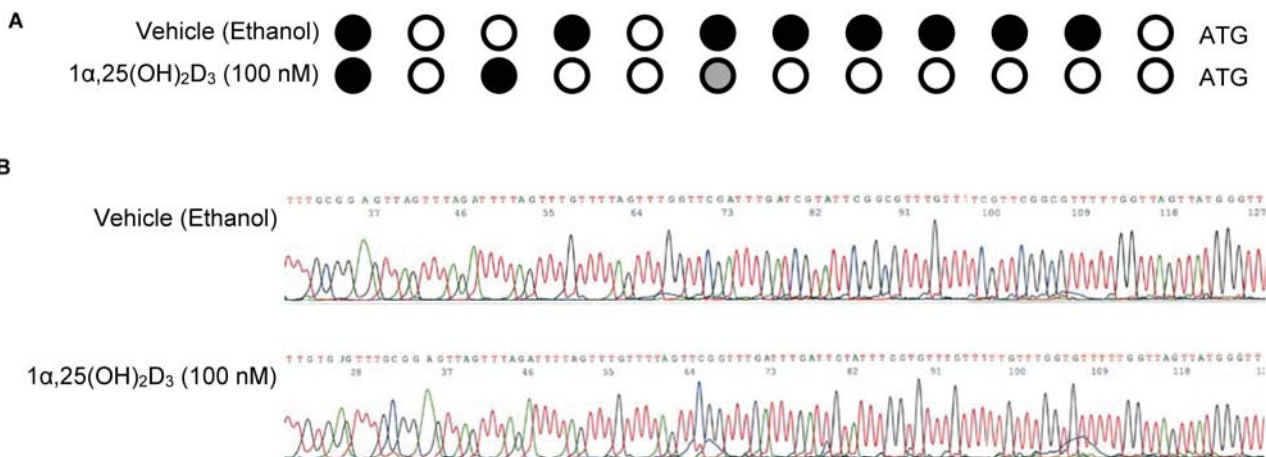

Figure 7. *Methylation analysis of CDH1 promoter in MDA-MB-231 breast cancer cells.* ● *– methylation,* ● *– hemimethylation,* ○ *– demethylation (A); Example of DNA sequences treated with ethanol or 1α,25(OH)$_2$D$_3$ (B).*

cadherin as a tumor suppressor protein in lobular breast carcinomas and other cancer models (19, 20) and since down-regulation of E-cadherin is required to initiate breast cancer metastatic growth (21). Furthermore, this effect was dependent on the duration of treatment and the quantity of $1\alpha,25(OH)_2D_3$ supplied to the cells. As far as we know, this is the first study demonstrating the *de novo* induction of E-cadherin expression in breast cancer cells by $1\alpha,25(OH)_2D_3$ due to *CDH1* promoter demethylation, although it has been reported that $1\alpha,25(OH)_2D_3$ can augment the expression of endogenous E-cadherin in mammary tumor cells (13). In addition, it has been demonstrated that a $1\alpha,25(OH)_2D_3$ analogue, increased the expression of E-cadherin in prostate cancer cells (15). In colon carcinoma cells, $1\alpha,25(OH)_2D_3$ is also known to promote differentiation by inducing E-cadherin expression and other adhesion proteins, an effect only observed in VDR positive cells (14). Likewise, in the MDA-MB-231 cells, E-cadherin expression was dependent on the presence of the VDR, suggesting it could mediate this effect.

In MDA-MB-231 cells, *CDH1* trancription is silenced due to promoter methylation (22). Interestingly, the levels of CDH1 expression upon $1\alpha,25(OH)_2D_3$ treatment in the MDA-MB-231 cells were 2- and 3-fold higher than those induced by the demethylating agent 5-aza-dC and by the HDAC inhibitor TSA, respectively, while the combination of $1\alpha,25(OH)_2D_3$ with either of these molecules promoted an additive effect, which was further confirmed by the protein expression. In gastric cancer cells, $1\alpha,25(OH)_2D_3$ has been shown to work in synergy with 5-aza-dC and TSA (23), thus supporting the effect obtained in the present study. Additionally, in colon cancer cells with silenced HDAC3, E-cadherin expression increased upon treatment with $1\alpha,25(OH)_2D_3$ (24), a result that mimics that observed in the MDA-MB-231 cells upon treatment with TSA and $1\alpha,25(OH)_2D_3$. In the other cells tested (Hs578T and BT-549) the results were not so encouraging when $1\alpha,25(OH)_2D_3$ was used alone; however, CDH1/E-cadherin expression was detectable when the cells were treated with $1\alpha,25(OH)_2D_3$ together with 5-aza-dC or TSA.

