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Vitamin D receptor (*VDR)* gene polymorphisms and genetic susceptibility to thyroid cancer

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Resumo Alargado

O cancro da tiróide é, de todas as neoplasias endócrinas, a mais comum, revelando ser uma patologia complexa e com uma etiologia desconhecida em parte. Tem uma incidência mundial que tem tendência a aumentar, contabilizando cerca de 1.7% dos cancros diagnosticados. Adicionalmente, o cancro da tiróide é mais prevalente em pacientes de meia idade e idosos, onde mais de metade dos indivíduos diagnosticados têm uma idade superior aos 45 anos. Ademais, esta neoplasia endócrina é mais comum nas mulheres, com uma incidência de 3 a 5 vezes maior. Os nódulos que surgem na tiróide são diagnosticados em cerca de 5% da população adulta mundial, e podem ser adenomas ou lesões malignas. Os carcinomas da tiróide derivam quer das células foliculares da tiróide, bem como das células C, porém, a grande maioria deles tem origem nas células foliculares. De todas as variantes de carcinomas da tiróide, os carcinomas papilar e folicular da tiróide são os mais predominantes, sendo a variante papilar a mais comum de entre todos, seguida da variante folicular. Apesar da elevada incidência mundial de cancro da tiróide, a taxa de mortalidade associada permanece estável. O tratamento do cancro da tiróide é um processo multifatorial, envolvendo a combinação de terapias cirúrgicas, hormonais ou de medicina nuclear.

Sabe-se que o cancro da tiróide, em especial os tumores diferenciados da tiróide, estão a aumentar de incidência em alguns países desenvolvidos. Existem muitos fatores de risco que aumentam a predisposição para este tipo de cancro, incluindo fatores genéticos com risco associado a esta patologia. A título de exemplo, o cancro diferenciado da tiróide está associado a uma forte hereditariedade, aumentando a suscetibilidade genética do indivíduo em desenvolver cancro de acordo com o seu historial familiar. Para além disso, a presença de polimorfismos genéticos podem determinar a suscetibilidade individual do indivíduo para o desenvolvimento de cancro da tiróide. Atualmente são conhecidos vários genes associados com a função tiroideia e que modulam o risco para a tumorigénese.

O VDR é um membro da superfamília de recetores nucleares, sendo a única proteína com afinidade para a 1α,25-dihidroxivitamina D, também conhecida como calcitriol. Nos mamíferos, a expressão do *VDR* encontra-se aumentada em tecidos metabólicos tais como o intestino, rins, pele e glândula da tiróide. O impacto biológico do *VDR* surge quando este se liga aos seus elementos localizados nas regiões promotores dos genes alvo, interferindo assim em muitas ações celulares e moleculares que vão desde a regulação do metabolismo de cálcio até à regulação de péptidos antimicrobiais. Desta forma, a ação molecular da vitamina D/ *VDR* está envolvida na regulação mineral e homeostase óssea, modulação do crescimento, eventos cardiovasculares, prevenção de cancro e regulação de respostas imunes. Uma disfunção do *VDR* ou défice de vitamina D podem levar a consequências no desenvolvimento e saúde óssea assim como aumentar a predisposição do indivíduo para o desenvolvimento de algumas doenças crónicas, incluindo o cancro.

Os polimorfismos associados ao gene *VDR* já provaram estar implicados como um fator principal de risco em vários tipos de cancro, tais como o cancro da próstata, mama ou cólon. Ao longo do tempo, estudos de associação têm sido feitos de modo a se poder correlacionar os polimorfismos genéticos e o seu impacto na saúde do indivíduo. Assim, no presente trabalho pretende-se estudar a suscetibilidade genética do cancro da tiróide associada aos polimorfismos do gene *VDR*. Neste trabalho, foram estudados quatro polimorfismos diferentes gene do *VDR*. Para tal, através do uso de enzimas de restrição, foi possível analisar áreas restritas do gene *VDR*, localizado no cromossoma 12q12-q14 de forma a se poder observar variações da sequência de DNA. Os quatro polimorfismos estudados no âmbito deste projeto foram o *Fok*I (rs10735810 C>T), localizado no exão 2 do *VDR*, *Bsm*I (rs1544410 G>A) e *Apa*I (rs7975232 G>T), localizados no intrão 8, e *Taq*I (rs731236 T>C), localizado no exão 9 do *VDR*. Estes quatro polimorfismos foram analisados com o objetivo de verificar de que forma influenciam a predisposição de um indivíduo para o desenvolvimento de cancro da tiróide.

Desta forma, este estudo realizado na população Portuguesa, fez a análise destas variantes do *VDR*, e o seu impacto no desenvolvimento de cancro da tiróide de acordo com os seguintes parâmetros: tipo de cancro, idade de diagnóstico, sexo, dimensões do carcinoma, metástases ganglionares e à distância, multicentricidade tumoral, e estádios de cancro. Todos os participantes deste estudo foram indivíduos caucasianos de origem Portuguesa. Estes indivíduos foram divididos em dois grupos distintos. Um dos grupos foi composto por indivíduos com cancro diferenciado da tiróide (N = 208), provenientes do Instituto Português de Oncologia de Coimbra. O grupo de indivíduos saudáveis (N = 248), que constituíam o grupo controlo, consistiram em dadores voluntários de sangue Portugueses caucasianos, que não possuíam um historial clínico de cancro da tiróide.

Após o recrutamento dos indivíduos e obtenção das amostras de sangue dos mesmos, procedeu-se a uma série de metodologias práticas que visaram como objetivo final genotipar as amostras recolhidas. A cada indivíduo, doente ou controlo, foi atribuído um número de código único, de forma a poder identificar e diferenciar a amostra em estudo. O processo clínico dos doentes foi registado com todos os dados necessários para este estudo. Quanto aos indivíduos saudáveis, estes permaneceram no anonimato, sendo apenas registado a idade, sexo, peso, altura e naturalidade. Após estes procedimentos de registo, o DNA genómico foi extraído das amostras de sangue recolhidas através do método de "*salting-out*". De seguida o DNA extraído foi quantificado e armazenado. Para efeitos de genotipagem, o DNA de cada indivíduo participante no estudo foi submetido à técnica "*polymerase chain reaction*", mais conhecida por PCR. Com este procedimento pretende-se amplificar o fragmento do gene *VDR* onde se encontra cada polimorfismo. Após a amplificação do fragmento do *VDR* que se pretendeu estudar, conforme o polimorfismo, procedeu-se à digestão enzimática utilizando a respetiva enzima. Desta forma, conseguimos determinar o genótipo do indivíduo, através da visualização desses produtos digeridos num gel de agarose de 3%. Para além deste método, a genotipagem foi também confirmada através da sequenciação de DNA, sendo utilizada uma

amostra representativa de cada genótipo para cada polimorfismo. Terminada a genotipagem de todos os indivíduos participantes deste estudo, para os quatro polimorfismos do *VDR*, procedeu-se ao tratamento estatístico dos dados analisando os parâmetros acima referidos.

Como resultados, verificaram-se, em alguns parâmetros, algumas diferenças de frequências dos polimorfismos. De entre esses resultados, nas comparações entre pacientes de sexo diferente, o genótipo GA do polimorfismo *Bsm*I foi mais frequente no sexo masculino (p = 0.044). Na análise de metástases ganglionares e à distância, o genótipo AA (p = 0.004) do *Bsm*I e o alelo A (p = 0.014) e o genótipo CC (p = 0.024) do *Taq*I foram mais frequentes no grupo de doentes com metástases. No estudo da multicentricidade tumoral, o alelo C (p = 0.041) do *Fok*I, o genótipo AA (p = 0.013) do *Bsm*I, e o genótipo CC (p = 0.017) do *Taq*I foram mais frequentes nos doentes com multicentricidade. No estudo dos estádios de cancro, os genótipos GT (p = 0.012) e TT (p = 0.004) do *Apa*I, e seu respetivo alelo T (p = 0.031) foram mais frequentes em doentes com estádios mais avançados.

A correção estatística de Bonferroni para comparações múltiplas revelou que os resultados foram estatisticamente significativos apenas para o genótipo AA do polimorfismo *Bsm*I, que parece estar envolvido na presença de metástases ganglionares em indivíduos com cancro diferenciado da tiróide. Para além disso, também o genótipo TT do polimorfismo *Apa*I revelou diferenças estatisticamente significativas, podendo estar associado a um estádio mais avançado de cancro da tiróide.

Desta forma, os polimorfismos do gene do *VDR* podem servir como marcadores de risco úteis para pacientes com cancro diferenciado da tiróide, uma vez que estes já forma associados em outros tipos de cancro. No entanto, não é possível retirar conclusões a partir destes resultados uma vez que são necessários mais estudos que permitam compreender as ações celulares e moleculares do *VDR*. Para tal, estudos funcionais genómicos serão necessários, para que se possa clarificar de que forma os polimorfismos deste gene podem influenciar a suscetibilidade genética para o cancro da tiróide.

Palavras-chave

Cancro da tiróide; carcinoma diferenciado da tiróide; gene *VDR*; polimorfismos *VDR*.

Abstract

Thyroid cancer is the most common endocrine malignancy and a complex disease with a largely unknown aetiology. Thyroid carcinomas are derived from thyroid follicular cells and parafollicular cells. The majority of thyroid cancer cases comprise both papillary (PTC) and follicular carcinomas (FTC). The evaluation of genetic susceptibility could give valuable information regarding the risk of thyroid cancer development. There are many genes associated with the thyroid function that modulates the risk of tumour development. Among them, is the vitamin D receptor gene (*VDR*), located on chromosome 12q12-q14, and includes eight protein coding exons (exons 2-9) and one untranslated exon (exons 1a-1f). The most common *VDR* polymorphisms investigated are *Fok*I (rs10735810 C>T), located in exon 2 of *VDR*, *Bsm*I (rs1544410 G>A) and *Apa*I (rs7975232 G>T), located in intron 8, and *Taq*I (rs731236 T>C), located in exon 9 of *VDR*.

The importance of vitamin D and its receptor *VDR*, in many signalling pathways is well known. Therefore we aim to verify in which way *VDR* polymorphisms influence the predisposition for thyroid cancer development. The contribution of four well-known *VDR* polymorphisms (*Fok*I, *Bsm*I, *Apa*I and *Taq*I) for the genetic susceptibility of thyroid cancer in the Portuguese population was analysed, including haplotypes comparisons. The following parameters were studied: thyroid cancer type differences (PTC vs. FTC), age (≤45 vs. >45 years), gender (male vs. female), carcinoma size (≤10mm vs. >10mm), lymph node metastasis and distant metastasis multicentricity, and stage of cancer (I-II vs. III-IV). All the participants in the study were Caucasian Portuguese inhabitants, being subdivided into two groups: patients with thyroid cancer (N = 208) and a control group (N = 248).

In conclusion, there were some statistically significant differences in some parameters assessed. However, the results considered were only those with a statistical significant p-value < 0.005, according to Bonferroni's correction. Therefore, the results suggest that *Bsm*I polymorphism genotype AA (p = 0.004) may influence lymph node metastasis or distant metastasis in patients with DTC. Moreover, the TT genotype (p = 0.004) of *Apa*I polymorphism may increase the predisposition for more aggressive phenotypes of DTC, since it is overrepresented in patients with more advanced cancer stages (III-IV).

Keywords

Thyroid cancer; Differentiated Thyroid Carcinoma; *VDR* gene; *VDR* polymorphisms.

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List of Acronyms

AKAP9 – A-kinase anchor protein 9

AKT – Protein kinase B

ATC – Anaplastic thyroid carcinoma

Bcl-2 – B-cell lymphoma 2

BRAF – V-Raf murine sarcoma viral oncogene homolog B1

BSA – Bovine serum albumin

C5 – Cervical vertebrae 5

cDNA – Complementary DNA

CT – X-ray computed tomography

CTNNB1 – Catenin (cadherin-associated protein) beta 1

DBD – DNA-binding domain

DNA – Deoxyribonucleic acid

ddNTPs – Dideoxynucleotides triphosphates

dNTPs – Deoxyribonucleotide triphosphates

DTC – Differentiated thyroid carcinoma

E2F – E2 promoter binding factor

EDTA – Ethylenediaminetetraacetic acid

EGF – Epidermal growth factor

EGF-R – Epidermal growth factor receptor

ERK – Extracellular-signal-regulated kinase

ExoI – Exonuclease I

FastAP – Thermosensitive alkaline phosphatase

FNAB - Fine needle aspiration biopsy

FOXE1 – Thyroid transcription factor 2

FTC – Follicular thyroid carcinoma

HCl – Hydrogen chloride

HRAS – V-HA-ras Harvey rat sarcoma viral oncogene homolog

IFN-γ – Interferon gamma

IGFBP – Insulin-like growth factor-binding protein

IL-1 – Interleukin 1

IL-12 – Interleukin 12

IGF – Insulin-like growth factor

HWE – Hardy-Weinberg equilibrium

$KHCO_3$ – Potassium bicarbonate

KRAS – V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

LBD – Ligand-binding domain

MAPK – Mitogen-activated protein kinase

MEK – Mitogen-activated protein kinase kinase

$MgCl_2$ – Magnesium chloride

mg – milligram

mL - millilitre

mM – millimole

MRI – Magnetic resonance imaging

mRNA – messenger RNA

MTC – Medullary thyroid carcinoma

NaCl – Sodium chloride

Na_2EDTA – Calcium disodium EDTA

NH_4Cl – Ammonium chloride

NKX2-1 – Thyroid transcription factor 1

NRAS – Neuroblastoma RAS viral (v-ras) oncogene homolog

OPN – Osteopontin

p107 – Retinoblastoma-like protein 1

p130 – Retinoblastoma-like protein 2

PAX8 – paired box gene 8

PCR – Polymerase chain reaction

PDTC – Poorly differentiated thyroid carcinoma

PI3K – Phosphatidylinositide 3-kinase

PPARγ - peroxisome proliferator-activated receptor γ

PTC – Papillary thyroid carcinoma

RAF - Rapid – accelerated fibrosarcoma

RANKL – Receptor activator of nuclear facto kappa-B ligand

RAS – Rat sarcoma

RBC – Red blood cells

RET – rearranged during transfection

RNA – Ribonucleic acid

rpm - Rotations per minute

RXR – Retinoid X receptor

SDS – Sodium dodecyl sulfate

SNP - Single nucleotide polymorphism

T1 – Thoracic vertebrae 1

T3 – Triiodothyronine

T4 – Thyroxine

TAE – Tris base, acetic acid and EDTA

TE – Tris-EDTA

TERT – Telomerase reverse transcriptase

TFC – Thyroid follicular cells

Tg – Thyroglobulin

TGF-α – Transforming growth factor alpha

TGF-β – Transforming growth factor beta

TNF-α – Tumour necrosis factor alpha

TP53 – Tumour protein p53

TRH – TSH-releasing hormone

trk – Tropomyosin-receptor kinase

TSH – Thyroid-stimulating hormone

UV – Ultraviolet

VDR – Vitamin D receptor

VDRE – Vitamin D response elements

VEGF – Vascular endothelial growth factor

µL - microlitre

1 – Introduction

1.1. Thyroid gland

The thyroid gland is the first endocrine gland to form in the developing human embryo, being the first endocrine structure to become recognizable during development. It has a dual origin that includes the endoderm of the primitive pharynx and the neural crest (1-3).

The thyroid gland is one of the largest endocrine glands (3, 4). In the adult, this gland is located in the neck region, deep to the cervical strap muscles anterior to the second and third tracheal rings at the C5 through T1 vertebral levels (1). It is composed of two lobes connected by a narrow band of thyroid tissue called the isthmus. The lobes are lateral to the upper portion of the trachea just inferior to the larynx (Figure 1) (3-5).

The thyroid gland is composed mainly by two cell types: the thyroid follicular cells (TFC), or thyrocytes, and the parafollicular cells, or C-cells. The thyrocytes produce and release thyroid hormones which are thyroxine (T4) and triiodothyronine (T3), and the C-cells produce and release calcitonin (3, 5, 6). The TFC are spherical structures serving as thyroglobulin (Tg) storage sites allowing controlled release of thyroid hormones, and the parafollicular cells are scattered among the interfollicular spaces in a parafollicular position (5, 6).

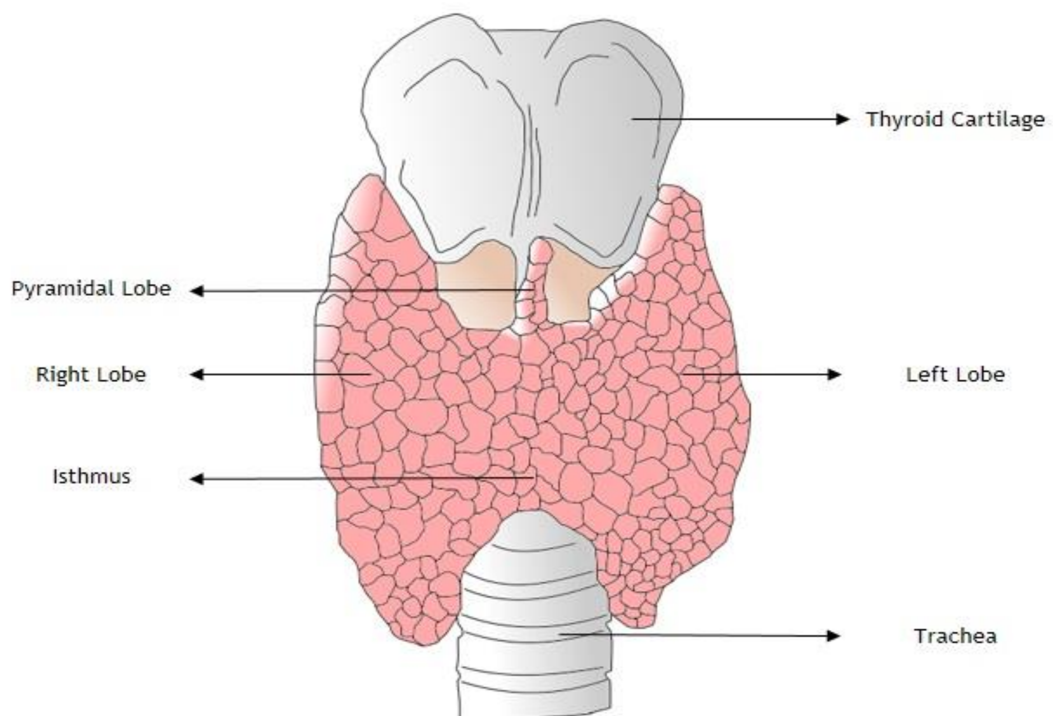

Figure 1 - Anterior thyroid gland anatomy. Frontal view of the thyroid gland showing both right and left lobes, the pyramidal lobe, isthmus, thyroid cartilage and trachea.

The thyroid-stimulating hormone (TSH or thyrotropin), produced by the pituitary gland, is considered to be the principal regulator of thyroid hormone biosynthesis and secretion, and is under the control of TSH-releasing hormone (TRH), secreted by the hypothalamus (3, 6, 7). T4 and T3 synthesis and secretion is regulated by a negative feedback loop involving inhibition of TSH and TRH synthesis. Moreover, a tissue-specific and hormone-regulated expression of the enzymes that metabolize the thyroid hormones contributes to this negative feedback that controls T4 and T3 synthesis and secretion (Figure 2). These hormones are crucial in many aspects of life such as regulation of different aspects of growth and correct maturation of the brain (3, 7).

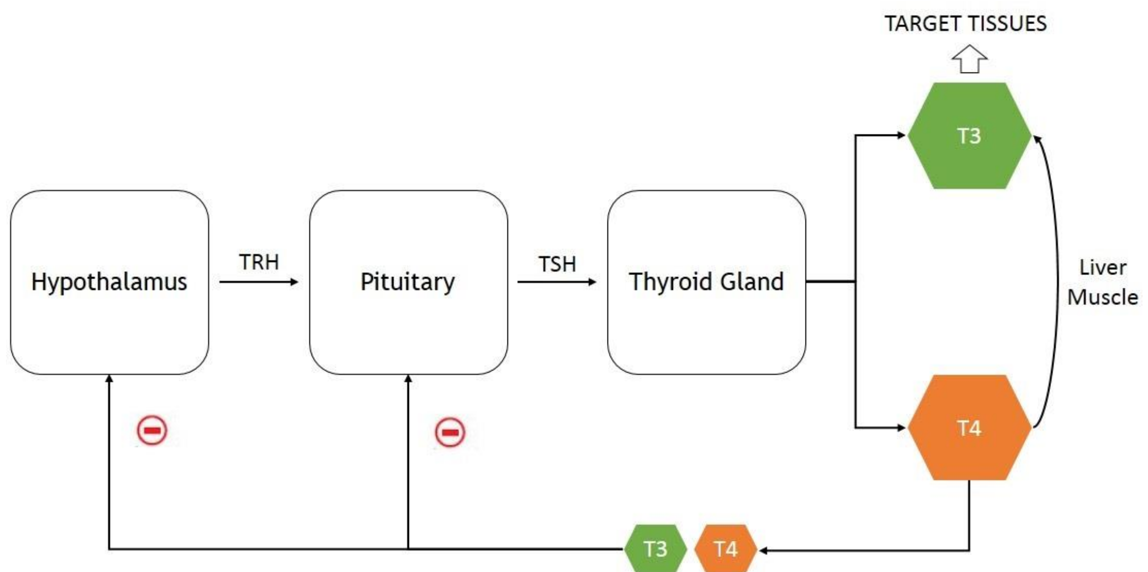

Figure 2 - Thyroid hormonal regulation mechanism. TRH is synthesized in the hypothalamus and stimulates synthesis and secretion of TSH from the thyrotroph cells in the anterior pituitary gland. TSH stimulates growth of thyroid follicular cells and synthesis and release of T4 and T3. These thyroid hormones inhibit synthesis and secretion of both TRH and TSH. This negative feedback mechanism maintains circulating thyroid hormones and TSH in a physiological inverse relationship (8).

When the thyroid endocrine function is compromised, there are some pathological features that develop in this organ. The clinical presentations of patients with thyroid lesions include hypothyroidism and hyperthyroidism, a nodule or goitre. Thyroid nodules occur commonly and sometimes they can represent more severe pathologies like thyroid carcinomas. (9, 10). Benign follicular epithelial lesions, like follicular thyroid adenomas, account for most of these nodules. The malignant thyroid neoplasms are subdivided into differentiated thyroid carcinomas (DTC), such as follicular and papillary carcinomas, poorly differentiated thyroid carcinoma (PDTC), anaplastic thyroid carcinoma (ATC) and medullary thyroid carcinomas (MTC) (9). Since the thyroid gland has an important role in metabolism, development and growth, it is important to study the thyroid disorders that can lead to thyroid dysfunction, and to neoplastic lesions (3).

1.2. Thyroid cancer

Thyroid cancer is the most common endocrine malignancy and a complex disease with a largely unknown aetiology (11). It has a worldwide incidence that continues to rise (11-15), accounting for 1.7% of total cancer diagnoses (16). This malignancy is characterized by one of the strongest familial relative risks in cancer, where first degree relatives of thyroid cancer patients are up to 8.6 times more likely to develop this cancer than the general population (17).

As described above, the incidence rates of thyroid cancer are growing in a fast rate. According to the analysed trends in the incidence of this disease across all the continents, thyroid cancer has increased in 19 populations in America, Asia, Europe, and Oceania, during which there was considerable variation in the thyroid cancer incidence rates (18). In general, thyroid cancer is more prevalent in the middle-aged and old patients, where 61% of the patients are older than 45 years (18, 19). Prevalence is also higher in females compared to males, with a ratio of 3:1 to 5.5:1 (19, 20). However, the incidence rates have also increased in children and adolescents at a rate of about 1.1% per year in the U.S.A. and in England, and have increased at an even greater rate of 3% per year across Europe. Therefore, the assessment of the age of onset for thyroid cancer is important because, despite the higher prevalence in older patients, the changing patterns relating age and incidence rates have important prognostic and treatment implications. According to statistical data from 1973 to 2006, the onset symptoms had a tendency to start later along the years, due to a worldwide population aging (18).

Thyroid nodules are diagnosed in over 5% of the adult population and can be benign adenomas or malignant lesions (16). Carcinomas are derived from thyroid follicular cells and parafollicular cells, however, most of them originate from thyroid follicular cells (14, 16). The majority of cases of thyroid cancers comprise both papillary and follicular carcinomas, where the papillary carcinoma is the most common thyroid cancer type, followed by follicular carcinoma (12, 14). The treatment of thyroid cancers is multifactorial, involving a combination of surgical, hormonal, and nuclear medicine therapies (12).

Although there has been a dramatic increase in the incidence of thyroid cancer worldwide, the mortality from the disease remains stable (13). The survival rate in thyroid cancer is generally good, because thyroid cancer has a better prognosis than many other cancers (19, 21). However, the survival rate drops to about 40% when distant metastasis is present in the patient (19). The various genetic alterations described in the last decades, and the molecular genetics of thyroid cancer has had an important role in clinical practice (11).

1.2.1. Papillary thyroid carcinoma

Papillary thyroid carcinoma (PTC) is the dominant form of thyroid cancer, accounting for more than two thirds of patients worldwide with this malignancy (22, 23). There are a number of histological subtypes of PTC which are named according to their dominant structural component. For example, the classic PTC is composed of papillary structures and is most commonly found in adults. Furthermore, the follicular variant has follicular structures and nuclear features characteristic for PTC (24). In the last years, the incidence of PTC increased, mainly those carcinomas with a size less than 1-2 cm. The incidence of larger tumours has remained stable (25).

This common malignant tumour is also prevalent in countries that have iodine sufficient or iodine excess diets, a parameter that appears to influence PTC development (26). Other risk factors, besides dietary habits, are related to radiation exposure, lifestyle, hormonal factors and genetic predisposition (23, 26, 27). For example, the individual exposure to radiation in some contaminated areas, where radio-isotopes like iodine are deposited, present a higher prevalence of PTC, even in children (28). In addition, some studies report findings regarding the association of PTC and lifestyle issues, like body weight, smoking, medical conditions and drugs (29).

The diagnosis of this carcinoma is based on the assessment of the papillary architecture in the classic type along with several well recognised nuclear features (30). Normally, the development of this type of carcinoma presents an asymptomatic slow-growing thyroid mass with a relatively indolent behaviour and a favourable prognosis (31). Patients with PTC usually present a palpable nodule in the thyroid site. The non-palpable nodules are frequently discovered incidentally, usually after an ultrasound, CT or MRI examination (23). Fine needle aspiration biopsy (FNAB) is used as a common method for the screening of thyroid nodules. This method is of easy availability, simplicity in performance and with an accuracy in the range of 70-97% in the detection of this malignancy (23, 27).

1.2.2. Follicular thyroid carcinoma

Follicular thyroid carcinoma (FTC), which is the second most common histological subtype of differentiated thyroid carcinoma (DTC), originates from thyroid follicular cells and accounts for about 10-15% of thyroid malignancies (32-35). The most common signs of initial presentation of FTC include a solitary thyroid nodule, a dominant nodule in a multinodular goitre and cervical lymphadenopathy (33). It occurs more often in older patients between the

ages of 45-70 years, being more prevalent in women, where the ratio comparative to males is 3:1 (35-37).

FTC has a propensity for haematogenous spread (35, 36). This thyroid carcinoma subtype is more likely to metastasize to distant organs, commonly to the lungs and bones, than regional lymph nodes, like PTC (32, 34). Normally, in 20% of the patients with FTC, lungs and bones metastasis occurs, but rare sites for distant metastasis are also the brain, skin, soft tissue, liver and other organs (35, 36).

The histology of this carcinoma displays a variable morphology, which range from small to medium follicles containing colloid, and trabecular or solid growth cellular patterns (38). Therefore, we can classify the degree of invasion of FTC through histopathology diagnosis into a minimally invasive carcinoma or a widely invasive carcinoma (32, 37, 39, 40). This histopathological study of thyroid follicular cells is critical because it has a significant prognostic impact, since widely invasive FTC is more aggressive and displays a poorer prognosis than minimally invasive (32, 37). Furthermore, the vascular invasion has an influence on prognosis (39). The prognosis of FTC depends also on several factors like age of the patients, size and staging of the tumours, and response to treatment (39, 41).

For the treatment, hemithyroidectomy is an adequate treatment for patients with minimally invasive FTC without vascular invasion. Total thyroidectomy followed by adjuvant therapy is recommended for patients with widely invasive FTC, based on histomorphologic assessment (37, 39).

1.3. Genetic alterations in thyroid cancer

As described above, thyroid carcinomas are divided into different subtypes, each one presenting characteristic features and distinct molecular alterations. Each subtype is characterized by a set of mutations that leads to an increased cellular proliferation and differentiation (42). For instance, PTC is associated with BRAF (V-Raf murine sarcoma viral oncogene homolog B1) and RAS (Rat sarcoma) point mutations, as well as RET/PTC (rearranged during transfection) and *trk* (Tropomyosin-receptor kinase) rearrangements. These type of mutations are identified in more than 70% of PTC cases (11). For FTC, about 70 to 75% of this thyroid carcinoma type carries mutually exclusive genetic alterations like RAS point mutations or PAX8/PPARγ rearrangements (11, 42). Other thyroid cancers of lesser differentiation could involve mutations in the PI3K/AKT signalling pathway, TP53 and CTNNB1 genes. These mutations are rare in well differentiated thyroid carcinomas (11). Therefore, several intracellular signalling pathways, including the PI3K/AKT and MAPK, are associated with the development and progression of thyroid carcinomas (43).