Also remarkably, the $1\alpha,25(OH)_2D_3$ treatment promoted the correct localisation of E-cadherin at the cell membrane in the MDA-MB-231 cells, suggesting a functional adhesion molecule, unlike the granular and dispersed pattern of expression induced by treatment with 5-aza-dC, which is suggestive of a non-functional protein. Similarly, in colon carcinoma, upon $1\alpha,25(OH)_2D_3$ treatment, E-cadherin expression was observed at the cell membrane (14). However, this $1\alpha,25(OH)_2D_3$ effect on E-cadherin induction is not exclusive of disease settings, as in normal keratinocytes, the treatment with $1\alpha,25(OH)_2D_3$ stimulates the assembly of adherens junctions, assessed by translocation of E-cadherin to the cell membrane (25). Surprisingly, when the MDA-MB-231 cells were treated with both $1\alpha,25(OH)_2D_3$ and 5-aza-dC, the effect induced by $1\alpha,25(OH)_2D_3$ prevailed over the 5-aza-dC-induced effect and there was a rescue of E-cadherin expression back to the membrane, hinting that $1,25(OH)_2D_3$ is indeed inducing not only the expression of E-cadherin, but, apparently, it is also important for the correct membrane localisation of the protein as a cell-cell adhesion molecule. Unlike the current results, 5-aza-dC was found to be necessary to sensitise leukaemia cells to differentiate in response to $1\alpha,25(OH)_2D_3$ treatment (26).

For the first time, $1\alpha,25(OH)_2D_3$ was found to promote partial *CDH1* promoter demethylation, suggesting that $1\alpha,25(OH)_2D_3$ can work as a demethylating agent in MDA-MB-231 breast cancer cells. To the best of our knowledge, only one study has correlated $1\alpha,25(OH)_2D_3$ with methylation and reported that it induced methylation of *CYP27B1* (the enzyme responsible for its synthesis) and, thus, silenced its expression (27). In colon cancer cells, where $1\alpha,25(OH)_2D_3$ induces E-cadherin expression, a new mechanism involving phosphoinositide signalling was recently proposed (28). Also in colonic cancer cells, a novel mechanism involving $1\alpha,25(OH)_2D_3$ in epigenetic events was reported, where the knockdown of *KDM6B/JMJD3*, a histone demethylase induced by $1\alpha,25(OH)_2D_3$, down-regulated E-cadherin expression (29). Studies addressing the importance of these mediators in breast cancer are still lacking.

In summary, the majority of metaplastic carcinomas examined were positive for VDR expression, hinting that this rare type of aggressive cancer may be responsive to the antitumor effects of $1\alpha,25(OH)_2D_3$. Furthermore, $1\alpha,25(OH)_2D_3$ induced the *de novo* expression of the epithelial differentiation marker E-cadherin in the highly metastatic, triple-negative MDA-MB-231 breast cancer cell line. To the best of our knowledge, this is the first report of the *de novo* induction of E-cadherin in breast cancer cells by $1\alpha,25(OH)_2D_3$ due to *CDH1* promoter demethylation, therefore, revealing a novel mechanism for the action of $1\alpha,25(OH)_2D_3$ in breast cancer cells. The induction of differentiation promoted by $1\alpha,25(OH)_2D_3$ in triple-negative metaplastic breast cancer may decrease the aggressiveness of this subtype of mammary carcinomas and improve patient outcome, but further studies are necessary to confirm this hypothesis.

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Vitamin D and the mammary gland: a review on its role in normal development and breast cancer

Vitamin D and the mammary gland: a review on its role in normal development and breast cancer

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Abstract

Vitamin D is classically associated with the physiological role of calcium regulation and phosphate transport in bone metabolism. Several studies demonstrate a range of functions for Vitamin D, which are particularly important in the field of cancer. Vitamin D thereby participates in cell growth regulation and cell differentiation. Additionally, Vitamin D has been implicated in the suppression of cancer cell invasion, angiogenesis and metastasis. In the present review we highlight research data concerning the role of this hormone in the mammary gland, with a special focus on its potential value as a breast cancer therapeutic agent or prophylactic.

Keywords

1. Introduction

Vitamin D was first identified in 1919 by Edward Mellanby as a lipid soluble substance with anti-rachitic properties [1]. Humans can obtain Vitamin D from two main sources: from the diet and from sunlight exposure. Few natural foods contain Vitamin D in significant amounts and among these, fatty fish, eggs and sun-exposed mushrooms can be highlighted. Still, the majority (90-95%) of the required Vitamin D is produced by the skin when exposed to sunlight (ultraviolet B radiation) [2], which has caused Vitamin D to be nicknamed "the sunshine Vitamin".

In this review, for simplicity purposes, whenever we state Vitamin D, we are referring to the biologically active form ($1\alpha,25$-dihydroxyvitamin D_3), unless otherwise mentioned. The same criterion is applied to the enzymes 1α-hydroxylase and 25-hydroxyvitamin D 24-hydroxylase, which will be termed here CYP27B1 and CYP24A1, respectively.

Vitamin D is a steroid hormone that exerts most of its biological activities by binding to a specific high-affinity receptor, the Vitamin D Receptor (VDR) [3]. VDR belongs to the superfamily of nuclear receptors for steroid hormones and regulates gene expression by acting as a ligand-activated transcription factor [4]. However, Vitamin D can also induce VDR-independent effects, indicated by the fact that the anti-proliferative effects of Vitamin D in MCF-7 cells are not exclusively dependent on the presence of the VDR [5]. Vitamin D is also known to exert rapid effects that are not dependent on gene transcription [6]. Presumably, these effects are mediated by cell surface membrane receptors. Two proteins have been implicated in expression-independent Vitamin D action: membrane VDR and the Membrane-Associated Rapid Response Steroid binding ($1,25D_3$-MARRS) protein. The evidence for the existence of a Vitamin D membrane receptor came from two observations: first, the existence of Vitamin D analogues that can cause rapid actions of Vitamin D, but show low levels of affinity to the VDR [7, 8]; and second, the existence of a Vitamin D binding protein that has been described in the basolateral membrane of rat and chick enterocytes [9]. Besides the role of $1,25D_3$-MARRS as a Vitamin D binding protein, the VDR can mediate non-transcriptional effects [6]. The most striking evidence supporting this hypothesis was the demonstration that the Vitamin D-induced rapid actions are lost in osteoblasts from *Vdr*

knockout mice [10]. Moreover, VDR has been identified within caveolae-enriched plasma membrane fractions from various cell types [11], suggesting that it can also work as a membrane receptor.

2. Vitamin D in breast carcinogenesis

2.1 Epidemiology of Vitamin D in breast cancer

There has been a great amount of information in the literature regarding a protective role of Vitamin D in breast cancer. Two major types of epidemiological studies have been conducted: first, the ones that focused on the association between solar radiation and breast cancer risk; and second, the ones that analysed the relationship between Vitamin D intake and breast cancer risk.