BRAF mutation is associated with the morphological and functional alterations characteristic of the PTC phenotype (44, 45). BRAF is a serine–threonine kinase of the RAF family of proteins located on chromosome 7. RAF is a member of RAS-RAF-MEK-ERK cell signalling pathway, also known as MAPK signalling pathway, and it plays an essential role in mediating cellular differentiation, proliferation, senescence, and survival in response to extracellular cues. Therefore, BRAF has a critical role in cell cycle and proliferation control because it is the strongest MAPK signaling pathway activator and the most frequently mutated human oncogene in the kinase superfamily (11, 43, 45, 46). BRAF activating point mutations are clustered in exons 11 and 15 of the gene (43). These point mutations are identified in 40–45% of PTC, being the most common molecular alteration in this malignancy (11, 23, 42, 43, 45-47). The detection of this mutation is a specific but not sensitive feature of PTC, since this mutation has not been observed in benign thyroid lesions (44). It is also identified in 24% of ATC (11, 23, 43, 45, 46). Besides, BRAF point mutations like the V600E results in a series of events like the activation BRAF kinase, leading to prolonged stimulation of the MAPK pathway and, ultimately, thyroid tumorigenesis (11, 23, 42, 45). This mutation represents more than 95% of all BRAF mutations (46). BRAF V600E mutation is typically identified in PTC with classical histology and a tall cell variant, being rare in the follicular variant (11, 44-46). Therefore, the study of this mutation could be a key for the treatment and the diagnosis of the most aggressive PTC, because the histological features presented by this mutation are specific for these early phenotypes of PTC, which plays an important role in tumorigenesis initiation (42, 45). Other BRAF mutations, including the K601E point mutation, small in-frame insertions or deletions around codon 600, and AKAP9/BRAF rearrangement, are present in a minority of PTC cases (1–2%) (11, 46). So far, BRAF mutations have not been identified in FTC, MTC, benign thyroid adenomas, or hyperplasia (46).

The human RAS gene family, which includes HRAS, KRAS, and NRAS, are involved in MAPK signalling pathways as well PI3K/AKT and other signaling pathways (11, 42). Activating point mutations in RAS genes are common in human tumours (11). The KRAS codon 12/13 mutation predominates in most cancers, while the NRAS codon 61 and HRAS codon 61 mutations are more often seen in thyroid tumours (11, 23). RAS mutations occur in all types of thyroid epithelial malignancies (11). They are identified in 40–50% of FTC cases and 20–40% in follicular adenomas (11, 23, 42, 44). Since they are found with some frequency in FTC cases and follicular adenomas, RAS mutations are considered an early event in follicular neoplasia (44). RAS mutations are also found in 10–20% of PTC, mostly as activating mutations of codons 12 and 13 of the NRAS gene (11, 23, 44). RAS mutation is also reported to be frequent in PDTC, where similarities between PTC and RAS-mutated PDTC are reported (44).

The RET proto-oncogene encodes a cell membrane receptor tyrosine kinase, which influences cell survival (11, 43). Located on chromosome 10q11.2, this oncogene activates several signal transduction pathways, including the MAPK signalling cascade and the PI3K/AKT cell signalling pathway, leading to increased cell growth, survival and proliferation (43). RET is highly

expressed in thyroid parafollicular or C-cells and can be activated by RET/PTC chromosomal rearrangement, an event that leads to the activation of the MAPK signaling pathway (11). RET/PTC rearrangements arise from the fusion of the 3' end of the RET gene that encodes the tyrosine kinase domain with the 5' domain of one of the several constitutively expressed genes (44, 46). There are 11 different types of RET/PTC rearrangements described, however only two common types, like RET/PTC1 and RET/PTC3, are largely found in thyroid carcinomas (11, 42, 44). RET/PTC1 is found in 60–70% of RET/PTC rearrangements, and RET/PTC3 is present in 20–30% (11). RET/PTC rearrangement is frequently found in PTC (50-80%) and also in children and young adults (40–70%) (11, 23, 43, 44). In children with PTC, which developed the pathology after exposure to radiation, RET/PTC3 is the dominant rearrangement (23). The RET/PTC1 is usually identified in PTCs with classic papillary histology, but may also be seen in papillary microcarcinoma and other histologic variants. In papillary carcinomas arising after radiation exposure, RET/PTC1 is associated with classic papillary histology, and RET/PTC3 is more common in solid variants (11). Currently, there is no available tyrosine kinase inhibitor specific to RET, but there are several multi-targeted tyrosine kinase inhibitors which have demonstrated significant activity against RET (43).

PAX8/PPARγ rearrangements are the result of a fusion between the 5' end of the PAX8 transcription factor and the 3' end of PPARγ, a member of the nuclear hormone receptor superfamily, constitutively expressed in thyrocytes (42). This fusion oncoprotein that results from PAX8/PPARγ rearrangements acts as a dominant negative inhibitor of wild-type PPARγ and has a unique transcriptional activity when compared with wild-type PAX8 or PPARγ (44). PAX8/PPARγ rearrangement is identified in 30–40% of FTCs and less often in oncocytic variants (11, 42, 44). PPARγ-dependent genes were overexpressed in FTCs bearing the rearrangement (44). PAX8/PPARγ-positive follicular carcinomas present at a younger age, are smaller in size, and more often show vascular invasion. PAX8/PPARγ rearrangement is also present in a minority of follicular variant PTC and follicular adenomas. PAX8/PPARγ and RAS point mutations are non-overlapping mutations in follicular carcinomas, which suggests that these malignancies may arise through two distinct molecular pathways, each initiated by either PAX8/PPARγ rearrangement or RAS mutation (11).

Chromosomal rearrangements involving the *trk* gene are found in about 10% of PTC. This results from the fusion of tyrosine kinase domain of *trk* on chromosome 1q22 to the tropomyosin gene (23).

1.4. Genetic susceptibility to thyroid cancer

It is well known that thyroid cancer, mostly DTC, is increasing in some developed countries. There are many risk factors for thyroid cancer, like ionising radiation, however, there are other genetic risk factors that could be implicated in the aetiology of this pathology (48). For

instance, DTC is characterised by a strong hereditability, where the individual susceptibility to develop thyroid cancer increases according to his familial clinical history. Therefore, an individual susceptibility is likely due to genetic factors modulating the environmental risk (17, 48). Furthermore, first degree relatives with this pathology increases the risk factor considerably (17).

The presence of genetic polymorphisms could determine the individual susceptibility to thyroid cancer. There are many genes associated with thyroid function that modulate the risk of tumour development (48). Several association studies have been done to correlate gene polymorphisms with the risk of thyroid cancer. Hsiao et al. (49) reported an association of *VEGF* gene and thyroid cancer. In other studies, Gudmundsson et al. (50) showed that the genes *FOXE1* and *NKX2-1* are associated with an increased risk for DTC. Therefore, it is of major importance to study gene polymorphisms and thyroid genetics in order to clarify thyroid carcinoma aetiology (51).

1.5. Vitamin D

Vitamin D is a unique vitamin in humans because it can be synthesized in human skin due to daily UV radiation exposure. This vitamin has four major forms, namely ergocalciferol, cholecalciferol, 25-hydroxyvitamin D and 1α,25-dihydroxyvitamin D. Ergocalciferol, also known as vitamin D_2, is produced via UV radiation from his precursor ergosterol found in plants. Cholecalciferol, or vitamin D_3, is synthesized via UV radiation from 7-dehydrocholesterol, that exists in the skin of vertebrates (52). This 7-dehydrocholesterol is formed by oxidation of cholesterol from food, being then transported to the skin, mainly to the epidermis (53). 25-hydroxyvitamin D and 1α,25-dihydroxyvitamin are both produced from cholecalciferol hydroxylation, being the two active forms that circulate in the human body (52).

Vitamin D plays an important role in calcium homeostasis and bone metabolism throughout life (54). It was identified in the early 20th century as a critical factor to prevent rickets (55), demonstrating vitamin D importance in bone structure. However, due to the presence of vitamin D in other tissues in human body, this vitamin is associated with other important roles beyond bone metabolism (52, 54).

During the past decade, data presented by many researchers demonstrated that vitamin D and its metabolites have an extreme importance in other human pathologies such as colon, breast and prostate cancer, revealing a potential use in the treatment and prevention of these diseases (52, 56). This is the “new” function of vitamin D that besides the classic involvement in bone metabolism and calcium homeostasis is also involved in other human systems such as the immune, cardiovascular or reproductive systems (54). According to

previous studies, vitamin D or its metabolites have inhibitory effects on development and progression of many tumour cells. Some population-related studies show that low serum vitamin D concentrations lead to an increased risk of cancer development (56).

1.6. Vitamin D Receptor (*VDR*)

Vitamin D receptor (*VDR*) is a member of the nuclear receptors superfamily, and it is the only protein that binds with high affinity to 1α,25-dihidroxivitamina D, also known as calcitriol (57, 58). The nuclear receptors represent a group of important transcription factors, where the 48 members of this superfamily belong to an identified group of mammalian genes involved in transcriptional regulation (57).

Like most of the nuclear receptors superfamily, VDR have two zing fingers which forms a DNA-binding domain (DBD) of 66 amino acids. Furthermore, the C-terminal of the protein contains a ligand-binding domain (LBD) of approximately 300 amino acids formed by 12 α-helices (58). Therefore, VDR is a transcription factor which creates an active signal transduction complex that forms a heterodimer of calcitriol-liganded VDR and unoccupied retinoid X receptor (RXR). The VDR ligand triggers a tight association between VDR and RXR, and this liganded VDR-RXR a heterodimer is able to recognize vitamin D response elements (VDREs) in the DNA sequence of vitamin D-regulated genes (59).

In mammals, *VDR* expression is higher in metabolic tissues such as intestine, kidneys, skin and thyroid gland, being moderately expressed in almost all tissues. Furthermore, *VDR* is expressed in most of malignant tissues of colon, breast and prostate (57, 59, 60). Active *VDR* binds to VDREs located in the promoter regions of target genes, having a great biological impact because it interferes in many actions from calcium metabolism to key antimicrobial peptides. Therefore, vitamin D/*VDR* signaling is involved in mineral and bone homeostasis, modulation of growth, cardiovascular events, cancer prevention and immune responses regulation, including autophagy. A *VDR* dysfunction or a vitamin D deficiency in the human body can cause poor bone development and health, as well as increase the risk of developing many chronic diseases, including cancer (61).

1.6.1. *VDR* gene and protein structure

After cloning human *VDR* cDNA in 1988, it took almost 10 years before all the *VDR* genomic structure became clear (Figure 3). These findings happened before the Human Genome Project appeared as a tool which would allow an easy access to databases where genomic sequences can be found. The location of *VDR* in the physical map of chromosome 12 was first elucidated by linkage mapping, being after that identified by fluorescent in situ hybridization (FISH) and radiation hybrid mapping. Though these studies defined the position of *VDR* in

general terms with a resolution of >100 kb, it was insufficient to a better understanding of what is the role of *VDR* in some diseases, requiring a more accurate resolution (62).

In a structural way, the LBD are formed by a three-layer anti-parallel α-helical sandwich composed of 11 to 13 α-helices. The internal structure of LBDs of their respective nuclear receptors shows a similar diversity. The interior surface of LDB is formed by side chains of non-polar amino acids that complement the lipophilic character of the ligands, with the specificity achieved through a limited number of stereo-specific polar contacts (63).

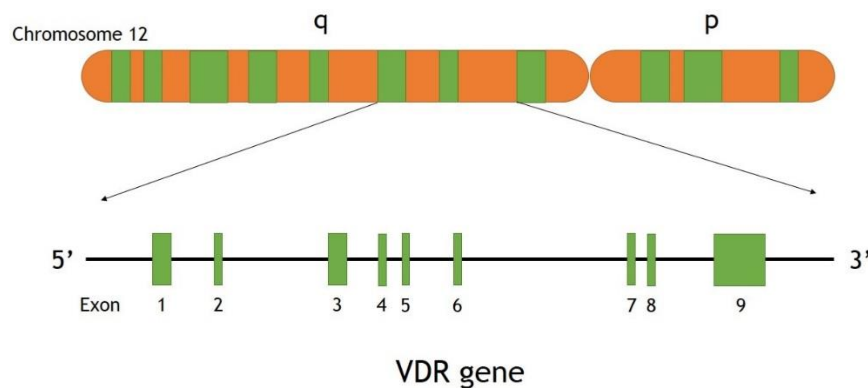

Figure 3 – Location and structure of the *VDR* gene. Scheme of the *VDR* gene representing the respective exons and introns, located on chromosome 12.

1.6.2. *VDR* gene polymorphisms

The pleiotropic biological effects that vitamin D causes in the human organism are mediated by its receptor, *VDR*. The function of this nuclear receptor is influenced by the presence of several genetic polymorphisms (64, 65). The *VDR* gene, located on chromosome 12q12-q14, includes eight protein coding exons (exons 2-9) and one untranslated exon (exons 1a-1f)(66, 67).

By screening with different restriction enzymes, only some restricted areas of *VDR* gene could be analysed to verify DNA sequence variations (68). The most commonly investigated *VDR* polymorphisms are *Fok*I (rs10735810 C>T), located in exon 2 of *VDR*, *Bsm*I (rs1544410 G>A) and *Apa*I (rs7975232 G>T), located in intron 8, and *Taq*I (rs731236 T>C), located in exon 9 of *VDR* (66). These are single nucleotide polymorphisms (SNPs), where *Fok*I is located at the 5' end of *VDR* gene and the other three SNPs are at the 3' end of the gene (Figure 4) (65, 66, 68).

According to specific data in the literature, the *VDR* polymorphisms are associated with plasma calcitriol levels, calcium variations in plasma and urinary calcium excretion. Besides,

these SNPs have an impact in human health, whereby individuals may have an increased risk of developing some diseases such as bone problems and cancer (64).

Exon 2

agctggccctggcactgactctggctctgaccgtggcctgcttgctgttcttacagGGATGGAGGCA
ATGGCGGCCAGCACTTCCCTGCCTGACCCTGGAGACTTTGACCGGAACGTGCCCC
GGATCTGTGGGGTGTGTGGAGACCGAGCCACTGGCTTTCACTTCAATGCTATGAC
CTGTGAAGGCTGCAAAGGCTTCTTCAGgtgagccctcctcccaggctctccccagtggaaag
ggagggagaagaagcaaggtgtttccat

(...)

Intron 8

agtgtgcaggcgattcgtagggggggattctgaggaactagataagcagggttcctggggccacagacag
gcctgcgcattcccaatactcaggctctgctcttgcgtgaactgggctcaacattcctgttatttgaggttt
cttgcgggcagggtacaaaactttggagcctgagagatggttctgcctat

(...)