The first set of epidemiological studies, demonstrated an inverse association between decreased sunlight exposure and, consequently, diminished Vitamin D production of the skin, correlated with higher breast cancer incidence and mortality [12]. One study described that women with breast cancer had, in average, lower Vitamin D blood levels than women without breast cancer [13]. In line with this observation, early stage breast cancer patients show higher serum levels of Vitamin D than those who have advanced bone metastatic disease [14]. Additional evidence is provided by an inverse association between the circulating levels of 25-hydroxyvitamin D_3 (the inactive circulating form of Vitamin D, which is used to measure the levels of Vitamin D in circulation) and the risk for developing breast cancer [15]. Furthermore, it has been described that patients harbouring the most aggressive subgroup of breast cancer (triple-negative) display the lowest levels of 25-hydroxyvitamin D_3 [16]. These results are supported by a recent study where it was observed that breast cancer patients with suboptimal Vitamin D levels are more likely to have tumours with aggressive profiles and worse prognostic markers [17].

Altogether, these studies demonstrate a protective role for Vitamin D in breast cancer, suggesting that disruption of the Vitamin D signalling pathway may be a predisposition to develop the disease.

2.2 Role of Vitamin D in normal breast development

The VDR is expressed in the normal mammary gland and Vitamin D has been shown to play an important role in the development and function of the mammary gland. Most studies conducted to elucidate the role of the Vitamin D hormone in breast development have been based on the use of *Vdr* knockout mice. Zinser and colleagues (2002) have published an elegant study regarding the role of the Vitamin D signalling pathway in the growth regulation of the mammary gland during pubertal development [18]. They have shown that *Vdr* knockout female mice display more extensive ductal elongation and branching, when compared with their wild-type counterparts. Furthermore, they observed that this enhanced morphogenesis was not associated with the deregulation of cell proliferation and apoptosis pathways [18].

Immunohistochemical studies have demonstrated that the VDR protein is expressed in samples from normal breast tissues [19, 20]. However, there are some discrepancies concerning the expression of the main enzymes involved in Vitamin D metabolism (CYP27B1 and CYP24A1) in normal breast, probably due to the use of different methodologies. Studies assessing the mRNA of the two genes confirmed their expression in the normal mammary gland [21-24] and demonstrated that both enzymes are functionally active in normal breast [24]. In contrast, our group showed, by immunohistochemistry, that both enzymes are detectable in normal breast but not in all cases. In fact, although the majority of the cases (over 60%) presented CYP27B1 expression, only a small percentage (about 30%) of them presented detectable CYP24A1 protein expression (Figure 1). This work provided evidence for an unbalance in the enzymes which favours the presence of Vitamin D in the normal mammary gland. Together with the fact that VDR has an important impact on breast development, it supports the role of the Vitamin D signalling pathway in the growth and development control of the mammary gland.

Collectively, these data indicate that VDR has an important impact on breast development and suggest that the Vitamin D signalling pathway participates in the negative growth regulation of the mammary gland.

2.3 Vitamin D in benign lesions of the mammary gland

Benign lesions of the breast can be associated with distinct clinical behaviours and their accurate classification applicable to patient management in terms of surgical treatment and prophylaxis [25]. In contrast to the extensive studies addressing the expression of Vitamin D signalling and metabolic pathways in breast cancer, the studies in benign lesions of the mammary gland have been ignored. Recently, we have evaluated the expression of VDR, CYP27B1 and CYP24A1 in a series of breast benign lesions [20]. This series consisted of usual and atypical ductal hyperplasias, columnar cell lesions, papillomatosis and adenosis. The results we have obtained indicate that the most benign lesions express VDR and CYP27B1 and less than 20% of these cases detectably express CYP24A1. A decrease in positive cases for the three proteins was observed when compared with the levels in normal breast, although the results were not statistically significant. This observation may indicate a disturbance in the levels of the proteins that regulate Vitamin D metabolism and signalling in early stages of breast cancer development (Figure 1).

2.4 Vitamin D in breast cancer models

Suppression of cell growth by Vitamin D was first reported in 1981, by Abe and colleagues [26], and paved the way for Vitamin D to be considered as a potential therapy in cancer research. Since then, numerous studies have been conducted in various cancer models, in order to identify the molecular mediators of such effects and these have shown the ability of Vitamin D to affect the different hallmarks of cancer [6, 27] (Figure 2). It was convincingly demonstrated in many of these studies that Vitamin D has a prominent role in tumour cell proliferation and consequently cell cycle genes have become the centre of attention to decrypt the molecular mechanisms of Vitamin D in cancer.