Exon 9

cagagcatggacagggagcaaggccaggcagggacagggccaggtgcgcccatggaaggacctaggtc
tggatcctaaatgcacggagaagtcactggagggctttggggccaggcagtggtatcaccggtcagcagt
catagaggggtggcctagggggtgctgccgttgagtgtctgtgtgggtggggggtggtgggattgagcagt
gagggcccagctgagagctcctgtgccttcttctctatccccgtgcccacagATCGTCCTGGGGT
GCAGGACGCCGCGCTGATTGAGGCCATCCAGGACCGCCTGTCCAACACACTGCAG
ACGTACATCCGCTGCCGCCACCCGCCCCCGGGCAGCCACCTGCTCTATGCCAAGAT
GATCCAGAAGCTAGCCGACCTGCGCAGCCTCAATGAGGAGCACTCCAAGCAGTAC
CGCTGCCTCTCCTTCCAGCCTGAGTGCAGCATGAAGCTAACGCCCCTTGTGCTCG
AAGTGTTTGGCAATGAGATCTCCTGACTAGGACAGCCTGTGGCGGTGCCTGGGTG
GGGCTGCTCCTCCAGGGCCACGTGCCAGGCCCGGGGCTGGCGGCTACTCAGCAG
CCCTCCTCACCCCGTCTGGGGTTCAGCCCCTCCTCTGCCACCTCCCCTATCCACCC
AGCCCATTCTCTCTCCTGTCCAACCTAACCCCTTTCCTGCGGGCTTTTCCCCGGTC
CCTTGAGACCTCAGCCATGAGGAGTTGC

Figure 4 – Location of the *VDR* polymorphisms. The *Fok*I, *Bsm*I, *Apa*I and *Taq*I single nucleotide polymorphisms are highlighted.

1.7. Molecular effects of vitamin D/*VDR*

Vitamin D has non-genetic and genetic effects, where the latter is mediated by its binding to the *VDR*. When vitamin D binds to *VDR*, conformational changes occur in the LBD which result in a modification of co-regulatory molecules and ultimately in both modification of chromatin and transcription. Transcription generally requires that *VDR* binds to the VDREs in target genes. *VDR* forms a heterodimer, especially with RXR, which also binds to 9-cis-retinoid acid, a metabolite of vitamin A present in the serum. The target genes on which *VDR* has a direct influence are many, so we can say that this gene has pleiotropic effects. The gene expression pathways are generated in different cell types, where there are some target genes that may be upregulated, such as osteopontin (OPN), RANKL, calbindin-9k, IGFBP and β3 integrin, and others downregulated, including IL-1, IL-12, TNF-α, IFN-γ, EGF-R and TERT. According to

target genes expression that depend on *VDR* actions, vitamin D produces anti-proliferative effects, increased apoptosis and increased differentiation of cells in a number of ways (69).

Based on the evidence above, there is an association between vitamin D/*VDR* biological effects and cancer, so the study of their cellular and molecular mechanisms is very important. In general, the effects of *VDR* and its agonists on cells lines from diverse human tumours are similar, resulting in biological modulation actions of cell cycle regulators, induction of differentiation markers and activation of cell death (70).

1.8. Vitamin D metabolism

Humans can obtain vitamin D through two major ways: diet and cutaneous synthesis after exposure to sunlight. The endogenous supply of cholecalciferol depends on exposure of the skin to UVB radiation. However, the vitamin D productions in humans can vary due to innumerous factors like different exposure to UV radiation, different cutaneous production and different diet habits (54).

Vitamin D synthesis is induced by UV radiation, where it catalyses a reaction in human epidermis. The precursor 7-dehydrocholesterol is converted to cholecalciferol in a two-step process (52). Then, cutaneous vitamin D enters circulation and is transported to the liver by the vitamin D binding protein (DBP). The DBP produced circulates at a much higher concentration than vitamin D and its metabolites (54, 71). After cholecalciferol transportation, occurs the first hydroxylation within the liver, leading to the formation of 25-hydroxyvitamin D, also known as calcidiol. Then, the calcidiol is transported to the kidneys, where the second hydroxylation occurs. Therefore, the 1α,25-dihydroxyvitamin D, or calcitriol, is formed, being the most biologically active hormonal form of vitamin D (Figure 5) (52, 71).

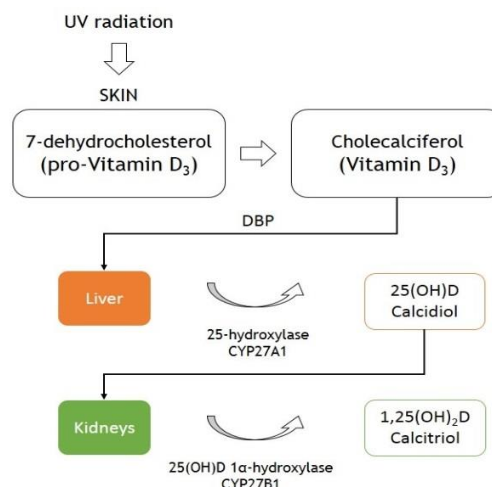

Figure 5 – Vitamin D synthesis. This scheme represents vitamin D production from its precursor 7-dehidrocholesterol to its active hormonal form calcitriol.

1.9. Mechanisms of vitamin D/*VDR* interactions

According to recent studies, we can visualize how DBD and LBD domains are arranged relative to one another, through the use of some techniques such as Small Angle X-ray Scattering and Fluorescence Resonance Energy Transfer. The *VDR* gene intervenes in the mediation of calcitriol-stimulated transcription, where RXR heterodimerization constitutes an obligatory initial step in *VDR* activation (59).

Therefore, and at a molecular level, calcitriol and its synthetic analogues modulate the gene expression through heterodimer *VDR*-RXR. The RXR, that is a nuclear receptor for 9-cis retinoic acid, is an obligate partner of *VDR* in mediating calcitriol action. In the absence of a ligand in cellular systems, most part of *VDR* is present in the cytoplasm. *VDR* ligand induces the heterodimerization and translocation of the complex *VDR*-RXR to the cell nucleus. The heterodimer *VDR*-RXR then binds to VDREs present in the promoter regions of responsive genes (72).

The hormonal ligand could influence *VDR* to interact more efficaciously with its heterodimeric partner, with VDREs and with its co-activators. There are several steps that are apparently started by the LBD. The presence of calcitriol ligand results in a conformational change in the position of helix 12 at the C-terminus of *VDR*, leading to a conformation that will work as a platform for co-activators binding. Therefore, ligand-intensified heterodimerization, VDREs docking and co-activators recruitment by *VDR* appear to be functionally inseparable events that occur to affect calcitriol-elicited gene transcription (59).

1.10. Vitamin D/*VDR* and cancer

In 1969, the *VDR* was discovered and since then, the role of *VDR* in the endocrine system and its presence and function in over 30 tissues and organs has been studied. *VDR* is involved in multiple signaling pathways, having points of convergence in these pathways which indicates the potential importance of *VDR* in the aetiology of cancer (73).

Epidemiologically, there are multiple evidences that vitamin D is an essential factor for prevention of age-related cancers, so we can say that vitamin D and cancer has major attention in many studies today (70, 74). The active hormonal form of vitamin D, calcitriol, interacts with *VDR*, having, as explained above, pleiotropic effects in the organism like regulation of calcium and phosphate metabolism, cell proliferation, differentiation, apoptosis, angiogenesis and potential metastasis of a varied number of cells. Therefore, calcitriol has a great potential as an “anti-cancer” compound. However, there is a possibility

that the use of calcitriol could result in hypercalcemic toxicity, which can restrain its clinical use (74).

In a general way, the functions of vitamin D and *VDR* appear to have a critical role in the development or regression of many cancers. Moreover, because of vitamin D/*VDR* involvement in many biological pathways, it is also important evaluate *VDR* associations with diet, lifestyle and environmental factors related to cancer (73).

In what concerns cancer development, vitamin D and *VDR* interactions in the human system may assume some antineoplastic activity. This fact was already proved through several in vitro and in vivo experiments in multiple malignancies in some cancers like prostate, breast, colorectal cancer, melanoma, and endocrine tumours (71, 75). The inhibition of cell proliferation and induction of cellular differentiation, along with the induction of apoptosis, are events that contribute for this antineoplastic activity of calcitriol, where the angiogenesis inhibition has a critical role in anti-tumoral activity (75).

Therefore, and considering cell proliferation and differentiation, the regulation of these cellular actions are not always linked. In most of cell lines that express a functional *VDR*, the presence of calcitriol results in an accumulation of these cells in G_0/G_1 phase of the cell cycle. The exact sequence events between *VDR*-mediated transactivation, repression of target genes, and the actual G_0/G_1 arrest state are probably a cell type-specific event. However, we can verify a convergence on signaling pathways at the level of complex formation between the retinoblastoma family of pocket proteins, the E2F family transcription factors, and the subsequent down-regulation of a large number of E2F target genes that are necessary for the normal cell cycle progression. The proteins p107 and p130, from retinoblastoma family of pocket proteins, are crucial for the anti-proliferative activity of calcitriol. Calcitriol inhibits also the cellular growth by induced effects in signaling pathways started by TGF-ß, epidermal growth factor, IGF, prostaglandins, Wnt-ligands, as well as other mitogenic signaling pathways (75).

Furthermore, calcitriol induces apoptosis in some tumours, including, for example, breast carcinomas, colon and prostate. Alterations in the expression of Bcl-2 family of pro- and anti-apoptotic proteins and subsequent release of cytochrome C from the mitochondria are mechanisms which contribute for apoptosis induction through calcitriol actions. However, depending on cell type, calcitriol has also shown to interfere with other signaling pathways, like IGF and TNF-α, which may lead to induction of programmed cell death (75).

Another critical action that results from the mechanic action of vitamin D/*VDR* in cancer is associated with the inhibition of angiogenesis and tumour invasive ability. Because angiogenesis is essential for the growth of solid tumours, an anti-angiogenic activity promoted from calcitriol is a very important mechanism to repress the tumoral activity. Calcitriol inhibits angiogenesis through the inhibition of the proliferation of tumour-derived endothelial

cells, and repression of angiogenic factors such as VEGF, TGF-α, and EGF. From the point of view of invasiveness, the possibility of metastasis in secondary sites requires the degradation of the extracellular matrix, a process that could be inhibited by calcitriol. The inhibition of serine proteinases and tenascin-C, the decreased activity of metalloproteinases and the increased expression of E-cadherin all contribute to the anti-invasive effects of calcitriol (75).

1.11. Aims of the study

Due to the major importance of vitamin D/*VDR* complex, we aimed to verify in which way *VDR* polymorphisms influence the predisposition for thyroid cancer development.

Thus, the aims of the present work were: a) to analyse the contribution of four well-known *VDR* polymorphisms (*Fok*I, *Bsm*I, *Apa*I and *Taq*I) to the genetic susceptibility of thyroid cancer in the Portuguese population; b) to analyse the effect of *VDR* haplotypes with DTC; c) to correlate these *VDR* polymorphisms/haplotypes with clinical parameters such as thyroid cancer type (PTC vs. FTC), age (≤45 vs. >45 years), gender (male vs. female), carcinoma size (≤10mm vs. >10mm), lymph node metastasis and distant metastasis, multicentricity, and cancer stage (stages I-II vs. III-IV).

2 - Materials and methods

2.1. Studied population

All the participants in the study were Caucasian Portuguese inhabitants. The individuals were subdivided into two groups: patients with thyroid cancer (N = 208) and a control group (N = 248). The thyroid cancer patients (171 females and 36 males; mean age ± SD, 47.5 ± 14.3 years) were recruited by the *Instituto Português de Oncologia de Coimbra*. The control group consisted of 105 male and 143 female blood donor volunteers with no clinical history of thyroid cancer (mean age ± SD, 31.5 ± 13.8 years). The clinical process of thyroid cancer patients was registered with all the necessary data for the identification of the patient. Informed consent was obtained from all the participants. All the blood donors remained anonymous, being only registered the age, sex, weight, height and place of birth. This study was approved by the institutions ethical committees.

2.2. DNA extraction

Blood samples were collected from patients and healthy donors in 10 mL EDTA tubes. In the laboratory, a code number was attributed to each sample, in order to identify the patient or the blood donor of the control group. After this process of data registration, genomic DNA was extracted from the collected blood. This step was based on the "salting-out" method described by Miller et al. (76). This DNA extraction method used proteinase K, for peptide hydrolysis, and saturated NaCl solution for cellular proteins dehydration and precipitation. Therefore, the salting-out of cellular proteins occurred, being the genomic DNA subsequently recovered by standard salt and ethanol precipitation (76).

The previously collected blood was transferred to a 50 mL Falcon tubes, identified with the same number given to the patient or healthy subject. After this procedure, Red Blood Cell (RBC) lysis buffer (155 mM NH_4Cl; 20 mM $KHCO_3$; 0,1 mM Na_2EDTA; pH 7,4) was added to the Falcon tube, completing a total volume of 40 mL. Then, the falcon tubes were vortexed and incubated 15 minutes on ice. During this incubation period, the tubes were inverted several times. After the incubation on ice, the Falcon tubes were centrifuged (AllegraTM X-22R Centrifuge, Beckman Coulter, USA) during 10 minutes under conditions of 2500 rpm and at 4ºC. The supernatant was discarded after this step, leaving a pellet with leucocytes. This whole procedure was repeated once again. The Falcon tubes were filled with a total volume of 30 mL of RBC, and then the pellet was vortexed and resuspended, performing a second wash of the leucocytes.

After this secondary cell wash up, the supernatant was discarded again, leaving the pellet with cells. The pellet was vortexed and resuspended, being added to the Falcon tube a 5 mL of a SE Buffer (75 mM NaCl; 25 mM Na2EDTA; pH 8,0), 12.5 µL of proteinase K (20 mg/mL) and 500 µL of SDS (Sodium dodecyl sulfate) 10%. Then the Falcon tubes were incubated overnight at a temperature of 55ºC. This process promotes the cell lysis overnight.

After the overnight incubation, 3 mL of saturated NaCl (6M) was added to the Falcon tubes, being after that incubated at 55ºC during 10 minutes. After this period, the samples were vortexed during 25 seconds. Then, they were centrifuged under conditions of 4000 rpm, during 30 minutes at a temperature of 15ºC, in order to avoid the precipitation of the SDS.

To precipitate the DNA, the supernatant was transferred to a new 50 mL Falcon tube identified with the same registration code of the previous one. Cold 100% ethanol was added at about 2x the volume of the supernatant and the tubes were inverted gently about 50 times, observing the precipitation of the DNA. After this, the samples were centrifuged at 4500 rpm, during 5 minutes at 4ºC. The supernatant was decanted and discarded, and 10 mL of cold 70% ethanol was added to the tubes, that were once again inverted. They were then incubated again under conditions of 4500 rpm, during 5 minutes and at a temperature of 4ºC. The supernatant was discarded again and the DNA pellet was transferred to a 1.5 mL microcentrifuge tube. Then the samples were air dried for about 30 minutes. After this period, the DNA was hydrated with 1 mL of Tris-EDTA and left in constant rotation (Programmable Rotator Mixer, Star Lab) overnight at room temperature.