Treatment of breast cancer cell lines and mice with Vitamin D elicits a change in the expression of proteins involved in cell cycle regulation, such as cyclins, Cyclin Dependent Kinases (CDK) and

CDK inhibitors (CKIs) [6]. The increased expression of p21 and p27, and the impairment in the expression of CDK2, CDK4, cyclin D1, cyclin D3, cyclin A1 and cyclin E1 led to cell cycle arrest at the G_0-G_1 transition, as well as to the inhibition of CDK activity and hypophosphorylation of the retinoblastoma protein, pRB [28-30]. Together with these effects in cell cycle proteins, a downregulation of c-Myc was reported [31]. Additionally, it has been proposed that the transcription factor CCAAT Enhancer Binding Protein alpha (C/EBPα) may be mediating Vitamin D growth inhibitory effects, since in C/EBPα-negative MDA-MB-231 cells transfected with C/EBPα a decrease in cell proliferation was observed, while the knockdown of C/EBPα suppressed the antiproliferative effects of Vitamin D in MCF-7 cells [32]. The breast cancer tumour suppressor TCF-4 is present at lower levels in *Vdr* knockout mice, which suggests a role for TCF-4 in the antiproliferative effects induced by Vitamin D [33]. Furthermore, Vitamin D induction of Breast Cancer 1 gene (BRCA1) has also been inversely correlated with cell proliferation [34], while it has been described that Vitamin D decreases aromatase expression [35] and, thus, can modulate ER-positive breast cancer growth. Interestingly, Vitamin D provokes a sharp inhibition of MCF-7 growth, together with a slight induction in the activity of antioxidant enzymes [36].

Vitamin D has a role in the induction of apoptosis in breast cancer cells (Figure 2), since cell shrinkage, chromatin condensation and DNA fragmentation are observed in MCF-7 cells treated with this hormone [37]. The most probable mechanism of such Vitamin D-induced apoptosis is through the downregulation of Bcl-2 [38]. Vitamin D is able to enhance Tumour Necrosis Factor alpha (TNF-α) through caspase-dependent and caspase-independent mechanisms [39]. In support of the role of caspase-independent cell death mediated by Vitamin D, it was shown that induction of apoptosis in MCF-7 cells was correlated with disruption of mitochondrial function, which was associated with Bax translocation from the cytosol to the mitochondria, cytochrome C release and production of ROS (reactive oxygen species). These mitochondrial effects did not require caspase activation, since these were not blocked by a specific caspase inhibitor [40]. Another caspase-independent cell death mechanism induced by Vitamin D relies upon cytosolic calcium accumulation associated with an increase in lysosomal protease activity [41]. Finally, Vitamin D was still described as a pro-oxidant in breast cancer cells, causing an increase in the overall cellular redox potential [42], which may also be an important mechanism underlying the pro-

apoptotic effects of this hormone. In combination with Tamoxifen, Vitamin D is able to potentiate and enhance the morphological effects of apoptosis when administered to MCF-7 cells [43]. Effects of other anticancer agents are enhanced by the administration of Vitamin D, namely doxorubicin [44], taxol [45] and cisplatin [46] . Importantly, a recent study demonstrates that the interaction between p53 and VDR provides a mechanism for mutant p53 (the most common genetic alteration in human cancers) gain-of-function. This may have clinical implications and suggests that p53 status should be considered when studying Vitamin D for cancer therapy [47].

Vitamin D plays an important role in the modulation of cancer invasion and metastasis (Figure 2). Hansen and collaborators (1994) demonstrated that Vitamin D has the ability to inhibit the invasive potential of human breast cancer cells *in vitro* [48]. This reduced invasiveness was found to be associated with diminished activity of the metalloproteinase MMP-9 and downregulation of the plasminogen-activator, simultaneously with increased tissue inhibitor of MMP-1 activity and the induction of plasminogen-activator inhibitor [49]. *In vitro* experiments, using Vitamin D analogues, have demonstrated that they can inhibit the invasive potential of mammary cancer cells [50], as well as prevent skeletal metastasis and prolong survival time in nude mice transplanted with human breast cancer cells [51]. This is in line with the observation that Vitamin D induces cell adhesion, as well as impairs *in vitro* motility [52]. Furthermore, Vitamin D is able to downregulate the expression of P-cadherin [52], an invasion promoter molecule in breast cancer cells [53]. We have addressed the effects of Vitamin D in E-cadherin negative breast cancer cell lines and have observed that treatment with Vitamin D induces the *de novo* expression of E-cadherin in MDA-MB-231 cells by *CDH1* promoter demethylation [54], providing further evidence for a Vitamin D role in invasion and metastasis.

Angiogenesis is yet another cancer hallmark that Vitamin D can modulate (Figure 2). An analogue of Vitamin D was able to inhibit angiogenesis at low concentrations *in vivo* [55]. Using xenografted mice with Vascular Endothelial Growth Factor (VEGF)-overexpressing MCF-7 breast cancer cells, it has been demonstrated that the administration of Vitamin D results in reduced tumours vascularisation [56]. The tumours formed in the treated animals displayed smaller capillaries when compared with their littermates, suggesting that Vitamin D may also inhibit vessel growth and

maturation. Additional evidence of Vitamin D and analogues in angiogenesis inhibition are through a decrease of VEGF and tenascin-C expression [57, 58].

The anti-proliferative, pro-apoptotic, anti-invasion and anti-angiogenic properties.of Vitamin D indicate that it could serve as a potential therapeutic agent. However, breast cancer is not one disease and is divided in many different molecular subtypes [59]. This may in part explain the many different functions described above for Vitamin D. In order to transform Vitamin D into a (targeted) therapy, a better understanding of the role and function of this hormone in solid tumours is required, accompanied by an upfront stratification of the different patient cohorts in the Vitamin D research field.

Conclusions

The data described provide good evidence for an essential role of Vitamin D in normal development of the mammary gland and breast cancer. The different functions and effects of Vitamin D on cell biology, such as in cell cycle, apoptosis, invasion and metastasis, as well as angiogenesis, virtually bring together the entire spectrum of tumour development.

Vitamin D has a dual role in breast cancer: epidemiological data points to its importance in cancer prevention, whereas its anti-carcinogenic effects show a promising value as a potential therapeutic agent. If not alone, at least in combination with other anticancer agents, the use of Vitamin D or its derivatives may constitute a potential treatment for those suffering from breast cancer.

Competing interests

The authors declare they have no competing interests.