2.3. DNA Quantification

After the DNA extraction from the peripheral blood samples, the quantity and the quality of the DNA was assessed by spectrophotometric quantification using Nanodrop (Nanophotometer™, Implen, Germany). The DNA absorbs the UV light that passes through the sample at a 260 nm. During the spectrophotometric quantification, the DNA concentration is evaluated in each DNA sample, based on the amount of UV light absorbed by the same sample, displaying the final DNA concentration value.

Contamination of the DNA samples with proteins is possible. To verify the purity of the DNA sample, the ratio of OD_{260}/OD_{280} was measured. A ratio with a range of values between 1.8 and 2.0 indicates that the UV light was absorbed by nucleic acids. An OD_{260}/OD_{280} ratio below 1.8 shows that the sample was contaminated with proteins. Ratio values above 2.0 indicates a possible contamination by RNA. After the DNA samples quantification, all the samples were diluted at a concentration of 100 ng/µL and stored at -20ºC.

2.4. Polymerase Chain Reaction

The polymerase chain reaction, commonly known as PCR, is a biochemical method, developed by Kary Mullis in 1983, used to amplify a specific DNA fragment through several repeated heating and cooling reactions, synthesizing a new strand of DNA complementary to prior DNA template. This method uses a thermal cycler, which performs a number of cycles with different temperatures for DNA melting and enzymatic replication of the DNA.

In this study, the *VDR* gene was amplified using the PCR method. The exon 2 was amplified to study the *Fok*I polymorphism, and exon 9 was amplified for the *Apa*I and *Taq*I polymorphisms assessment. The intron 8 was amplified to study the *Bsm*I polymorphism.

The genomic DNA was amplified using specific primers and according to a specific PCR program (T100TM Thermal Cycler, Bio Rad). The initial denaturation step lasted for 5 minutes at 95ºC, followed by 35 amplification cycles: a denaturation step at 95ºC for 30 seconds, an annealing step for 30 seconds with specific temperature conditions for each exon or intron (see Table 1), and an extension step for 30 seconds at 72ºC. After these 35 amplification cycles, there was a final extension step for 10 minutes at 72ºC. The final hold step was performed at 15ºC with undefined time.

The PCR reaction of the exons 2 and 9 were made with a final volume mix of 25 µL. This mix contained 1X Buffer (20 mM Tris HCL; pH 7.5; 100 mM NaCl; 0.1 mM EDTA; 1mM dithiothreitol; 50% (v/v) glycerol), 0.2 mM of dNTPs, optimized concentration of $MgCl_2$ for each fragment, 0.3 mM of forward and reverse primers for their respective DNA fragment, 1U of Dream Taq (NZYtech Lda., Lisbon, Portugal), and about 200 ng of genomic DNA. The PCR reaction of intron 8 was made with a final volume mix of 15 µL containing Supreme NZYTaq 2X Green Master Mix (NZYtech Lda., Lisbon, Portugal), optimized conditions of $MgCl_2$ and the respective forward and reverse primers for the intron. About 200 ng of genomic DNA was added to the final mix.

Table 1 – *VDR* PCR primers. Primers and specific PCR reaction conditions for *VDR* exons 2 and 9 and intron 8.

VDR Exon/Intron	Primers (5'-3')	$MgCl_2$ (mM)	Annealing temperature (C°)	Fragment (bp)
2	F:AGCTGGCCCTGGCACTGACTCTGGCTCT R:GAGGGAGAAGAAGCAAGGTGTTTCCAT	1.5	60	267

8	F:AGTGTGCAGGCGATTCGTAG R:CTGAGAGATGGTTCTGCCTAT	1.0	65	191
9	F:CAGAGCATGGACAGGGAGCAA R:GAGACCTCAGCCATGAGGAGTTGC	1.5	60	745

2.5. Enzymatic Digestion

After the amplification of the *VDR* fragments with the polymerase chain reaction method, all the amplified fragments were digested with specific enzymes under specific time and temperature conditions, allowing to assess the genotype of each individual used in the study.

Four *VDR* polymorphisms were studied: the FokI (rs10735810 T>C), BsmI (rs1544410 A>G), ApaI (rs7975232 G>T) and TaqI (rs731236 C>T). To perform the enzymatic digestion, the respective enzymes *Fok*I, *Bsm*I, *Apa*I and *Taq*I were used under specific conditions.

To verify the *VDR* polymorphisms in the exon 2, *Fok*I enzyme (New England BioLabs®, R0109S) was used. After the amplification of this *VDR* fragments, 10 µL of PCR product was used to proceed with the respective fragment digestion, adding to this PCR product a mix of a 5 µL containing 4 Units of *Fok*I enzyme and a 1X NEbuffer 4 (50 mM potassium acetate; 20 mM Tris-acetate; 10 mM magnesium acetate; 1 mM dithiothreitol; pH 7.9). This digestion occurred in an incubator under temperature conditions of 37ºC for 1 hour. After the digestion of *VDR* exon 2 with *Fok*I enzyme, three genotypes could be distinguished: the homozygous TT and CC genotype and the heterozygous TC genotype. The homozygous TT genotype resulting from this digestion produced 2 fragments (70 and 197 bp), and the CC genotype produced a single fragment (267 bp) due to absence of digestion. The heterozygous CT genotype produced 3 fragments (70, 197 and 267 bp).

For the *Bsm*I polymorphism, a 10 µL of PCR product with the amplified *VDR* intron 8 was used. A 5 µL mix containing 5 Units of *Bsm*I enzyme (New England BioLabs®, R0134S) and 1X NEbuffer 4 (50 mM potassium acetate; 20 mM Tris-acetate; 10 mM magnesium acetate; 1 mM dithiothreitol; pH 7.9) was added to the 10 µL of PCR product. For this digestion samples were incubated at temperature conditions of 65ºC for 1 hour. After the digestion with *Bsm*I, three genotypes could be distinguished. Homozygous GG genotype produced 2 fragments (76

and 115 bp), and homozygous AA genotype produced a single fragment (191 bp). Heterozygous genotype GA produced 3 fragments (76, 115 and 191 bp).

To study the *Apa*I and *TaqI* polymorphisms, the same amplified PCR product of *VDR* exon 9 was used. For the *Apa*I polymorphism, a 5 µL mix containing 5 units of *Apa*I enzyme (New England BioLabs®, R0114S), a 1X NEbuffer 4 (50 mM potassium acetate; 20 mM Tris-acetate; 10 mM magnesium acetate; 1 mM dithiothreitol; pH 7.9) and BSA was added to the PCR product. The digestion of *Apa*I occurred under temperature conditions of 25ºC for 1 hour, producing three distinct genotypes. The homozygous GG genotype produced 2 fragments (217 and 528 bp), and homozygous TT genotype produced 1 fragment (745 bp). Heterozygous GT genotype produced 3 distinct fragments (217, 528 and 745 bp). For the *Taq*I polymorphism, a 5 µL mix containing 5 units of *Taq*I enzyme (New England BioLabs®, R0149S), a 1X NEbuffer 4 (50 mM potassium acetate; 20 mM Tris-acetate; 10 mM magnesium acetate; 1 mM dithiothreitol; pH 7.9) and BSA was added to the PCR product. Samples were digested under temperature conditions of 65ºC for 1 hour, resulting in three different genotypes. Homozygous CC genotype produced 3 fragments (201, 251 and 293 bp), and homozygous TT genotype produced 2 fragments (251 and 494 bp). Heterozygous TC genotype produced 4 fragments (201, 251, 293 and 494 bp).

2.6. Electrophoresis

To assess the PCR amplification and enzymatic digestion, electrophoresis technique was used to verify the migration of DNA fragments in an agarose gel. Agarose gel was prepared by boiling agarose with 1X TAE buffer and stained with Green Safe Buffer (1 µL/mL) (NZYtech Lda., Lisbon, Portugal). For PCR products electrophoresis, an agarose gel was made with a concentration of 1% (m/v). In the agarose gel, 5 µL of PCR product were loaded and the migration of fragments in the agarose gel was performed under voltage conditions of 130V for 20 minutes. For electrophoresis of enzymatic digestion, an agarose gel with a concentration of 3% (m/v) was made, with the total volume of enzymatic digestion products loaded in the gel. The migration in the agarose gel was performed under voltage conditions of 110V for 35 minutes.

To compare the molecular weight of the DNA fragments, a molecular weight marker (HyperLadder II, Bioline or VC 100 bp Plus DNA Ladder, Vivantis) was used. For agarose gel visualization, UV light using UVITEC system (Uvitec Cambridge) was used.

2.7. DNA Sequencing

DNA sequencing of selected samples was used to confirm the genotypes of different *VDR* polymorphisms. DNA sequencing was developed in the mid-seventies by Frederick Sanger (77). Since then, the sequencing of DNA has been revolutionized by the advent of dideoxynucleotides (ddNTPs). The ddNTPs are readily incorporated into a growing DNA chain, but lack the 3' hydroxyl group that allows the chain to continue and terminate the polymerization effectively. DNA sequencing occurs in multiple steps (78).

However, before DNA sequencing, a previous step takes place. After the amplification of *VDR* fragments through PCR, the resultant products had to be purified. The PCR purification step eliminates and neutralizes PCR residuals. In this reaction, a 10 µL of PCR product was used, and 1U of *FastAP* (thermosensitive Alkaline Phosphatase, Thermo Scientific) enzyme and 10U of *Exo*I (Exonuclease I, Thermo Scientific) were added. Then, the reaction was incubated during 40 minutes, in two steps of 20 minutes at 37ºC, for activation, and 20 minutes at 80ºC, for inactivation.

After purification of PCR products, the DNA sequencing was done according to the GenomeLab™ Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter). The equipment used for DNA sequencing was GenomeLab™ GeXP sequencer, Genetic Analysis System (Beckman Coulter). The genotyping analysis was carried out using GenomeLab system version 10.2 software (Beckman Coulter).

2.8. Statistical analysis

Statistical analysis was performed in this association study for the *VDR* polymorphisms in order to assess these polymorphisms contribution to thyroid cancer development. All the allelic and genotypic frequencies were assessed through direct counting, after electrophoresis visualization of digested PCR fragments on a 3% agarose gel. This counting was performed in both patients and control groups. Then, the Hardy-Weinberg equilibrium (HWE) was performed in the control group for all *VDR* polymorphisms and their genotypic frequencies. The HWE law states that the alleles of a locus are independent and therefore in an infinite population the expected genotype frequencies remain constant and can be predicted by the allele frequencies according to the binomial distribution (79). The HWE assumptions states that there are no input of new genetic material, individuals mate randomly, the population is effectively infinite, all mated pairs produce the same number of offspring and all genotypes survive with the same probability (80). Therefore, a comparison of the expected and observed

genotype frequencies in the control group provides a test of HWE. To verify this comparison, a chi-square test was used (X^2).

After the direct counting of all genotypes of *VDR Fok*I, *Bsm*I, *Apa*I and *Taq*I polymorphisms, the respective haplotypes were constructed. These *VDR* haplotypes result from the combination of all genotypes between the four *VDR* polymorphisms. The considered haplotypes for this study were only those with homozygous genotypes (e.g. CC/AA/GG/TT) or those with a maximum of one heterozygous genotype among the four polymorphisms (e.g. CT/AA/GG/TT). Haplotypes with more than one heterozygous genotype in the *VDR* sequence (e.g. TT/GA/GG/TC) were not considered because it is uncertain which haplotype is present in the *VDR* sequence from four possible different haplotypes. Therefore, for this study, only 48 different haplotypes were considered.

From the available patients data, different parameters for thyroid cancer were assessed. Patients were divided into different subgroups in order to study thyroid cancer type differences (PTC vs. FTC), age (≤45 vs. >45 years), gender (male vs. female), carcinoma size (≤10mm vs. >10mm), lymph node metastasis or distant metastasis, multicentricity, and stage of cancer (stages I-II vs. III-IV). For these comparisons, Pearson's X^2-test of independence was used. However, X^2 approximation can be poor when there are low genotype counts, so when cases less than five, Fisher's exact test was used. The results were considered statistically significant when p-value < 0.05. The odds ratio (OR) was also estimated, with an associated 95% confidence interval. Since multiple parameters were considered in this study, Bonferroni's correction was used in order to avoid false positive results, by dividing the threshold for statistical significance (p-value < 0.05) by the number of comparisons.

3 – Results

3.1. *VDR* genotyping

3.1.1. *Fok*I polymorphism

To analyse the *Fok*I polymorphism, exon 2 of *VDR* was amplified using the PCR technique. After the PCR, a 267 bp fragment of genomic DNA was obtained. To view this fragment, the PCR product was loaded onto a 1% agarose gel, and electrophoresis was performed (Figure 6a). Then, after the verification of absence of contamination in the PCR products, the genotyping of *VDR Fok*I polymorphism was conducted using enzymatic digestion technique. From that enzymatic digestion, three different genotypes for *Fok*I polymorphism could be distinguished. The homozygous CC genotype produces a single fragment (267 bp), the homozygous TT genotype produces two different fragments (70 and 197 bp), and the CT genotype produces three different fragments (70, 197 and 267 bp) (Figure 6b). For further confirmation of these genotypes, DNA sequencing was also performed on a representative individual of each genotype (Figure 6c).

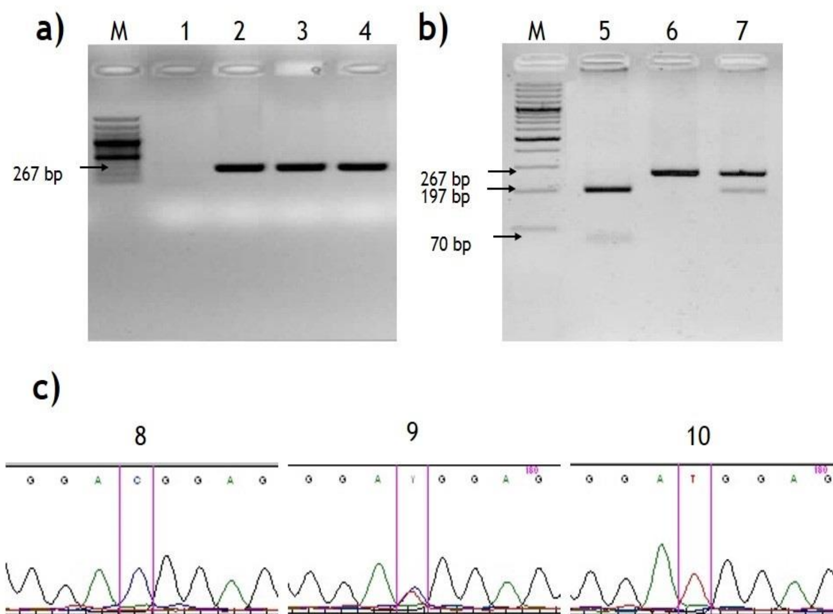

Figure 6 – Genotyping of the *Fok*I polymorphism. a) Electrophoresis of a 1% agarose gel with exon 2 PCR product loaded; 1 – negative control; 2, 3 and 4 – exon 2 fragment with 267 bp. b) Electrophoresis of a 3% agarose gel with *Fok*I enzymatic digestion of *VDR* exon 2; 5 – homozygous TT genotype (70 and 197 bp); 6 – homozygous CC genotype (267 bp); 7 – heterozygous CT genotype (70, 197 and 267 bp). c) DNA sequencing of *Fok*I polymorphism; 8 - homozygous CC genotype; 9 - heterozygous CT genotype; 10 - homozygous TT genotype.