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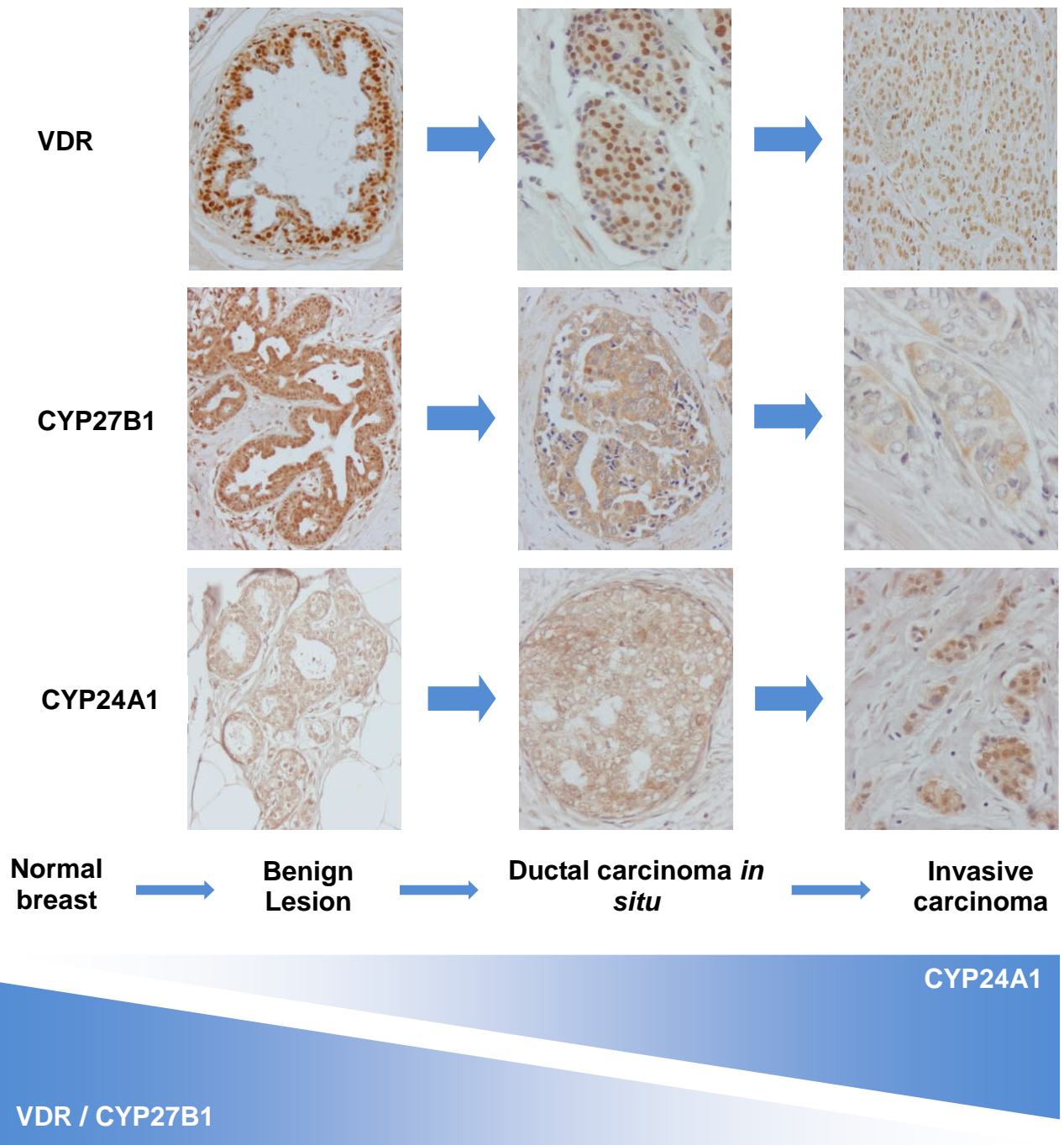
Figures