3.1.2. *Bsm*I polymorphism

The *Bsm*I polymorphism, located in *VDR* intron 8, was also amplified through the PCR technique under specific conditions. The amplified fragment, with a size of 191 bp, could be viewed in the agarose gel after electrophoresis (Figure 7a). Then, the PCR products with amplified intron 8, were submitted to enzymatic digestion with the *Bsm*I enzyme, producing three distinct genotypes. The homozygous GG genotype presented two fragments (76 and 115 bp), the homozygous AA genotype presented a single fragment (191 bp), and the heterozygous GA genotype presented three fragments (76, 115 and 191 bp) (Figure 7b). *Bsm*I polymorphism genotypes were also confirmed through DNA sequencing on a representative individual of each genotype (Figure 7c).

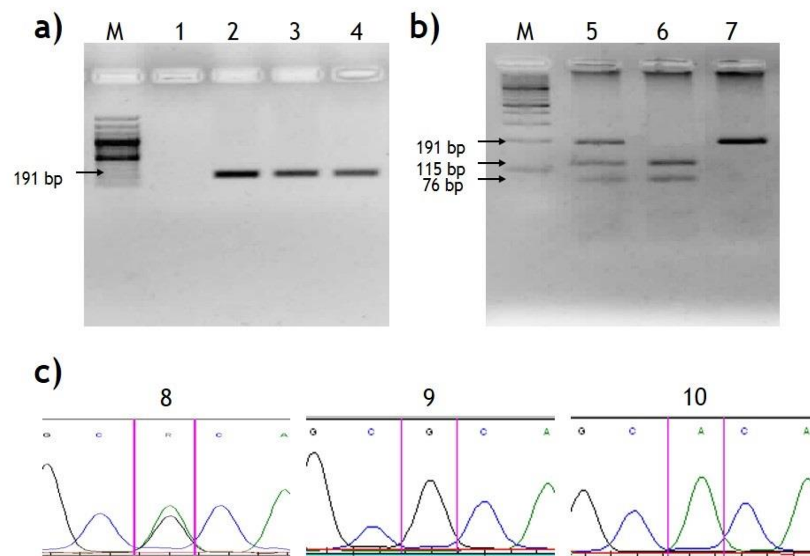

Figure 7 – Genotyping of the *Bsm*I polymorphism. a) Electrophoresis of a 1% agarose gel with intron 8 PCR product loaded; 1 – negative control; 2, 3 and 4 – intron 8 fragment with 191 bp. b) Electrophoresis of a 3% agarose gel with *Bsm*I enzymatic digestion of *VDR* intron 8; 5 – heterozygous GA genotype (76, 115 and 191 bp); 6 – homozygous GG genotype (76 and 115 bp); 7 – homozygous AA genotype (191 bp). c) DNA sequencing of *Bsm*I polymorphism; 8 - heterozygous GA genotype; 9 - homozygous GG genotype; 10 - homozygous AA genotype.

3.1.3. *Apa*I polymorphism

To assess the *Apa*I polymorphism genotypes, *VDR* exon 9 was amplified using PCR technique. The PCR product obtained was loaded on an agarose gel and an electrophoresis was performed. From the agarose gel, we could see the *VDR* exon 9 fragment, with 745 bp (Figure 8a). Then, using the *Apa*I enzyme, the enzymatic digestion was performed in order to verify the three distinct genotypes. The homozygous GG genotype showed two fragments (217 and 528 bp), the homozygous TT genotype showed one fragment (745 bp), and the heterozygous

GT genotype showed three fragments (217, 528 and 745 bp) (Figure 8b). DNA sequencing was also performed for further confirmation of *Apa*I genotype on a representative individual for each genotype (Figure 8c).

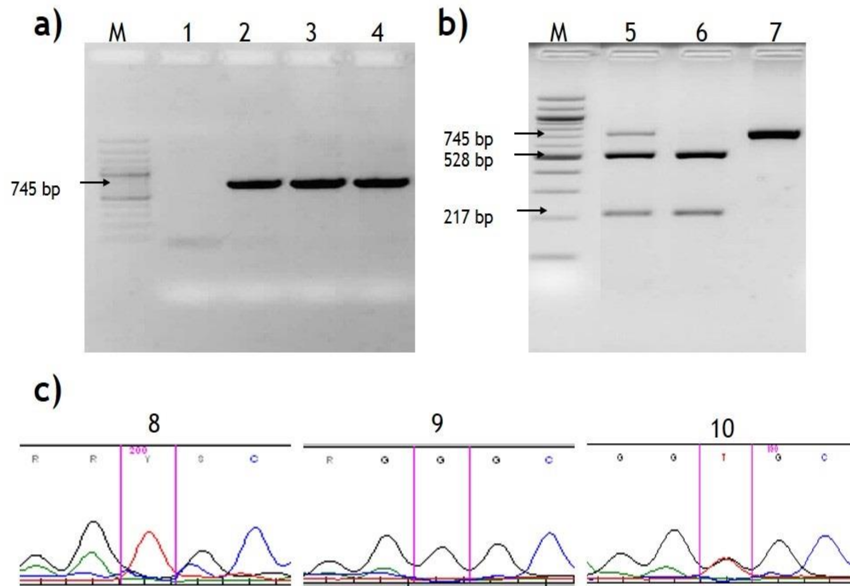

Figure 8 – Genotyping of the *Apa*I polymorphism. a) Electrophoresis of a 1% agarose gel with exon 9 PCR product loaded; 1 – negative control; 2, 3 and 4 – exon 9 fragment with 745 bp. b) Electrophoresis of a 3% agarose gel with *Apa*I enzymatic digestion of *VDR* exon 9; 5 – heterozygous GT genotype (217, 528 and 745 bp); 6 – homozygous GG genotype (217 and 528 bp); 7 – homozygous TT genotype (745 bp). c) DNA sequencing of *Apa*I polymorphism; 8 - homozygous TT genotype; 9 - homozygous GG genotype; 10 - heterozygous GT genotype.

3.1.4. *Taq*I polymorphism

The *Taq*I polymorphism, located in *VDR* exon 9, is in the same PCR fragment as the *Apa*I polymorphism. Therefore, the same PCR product for *Apa*I was used to assess *Taq*I genotypes. The fragment of exon 9, loaded on an agarose gel and submitted to electrophoresis, also presents a fragment of 745 bp (Figure 9a). The genotyping was carried out by enzymatic digestion, where three different genotypes of *Taq*I could be assessed. The homozygous TT genotype produced two fragments (251 and 494 bp), the homozygous CC genotype produced three different fragments (201, 251 and 293), and the heterozygous TC genotype produced four distinct fragments (201, 251, 293 and 494 bp) (Figure 9b). To confirm these *Taq*I genotypes, DNA sequencing was used on a representative individual for each genotype (Figure 9 c).

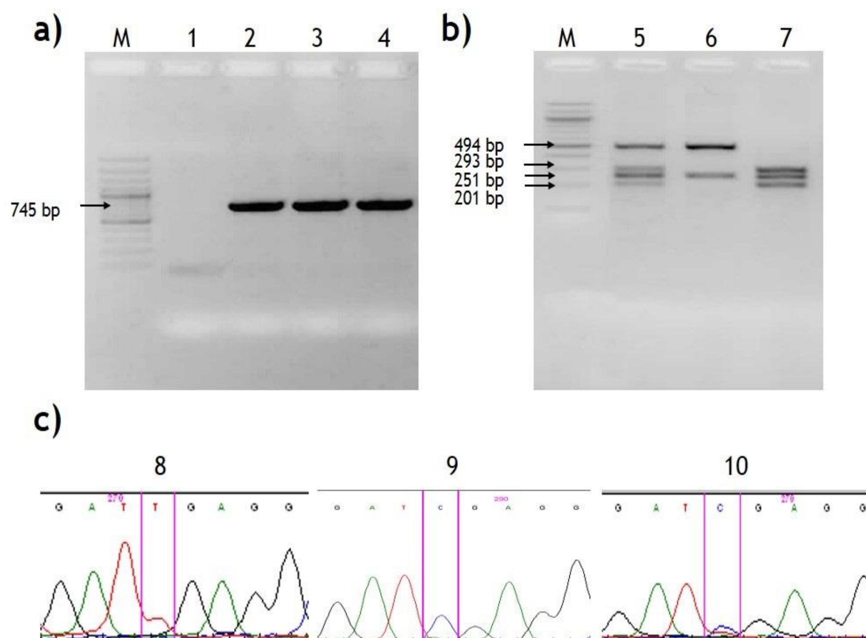

Figure 9 – Genotyping of the *Taq*I polymorphism. a) Electrophoresis of a 1% agarose gel with exon 9 PCR product loaded; 1 – negative control; 2, 3 and 4 – exon 9 fragment with 745 bp. b) Electrophoresis of a 3% agarose gel with *Taq*I enzymatic digestion of *VDR* exon 9; 5 – heterozygous TC genotype (201, 251, 293 and 494 bp); 6 – homozygous TT genotype (251 and 494 bp); 7 – homozygous CC genotype (201, 251 and 293 bp). c) DNA sequencing of *Taq*I polymorphism; 8 - homozygous TT genotype; 9 - homozygous CC genotype; 10 - heterozygous TC genotype.

3.2. Statistical analysis

3.2.1. The Hardy-Weinberg equilibrium (HWE)

The HWE was performed in order to verify if the control group of the present study was under the assumptions of this law. Therefore, a direct counting of the observed genotype frequencies in this group was made, and then the expected values calculated. For example, if the alleles G and T are present in a genotype, where the allele frequency for G allele is p and for T allele is q = (1-p), the expected values for the GG, GT and TT genotypes will be p^2, $2pq$ and q^2 (81). Following these conditions, a comparison between the observed and expected genotype frequencies in all four *VDR* polymorphisms was carried out based on a X^2-test. The p values verified for the HWE equilibrium weren't significant for any of the four *VDR* polymorphisms, indicating that the genotypes frequencies did not deviate significantly from these expected under the assumptions of HWE (Table 2).

Table 2 – HWE results. The four *VDR* polymorphisms and their respective genotypes in the control group. The observed and expected values for each genotype are represented and, under X2-test, p-values are calculated and presented.

Polymorphism	Genotype	Observed values	Expected values	X^2	p-value
***Fok*I**	CC	110	115,17	0,01	0,997
	CT	118	107,67		
	TT	20	25,17		
***Bsm*I**	GG	65	69,20	3,02	0,221
	GA	132	123,60		
	AA	51	55,20		
***Apa*I**	GG	49	50,58	0,05	0,975
	GT	126	122,84		
	TT	73	74,58		
***Taq*I**	TT	82	87,13	0,69	0,707
	TC	130	119,73		
	CC	36	41,13		

3.2.2. Analysis of *VDR* polymorphisms and haplotypes

Before the analysis of the data subdivided into diverse parameters, like, for example, different cancer types, age or gender, a raw comparison between patients and control group was made. For this analysis, all individuals from each group were considered, accounting for 208 patients and 248 controls. Therefore, all parameters were considered in this comparison. Furthermore, all the possible haplotypes present in each group were compared.

Table 3 – Analysis of Patients vs. Controls. *VDR* polymorphisms and respective genotypes, allele and haplotype frequencies for each patients and control group are represented. The odds ratio values (OR) and respective 95% confidence interval (95% CI) are also represented.

Polymorphism	Genotype /Alleles	Patients, n (%) N=208	Controls, n (%) N=248	OR	95% CI	p-value
***Fok*I**	CC	96 (46.15)	110 (44.35)	1.08	0.74-1.56	0.701
	CT	95 (45.67)	118 (47.58)	0.93	0.64-1.34	0.684
	TT	17 (8.17)	20 (8.06)	1.01	0.52-1.99	0.966
	C	287 (68.99)	338 (68.15)	1.04	0.79-1.38	0.784
	T	129 (31.01)	158 (31.85)	0.96	0.73-1.27	

***Bsm*I**	GG	58 (27.88)	65 (26.21)	1.09	0.72-1.65	0.688
	GA	96 (46.15)	132 (53.23)	0.75	0.52-1.09	0.132
	AA	54 (25.96)	51 (20.56)	1.35	0.88-2.10	0.173
	G	212 (50.96)	262 (52.82)	0.93	0.72-1.20	0.575
	A	204 (49.04)	234 (47.18)	1.08	0.83-1.40	
***Apa*I**	GG	34 (16.35)	49 (19.76)	0.79	0.49-1.29	0.347
	GT	100 (48.08)	126 (50.81)	0.90	0.62-1.30	0.561
	TT	74 (35.58)	73 (29.44)	1.32	0.89-1.96	0.162
	G	168 (40.38)	224 (45.16)	0.82	0.63-1.07	0.147
	T	248 (59.62)	272 (54.84)	1.22	0.93-1.58	
***Taq*I**	TT	67 (32.21)	82 (33.06)	0.96	0.65-1.43	0.847
	TC	101 (48.56)	130 (52.42)	0.86	0.59-1.24	0.411
	CC	40 (19.23)	36 (14.52)	1.40	0.86-2.30	0.178
	T	235 (56.49)	294 (59.27)	0.89	0.69-1.16	0.396
	C	181 (43.51)	202 (40.73)	1.12	0.86-1.46	
Haplotypes	C/G/G/T	46 (25.00)	57 (30.00)	0.78	0.49-1.23	0.279
	C/G/T/T	19 (10.30)	11 (5.80)	1.87	0.87-4.06	0.106
	C/G/T/C	3 (1.60)	1 (0.50)	3.13	0.32-30.39	0.299
	C/A/T/T	4 (2.20)	3 (1.60)	1.39	0.31-6.28	0.671
	C/A/T/C	58 (31.50)	45 (23.70)	1.48	0.94-2.34	0.090
	T/G/G/T	22 (12.00)	33 (17.40)	0.65	0.36-1.16	0.140
	T/G/T/T	5 (2.70)	3 (1.60)	1.74	0.41-7.39	0.447
	T/A/G/T	1 (0.50)	4 (2.10)	0.25	0.03-2.30	0.189
	T/A/T/C	23 (12.50)	27 (14.20)	0.86	0.47-1.57	0.627

Comparisons between patient and control groups showed no significant differences for any *VDR* genotype, allele, or haplotype (Table 3).

3.2.3. Analysis of thyroid cancer types

For the analysis of cancer type, the patients group were divided into two subgroups: those with papillary thyroid cancer (N = 182) and those with follicular thyroid cancer (N = 25). In these two groups, genotypes, allele and haplotype frequencies were compared.

Table 4 – Analysis of thyroid cancer type. *VDR* polymorphisms and respective genotypes, allele and haplotype frequencies for the PTC and FTC groups. The odds ratio values (OR) and respective 95% confidence interval (95% CI) are also represented.