Figure 1 – Representation of the differences in the expression of VDR, CYP27B1 and CYP24A1 during breast carcinogenesis (Adapted from [20]). VDR and CYP27B1 expression decreases with breast carcinogenesis, while CYP24A1 expression is augmented (brown represents positive staining).
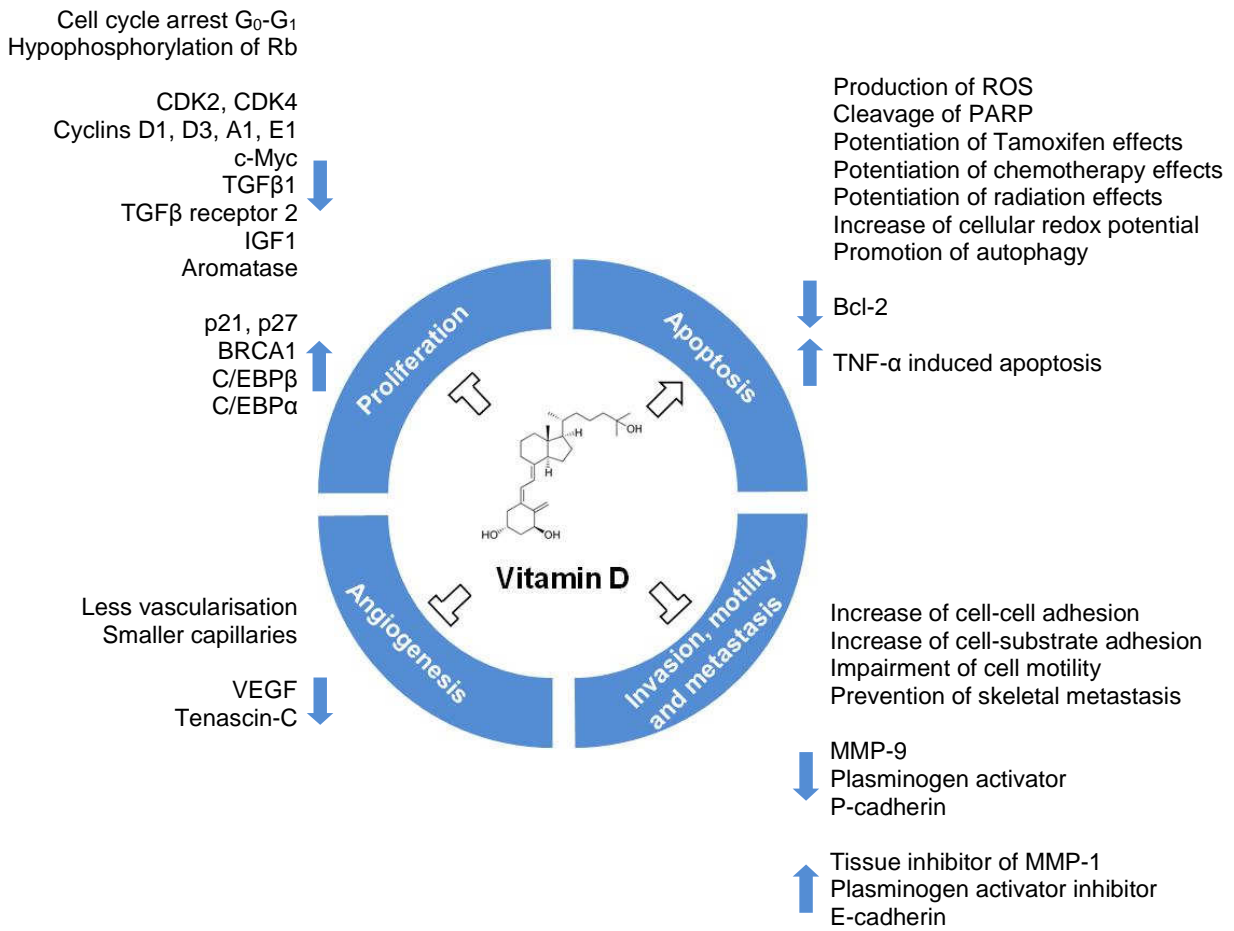
Cell cycle arrest G_0-G_1
Hypophosphorylation of Rb

CDK2, CDK4
Cyclins D1, D3, A1, E1
c-Myc
TGFβ1
TGFβ receptor 2
IGF1
Aromatase

p21, p27
BRCA1
C/EBPβ
C/EBPα

Production of ROS
Cleavage of PARP
Potentiation of Tamoxifen effects
Potentiation of chemotherapy effects
Potentiation of radiation effects
Increase of cellular redox potential
Promotion of autophagy

Bcl-2

TNF-α induced apoptosis

Proliferation

Apoptosis

Vitamin D

Angiogenesis

Invasion, motility and metastasis

Less vascularisation
Smaller capillaries

VEGF
Tenascin-C

Increase of cell-cell adhesion
Increase of cell-substrate adhesion
Impairment of cell motility
Prevention of skeletal metastasis

MMP-9
Plasminogen activator
P-cadherin

Tissue inhibitor of MMP-1
Plasminogen activator inhibitor
E-cadherin

Figure 2 – Schematic view of Vitamin D effects in breast cancer.

Appendix

The following articles, although not relevant for the work discussed in this thesis, were published during the course of the development of this study:

1. Rodrigues LR, **Lopes N**, Sousa B, Vieira D, Milanezi F, Paulsson M, Lindmark-Mänsson H, Teixeira JA, Schmitt F. Significance of Osteopontin Expression in Human Invasive Breast Tumour Stroma. *The Open Breast Cancer Journal*, 1:1-9, 2008.

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