Polymorphism	Genotype /Alleles	Patients PTC, n (%) N=182	Patients FTC, n (%) N=25	OR	95% CI	p-value
***Fok*I**	CC	86 (47.25)	9 (36.00)	1.59	0.67-3.79	0.290
	CT	82 (45.05)	13 (52.00)	0.76	0.33-1.75	0.513
	TT	14 (7.69)	3 (12.00)	0.61	0.16-2.30	0.462
	C	254 (69.78)	31 (62.00)	1.42	0.77-2.61	0.265
	T	110 (30.22)	19 (38.00)	0.71	0.38-1.30	
***Bsm*I**	GG	51 (28.02)	7 (28.00)	1.00	0.39-2.54	0.998
	GA	82 (45.05)	13 (52.00)	0.76	0.33-1.75	0.513
	AA	49 (26.92)	5 (20.00)	1.47	0.52-4.14	0.460
	G	184 (50.55)	27 (54.00)	0.87	0.48-1.58	0.647
	A	180 (49.45)	23 (46.00)	1.15	0.63-2.08	
***Apa*I**	GG	29 (15.93)	5 (20.00)	0.76	0.26-2.18	0.607
	GT	88 (48.35)	11 (44.00)	1.19	0.51-2.76	0.683
	TT	65 (35.71)	9 (36.00)	0.99	0.41-2.36	0.978
	G	146 (40.11)	21 (42.00)	0.92	0.51-1.68	0.798
	T	218 (59.89)	29 (58.00)	1.08	0.59-1.97	
***Taq*I**	TT	60 (32.97)	7 (28.00)	1.26	0.50-3.19	0.619
	TC	85 (46.70)	15 (60.00)	0.58	0.25-1.37	0.212
	CC	37 (20.33)	3 (12.00)	1.87	0.53-6.59	0.323
	T	205 (56.32)	29 (58.00)	0.93	0.51-1.70	0.822
	C	159 (43.68)	21 (42.00)	1.07	0.59-1.95	
Haplotypes	C/G/G/T	39 (23.50)	7 (31.80)	0.66	0.25-1.73	0.393
	C/A/T/T	5 (3.00)	1 (4.50)	0.65	0.07-5.85	0.701
	C/A/T/C	52 (31.30)	6 (27.30)	1.22	0.45-3.29	0.699
	T/G/G/T	18 (10.80)	4 (18.20)	0.55	0.17-1.80	0.314
	T/G/T/T	4 (2.40)	1 (4.50)	0.52	0.06-4.86	0.559
	T/A/T/T	3 (1.80)	1 (4.50)	0.39	0.04-3.89	0.403
	T/A/T/C	21 (12.70)	2 (9.10)	1.45	0.32-6.65	0.632

When genotypic and allelic frequencies between PTC and FTC patients were compared, there were no significant results. The haplotype analysis also showed no statistically significant differences (Table 4).

3.2.4. Analysis of age of diagnosis

To assess the age of diagnosis differences, the patients group were divided into two different groups. For this comparison, we considered a patients group with age of diagnosis ≤45 years (N = 98), and another group with patients with age of diagnosis >45 years (N= 109). For this

purpose, genotypic and allelic frequencies of the four *VDR* polymorphism were compared. Common haplotypes from both groups were also compared.

Table 5 – Analysis of age of diagnosis. *VDR* polymorphisms and respective genotypes, allele and haplotype frequencies for each patient groups with age of diagnosis ≤45 and >45. The odds ratio values (OR) and respective 95% confidence interval (95% CI) are also represented.

Polymorphism	Genotype /Alleles	Patients ≤45 years old, n (%) N=98	Patients >45 years old, n (%) N=109	OR	95% CI	p-value
***Fok*I**	CC	41 (41.84)	54 (49.54)	0.73	0.42-1.27	0.267
	CT	49 (50.00)	46 (42.20)	1.37	0.79-2.37	0.261
	TT	8 (8.16)	9 (8.26)	0.99	0.37-2.67	0.980
	C	131 (66.84)	154 (70.64)	0.84	0.55-1.27	0.404
	T	65 (33.16)	64 (29.36)	1.19	0.79-1.81	
***Bsm*I**	GG	28 (28.57)	30 (27.52)	1.05	0.57-1.93	0.867
	GA	46 (46.94)	49 (44.95)	1.08	0.63-1.87	0.775
	AA	24 (24.49)	30 (27.52)	0.85	0.46-1.59	0.620
	G	102 (52.04)	109 (50.00)	1.09	0.74-1.6	0.678
	A	94 (47.96)	109 (50.00)	0.92	0.63-1.36	
***Apa*I**	GG	16 (16.33)	18 (16.51)	0.99	0.47-2.06	0.971
	GT	51 (52.04)	48 (44.04)	1.38	0.8-2.38	0.250
	TT	31 (31.63)	43 (39.45)	0.71	0.4-1.26	0.241
	G	83 (42.35)	84 (38.53)	1.17	0.79-1.74	0.430
	T	113 (57.65)	134 (61.47)	0.85	0.58-1.26	
***Taq*I**	TT	32 (32.65)	35 (32.11)	1.03	0.57-1.84	0.934
	TC	48 (48.98)	52 (47.71)	1.05	0.61-1.82	0.855
	CC	18 (18.37)	22 (20.18)	0.89	0.44-1.78	0.741
	T	112 (57.14)	122 (55.96)	1.05	0.71-1.55	0.809
	C	84 (42.86)	96 (44.04)	0.95	0.65-1.41	
Haplotypes	C/G/G/T	23 (25.60)	23 (23.50)	1.12	0.58-2.18	0.740
	C/G/T/T	10 (11.10)	9 (9.20)	1.24	0.48-3.20	0.661
	C/A/G/T	1 (1.10)	1 (1.00)	1.09	0.07-17.69	0.952
	C/A/T/T	2 (2.20)	2 (2.00)	1.09	0.15-7.91	0.931
	C/A/T/C	23 (25.60)	35 (35.70)	0.62	0.33-1.16	0.132
	T/G/G/T	14 (15.60)	8 (8.20)	2.07	0.83-5.20	0.115
	T/G/T/T	1 (1.10)	4 (4.10)	0.26	0.03-2.41	0.206
	T/A/G/T	2 (2.20)	1 (1.00)	2.20	0.20-24.74	0.511
	T/A/T/C	10 (11.10)	13 (13.30)	0.82	0.34-1.97	0.653

In this analysis, there were no statistically significant differences between the genotypic and allelic frequencies in both groups. In the haplotypes common to both groups there were also no significant results (Table 5).

3.2.5. Analysis of gender

The gender analysis was carried out by dividing the patients group into the male patients group (N = 36) and the female patients group (N = 171). The genotypic and allelic frequencies were compared for the *Fok*I, *Bsm*I, *Apa*I and *Taq*I polymorphisms in both groups. Furthermore, the common haplotypes of these two groups were also compared.

Table 6 – Analysis of gender. *VDR* polymorphisms and respective genotypes, allele and haplotype frequencies for each male and female patient group. The odds ratio values (OR) and respective 95% confidence interval (95% CI) are also represented.

Polymorphism	Genotype /Alleles	Male patients, n (%) N=36	Female patients, n (%) N=171	OR	95% CI	p-value
***Fok*I**	CC	16 (44.44)	79 (46.20)	1.07	0.52-2.21	0.848
	CT	16 (44.44)	79 (46.20)	1.07	0.52-2.21	0.848
	TT	4 (11.11)	13 (7.60)	0.66	0.20-2.15	0.486
	C	48 (66.67)	237 (69.30)	1.13	0.66-1.94	0.661
	T	24 (33.33)	105 (30.70)	0.89	0.52-1.52	
***Bsm*I**	GG	8 (22.22)	50 (29.24)	1.45	0.62-3.39	0.394
	GA	22 (61.11)	73 (42.69)	0.47	0.23-0.99	0.044
	AA	6 (16.67)	48 (28.07)	1.95	0.76-4.98	0.157
	G	38 (52.78)	173 (50.58)	0.92	0.55-1.52	0.735
	A	34 (47.22)	169 (49.42)	1.09	0.66-1.82	
***Apa*I**	GG	3 (8.33)	31 (18.13)	2.44	0.70-8.45	0.149
	GT	21 (58.33)	78 (45.61)	0.60	0.29-1.24	0.165
	TT	12 (33.33)	62 (36.26)	1.14	0.53-2.43	0.739
	G	27 (37.50)	140 (40.94)	1.16	0.68-1.95	0.589
	T	45 (62.50)	202 (59.06)	0.87	0.51-1.46	
***Taq*I**	TT	9 (25.00)	58 (33.92)	1.54	0.68-3.49	0.299
	TC	22 (61.11)	78 (45.61)	0.53	0.26-1.11	0.091
	CC	5 (13.89)	35 (20.47)	1.60	0.58-4.40	0.364
	T	40 (55.56)	194 (56.73)	1.05	0.63-1.75	0.856
	C	32 (44.44)	148 (43.27)	0.95	0.57-1.59	
Haplotypes	C/G/G/T	4 (18.20)	42 (25.30)	1.52	0.49-4.76	0.465
	C/G/T/T	3 (13.60)	16 (9.60)	0.68	0.18-2.53	0.559
	C/G/T/C	1 (4.50)	2 (1.20)	0.61	0.05-6.87	0.686
	C/A/T/T	1 (4.50)	3 (1.80)	0.39	0.04-3.89	0.403
	C/A/T/C	5 (22.70)	53 (31.90)	1.59	0.56-4.55	0.380
	T/G/G/T	3 (13.60)	19 (11.40)	0.82	0.22-3.03	0.764
	T/G/T/T	1 (4.50)	4 (2.40)	0.52	0.06-4.86	0.559
	T/A/T/C	3 (13.60)	20 (12.00)	0.87	0.24-3.20	0.831

In the gender analysis, there was no statistical significance for all genotypes and allele frequencies except for the *Bsm*I polymorphism genotype GA (p = 0.044). In the haplotypes, this comparison between males and females showed no significant results (Table 6). However, when corrected for multiple comparisons, all the results showed no significant differences.

3.2.6. Analysis of carcinoma size

Considering the patients group, the carcinoma size analysis was made by dividing them into two distinct groups: those with a carcinoma size ≤10 mm (N = 63) and those with a carcinoma size >10 mm (N = 133). Once again, the genotypic and allelic frequencies obtained by direct counting were considered for this comparison. The haplotypes common to both groups were also considered in this analysis.

Table 7 – Analysis of carcinoma size analysis. *VDR* polymorphisms and respective genotypes, allele and haplotype frequencies for groups with a carcinoma size ≤10 mm and >10 mm. The odds ratio values (OR) and respective 95% confidence interval (95% CI) are also represented.

Polymorphism	Genotype /Alleles	≤10 mm, n (%) N=63	>10 mm, n (%) N=133	OR	95% CI	p-value
***Fok*I**	CC	33 (52.38)	58 (43.61)	1.42	0.78-2.60	0.25
	CT	26 (41.27)	63 (47.37)	0.78	0.43-1.43	0.423
	TT	4 (6.35)	12 (9.02)	0.68	0.21-2.21	0.523
	C	92 (73.02)	179 (67.29)	1.32	0.82-2.10	0.252
	T	34 (26.98)	87 (32.71)	0.76	0.48-1.22	
***Bsm*I**	GG	19 (30.16)	37 (27.82)	1.12	0.58-2.16	0.735
	GA	29 (46.03)	60 (45.11)	1.04	0.57-1.89	0.904
	AA	15 (23.81)	36 (27.07)	0.84	0.42-1.69	0.627
	G	67 (53.17)	134 (50.38)	1.12	0.73-1.71	0.605
	A	59 (46.83)	132 (49.62)	0.89	0.58-1.37	
***Apa*I**	GG	11 (17.46)	22 (16.54)	1.07	0.48-2.36	0.872
	GT	33 (52.38)	60 (45.11)	1.34	0.73-2.44	0.341
	TT	19 (30.16)	51 (38.35)	0.69	0.37-1.32	0.264
	G	55 (43.65)	104 (39.10)	1.21	0.79-1.85	0.391
	T	71 (56.35)	162 (60.90)	0.83	0.54-1.27	
***Taq*I**	TT	20 (31.75)	45 (33.83)	0.91	0.48-1.73	0.772
	TC	34 (53.97)	59 (44.36)	1.47	0.81-2.68	0.208
	CC	9 (14.29)	29 (21.80)	0.60	0.26-1.35	0.214
	T	74 (58.73)	149 (56.02)	1.12	0.73-1.72	0.612
	C	52 (41.27)	117 (43.98)	0.89	0.58-1.37	
Haplotypes	C/G/G/T	16 (29.60)	28 (21.90)	1.50	0.73-3.09	0.264
	C/G/T/T	9 (16.70)	10 (7.80)	2.36	0.9-6.19	0.074
	C/G/T/C	2 (3.70)	1 (0.80)	4.88	0.43-55.05	0.157
	C/A/G/T	1 (1.90)	1 (0.80)	2.40	0.15-39.03	0.527
	C/A/T/C	12 (22.20)	44 (34.40)	0.55	0.26-1.14	0.105
	T/G/G/T	6 (11.10)	16 (12.50)	0.88	0.32-2.37	0.793
	T/A/G/T	2 (3.70)	1 (0.80)	4.88	0.43-55.05	0.157
	T/A/T/T	1 (1.90)	1 (0.80)	2.40	0.15-39.03	0.527
	T/A/T/C	5 (9.30)	16 (12.50)	0.71	0.25-2.06	0.532

From this analysis, no significant differences for the polymorphism genotypes and alleles were shown. The same occurred with the haplotypes (Table 7).

3.2.7. Analysis of lymph nodes metastasis and distant metastasis

The development of lymph node and distant metastasis was also analysed, were the patients group with thyroid cancer were divided into two distinct subgroups. For this analyses, the groups considered were the patients with metastasis (N = 54) and the patients without (N = 154). The genotype, allele and haplotype distribution of the four *VDR* polymorphism were compared between both groups.

Table 8 - Analysis of metastasis. *VDR* polymorphisms and respective genotypes, allele and haplotype frequencies for the group of patients that present metastasis and without metastasis. The odds ratio values (OR) and respective 95% confidence interval (95% CI) are also represented.

Polymorphism	Genotype /Alleles	Metastasis, n (%) N=54	Without Metastasis, n (%) N=154	OR	95% CI	p-value
***Fok*I**	CC	27 (50.00)	69 (44.81)	1.23	0.66-2.29	0.510
	CT	23 (42.59)	72 (46.75)	0.84	0.45-1.58	0.597
	TT	4 (7.41)	13 (8.44)	0.87	0.27-2.79	0.811
	C	71 (71.30)	210 (68.18)	1.16	0.72-1.88	0.547
	T	31 (28.70)	98 (31.82)	0.86	0.53-1.40	
***Bsm*I**	GG	12 (22.22)	46 (29.87)	0.67	0.32-1.39	0.281
	GA	20 (37.04)	76 (49.35)	0.60	0.32-1.14	0.118
	AA	22 (40.74)	32 (20.78)	2.62	1.34-5.11	0.004
	G	44 (40.74)	168 (54.55)	0.57	0.37-0.89	0.014
	A	64 (59.26)	140 (45.45)	1.75	1.12-2.72	
***Apa*I**	GG	7 (12.96)	27 (17.53)	0.70	0.29-1.72	0.435
	GT	24 (44.44)	76 (49.35)	0.82	0.44-1.53	0.535
	TT	23 (42.59)	51 (33.12)	1.50	0.79-2.83	0.211
	G	38 (35.19)	130 (42.21)	0.74	0.47-1.17	0.201
	T	70 (64.81)	178 (57.79)	1.35	0.85-2.12	
***Taq*I**	TT	15 (27.78)	52 (33.77)	0.75	0.38-1.49	0.418
	TC	23 (42.59)	78 (50.65)	0.72	0.39-1.35	0.308
	CC	16 (29.63)	24 (15.58)	2.28	1.10-4.73	0.024
	T	53 (49.07)	182 (59.09)	0.67	0.43-1.04	0.071
	C	55 (50.93)	126 (40.91)	1.50	0.97-2.33	
Haplotypes	C/G/G/T	11 (19.00)	35 (26.90)	0.64	0.30-1.36	0.241
	C/G/T/T	4 (6.90)	15 (11.50)	0.57	0.18-1.79	0.329
	C/G/T/C	1 (1.70)	2 (1.50)	1.12	0.10-12.64	0.925
	C/A/T/T	2 (3.40)	2 (1.50)	2.29	0.31-16.64	0.402
	C/A/T/C	21 (36.20)	37 (28.50)	1.43	0.74-2.75	0.288
	T/G/G/T	4 (6.90)	18 (13.80)	0.46	0.15-1.43	0.171
	T/G/T/T	2 (3.40)	3 (2.30)	1.51	0.25-9.30	0.653
	T/A/T/T	1 (1.70)	1 (0.80)	2.26	0.14-36.82	0.556
	T/A/T/C	11 (19.00)	12 (9.20)	2.30	0.95-5.58	0.060

Table 8 gives the comparisons results between patients with metastasis and the patients without. The *Bsm*I polymorphism genotype AA (p = 0.004) and alleles G and A (p = 0.014), and the *Taq*I polymorphism genotype CC (p = 0.024) are overrepresented in the group of patients with metastasis. Furthermore, with the Bonferroni's correction, the *Bsm*I genotype AA still presents statistical significance (Table 8). In the haplotypes frequencies comparisons, there were no statistical significant results.

3.2.8. Analysis of multicentric patterns

Multicentricity of thyroid cancer was also studied. The comparisons of multicentric patterns in the patients group were made. Two subgroups from the patients were created: those that present tumoral multicentricity (N = 53) and those that did not showed tumoral multicentricity (N = 129).

To perform this analysis, the four *VDR* polymorphisms genotypic and allelic frequencies from both groups were compared. Furthermore, all common haplotypes between both groups were also assessed. The final results of this analysis showed the OR values, with a 95% confidence interval, and all the p-values for the genotype, allele a haplotype distribution.

Table 9 – Analysis of multicentricity. *VDR* polymorphisms and respective genotypes, allele and haplotype frequencies for the group of patients that present tumour multicentricity and for the patients without multicentric patterns. The odds ratio values (OR) and respective 95% confidence interval (95% CI) is also represented.

Polymorphism	Genotype /Alleles	With multicentricity, n (%) N=53	Without multicentricity, n (%) N=129	OR	95% CI	p-value
***Fok*I**	CC	29 (54.72)	54 (41.86)	1.68	0.88-3.20	0.114
	CT	23 (43.40)	61 (47.29)	0.85	0.45-1.63	0.632
	TT	1 (1.89)	14 (10.85)	0.16	0.02-1.23	0.071
	C	81 (76.42)	169 (65.50)	1.71	1.02-2.86	0.041
	T	25 (23.58)	89 (34.50)	0.59	0.35-0.98	
***Bsm*I**	GG	14 (26.42)	37 (28.68)	0.89	0.43-1.83	0.757
	GA	18 (33.96)	64 (49.61)	0.52	0.27-1.02	0.054
	AA	21 (39.62)	28 (21.71)	2.37	1.19-4.73	0.013
	G	46 (43.40)	138 (53.49)	0.67	0.42-1.05	0.080
	A	60 (56.60)	120 (46.51)	1.50	0.95-2.37	
***Apa*I**	GG	4 (7.55)	24 (18.60)	0.36	0.12-1.09	0.060
	GT	27 (50.94)	61 (47.29)	1.16	0.61-2.20	0.654
	TT	22 (41.51)	44 (34.11)	1.37	0.71-2.64	0.345
	G	35 (33.02)	109 (42.25)	0.67	0.42-1.08	0.102
	T	71 (66.98)	149 (57.75)	1.48	0.92-2.38	

***Taq*I**	TT	17 (32.08)	41 (31.78)	1.01	0.51-2.01	0.969
	TC	19 (35.85)	67 (51.94)	0.52	0.27-1.00	0.048
	CC	17 (32.08)	21 (16.28)	2.43	1.16-5.10	0.017
	T	53 (50.00)	149 (57.75)	0.73	0.46-1.15	0.176
	C	53 (50.00)	109 (42.25)	1.37	0.87-2.15	
Haplotypes	C/G/G/T	8 (14.30)	30 (27.80)	0.43	0.18-1.02	0.052
	C/G/T/T	7 (12.50)	10 (9.30)	1.40	0.50-3.90	0.519
	C/A/T/T	1 (1.80)	1 (0.90)	1.95	0.12-31.70	0.634
	C/A/T/C	23 (41.10)	30 (27.80)	1.81	0.92-3.57	0.084
	T/G/G/T	3 (5.40)	14 (13.00)	0.38	0.10-1.38	0.130
	T/G/T/T	1 (1.80)	4 (3.70)	0.47	0.05-4.33	0.498
	T/A/T/T	1 (1.80)	1 (0.90)	1.95	0.12-31.70	0.634
	T/A/T/C	8 (14.30)	14 (13.00)	1.12	0.44-2.85	0.814

As shown in Table 9, the comparisons between both groups showed that *Fok*I allele C, the *Bsm*I genotype AA, and the *Taq*I polymorphism genotype CC are overrepresented in patients with multicentricity. The haplotypes distribution showed no statistically significant differences. However, according to Bonferroni's correction, there are no statistical significant values.

3.2.9. Analysis of thyroid cancer stages

For the thyroid cancer stages analysis, four different stages of thyroid cancer were analysed: I, II, III and IV stages. For that purpose, the patients were divided into two different subgroups. One comprises the patients with cancer stages I and II (N = 150), and the other group accounts the patients with cancer stages III and IV (N = 54). This analysis was made by the comparisons between genotype, allele and haplotype distribution in both groups.

Table 10 – Analysis of thyroid cancer stages. *VDR* polymorphisms and respective genotypes, allele and haplotype frequencies for the group of patients that present thyroid cancer stages I-II and for the patients that present stages III-IV. The odds ratio values (OR) and respective 95% confidence interval (95% CI) are also represented.

Polymorphism	Genotype /Alleles	Stages I-II, n (%) N=150	Stages III-IV, n (%) N=54	OR	95% CI	p-value
***Fok*I**	CC	67 (44.67)	26 (48.15)	0.87	0.47-1.62	0.660
	CT	73 (48.67)	21 (38.89)	1.49	0.79-2.81	0.216
	TT	10 (6.67)	7 (12.96)	0.48	0.17-1.33	0.151
	C	207 (69.00)	73 (67.59)	1.07	0.67-1.71	0.787

	T	93 (31.0)	35 (32.41)	0.94	0.58-1.50	
***Bsm*I**	GG	44 (29.33)	13 (24.07)	1.31	0.64-2.68	0.460
	GA	70 (46.67)	23 (42.59)	1.18	0.63-2.21	0.606
	AA	36 (24.00)	18 (33.33)	0.63	0.32-1.24	0.183
	G	158 (52.67)	49 (45.37)	1.34	0.86-2.08	0.193
	A	142 (47.33)	59 (54.63)	0.75	0.48-1.16	
***Apa*I**	GG	25 (16.67)	8 (14.81)	1.15	0.48-2.73	0.751
	GT	80 (53.33)	18 (33.33)	2.29	1.19-4.38	0.012
	TT	45 (30.00)	28 (51.85)	0.40	0.21-0.75	0.004
	G	130 (43.33)	34 (31.48)	1.66	1.04-2.65	0.031
	T	170 (56.67)	74 (68.52)	0.60	0.38-0.96	
***Taq*I**	TT	51 (34.00)	15 (27.78)	1.34	0.68-2.66	0.402
	TC	73 (48.67)	25 (46.30)	1.10	0.59-2.05	0.765
	CC	26 (17.33)	14 (25.93)	0.60	0.29-1.26	0.173
	T	175 (58.33)	55 (50.93)	1.35	0.87-2.10	0.183
	C	125 (41.67)	53 (49.07)	0.74	0.48-1.15	
Haplotypes	C/G/G/T	35 (26.90)	9 (16.10)	1.92	0.85-4.33	0.110
	C/G/T/T	15 (11.50)	4 (7.10)	1.70	0.54-5.36	0.364
	C/A/T/T	3 (2.30)	1 (1.80)	1.30	0.13-12.77	0.822
	C/A/T/C	37 (28.50)	21 (37.50)	0.66	0.34-1.29	0.222
	T/G/G/T	17 (13.10)	5 (8.90)	1.53	0.54-4.39	0.422
	T/G/T/T	1 (0.80)	4 (7.10)	0.10	0.01-0.92	0.032
	T/A/T/C	13 (10.00)	10 (17.90)	0.51	0.21-1.25	0.135

According to Table 10, the *Apa*I polymorphism genotypes GT and TT, and the allele T, are overrepresented in patients with cancer stages III-IV. In the haplotypes distribution, only the haplotype T/G/T/T appears to be overrepresented in patients with stages III-IV. However, after Bonferroni's statistical correction, only the *Apa*I TT genotype remained significant for cancer stages III-IV

4 - Discussion and conclusion

In this case-control study, four *VDR* polymorphisms, namely *Fok*I, *Bsm*I, *Apa*I, and *Taq*I, were assessed. The purpose of the present work was to study how genotypes, alleles and haplotypes distribution of the *VDR* gene influence the prevalence of thyroid cancer in the Portuguese population. Therefore, two different major groups were considered: the patients group and the control group.

The patients group, composed by Portuguese patients with DTC (PTC and FTC), accounted for 208 patients. In this group, various parameters were assessed (cancer type, age, gender, carcinoma size, stage and metastasis) in order to verify how *VDR* polymorphisms influence the individual predisposition to thyroid cancer. In addition, this study relied only on patients with the DTC variant, since it is the most common thyroid carcinoma variant among all.

The control group, which accounted for 248 Portuguese Caucasian individuals, had no clinical history related to thyroid pathologies. This sample of the Portuguese population did not deviate significantly from the Hardy-Weinberg equilibrium (HWE), and therefore we can assume that no significant bias occurred in our control population.

After both patients and controls were recruited, the *VDR* polymorphisms were analysed. The *Fok*I polymorphism, located at the 5' end of the *VDR* gene, has been associated with a frameshift in the VDR protein (82). Therefore, the polymorphic *Fok*I site in exon 2 results in an alternative translation initiation site, which leads to the addition of three amino acids to the VDR protein in individuals that carry the T allele (64). Since the *Fok*I polymorphism leads to a different protein, it could alter VDR protein function and, therefore, have some implications in thyroid cancer susceptibility. The polymorphisms *Bsm*I, *Apa*I and *Taq*I, located in the 3' end region of *VDR* gene, do not alter VDR protein structure. However, these polymorphisms are important and could be associated with tumorigenesis. Although the VDR protein structure is not altered, these polymorphisms may change the expression levels of this protein. Moreover, some of these polymorphisms could be in linkage disequilibrium with other non-synonymous polymorphisms, that may contribute to an increased risk of thyroid cancer.

For the statistical analysis, several parameters were assessed in order to verify in which way these four *VDR* polymorphisms influence thyroid cancer genetic susceptibility. Therefore, the first analysis made compared the genotype, allele and haplotype distribution in patients with DTC and healthy subjects (control group). In this comparison, the four *VDR* polymorphism and respective haplotypes were assessed. Despite the influence of *Fok*I polymorphism on the translational activity of *VDR*, which alters the VDR protein structure in three amino acids longer, there were no statistical significant results for this polymorphism. This data correlates with the results shown by Haghpanah et al. (83). For the *Bsm*I polymorphism, there was no

genotypic and allelic association found for genetic susceptibility for DTC. The *Apa*I polymorphism also showed no statistical significant results for genotype and allele distribution. However, these findings go against the data presented by Penna-Martinez et al. (84) that showed statistical significant data for the *Apa*I polymorphism TT genotype, which appears to be less frequent in patients from Germany. The comparisons for *Taq*I polymorphism also showed no statistical significant results.

Then, the haplotypes were assessed. A haplotype was considered to be the combination of the alleles of the four different *VDR* polymorphisms that are transmitted on a chromosome. The haplotypes results showed no statistically significant differences between patients and controls.

After the comparison analysis between patients and controls, other parameters were set for further comparisons in the thyroid cancer genetic susceptibility. The following comparisons were made: thyroid cancer type differences (PTC vs. FTC), age (≤45 vs. >45 years), gender (male vs. female), carcinoma size (≤10mm vs. >10mm), lymph node metastasis and distant metastasis, multicentricity, and stage of cancer (stages I-II vs. III-IV).

One of the parameters assessed was the thyroid cancer type. For this comparison, the patients with PTC variant were compared with FTC patients, the two most common thyroid malignancies. The total number of patients with PTC was much superior to the FTC patients group, reflecting the real proportion of DTC incidence throughout the world. The results for the *FokI* non-synonymous polymorphism showed, despite the VDR protein alteration, no significant results. The other three polymorphisms, *Bsm*I, *Apa*I and *Taq*I, also showed no significant statistical results. In other association studies from Penna-Martinez et al. (85), in the German population, the CC genotype of *Fok*I and the GG genotype of *Apa*I were significantly less frequent in patients that presented the FTC variant. Furthermore, the haplotype analysis for the assessment of this parameter showed no statistical significant results.

In addition, another parameter analysed for this genetic susceptibility was the age of diagnosis. To perform this analysis, the patients group were divided into two groups with a 45 years old cut-off. The cut-off was used at the age of 45 because this is the age limit used for cancer staging. It is important to assess this parameter in order to verify if the age of diagnosis influences the predisposition to cancer. Both groups ≤45 years old and >45 years old had a similar number of patients. None of the polymorphisms showed statistically significant differences. The same occurred in the common haplotypes to both groups. These results could not be compared with previous studies since there are no previous case-control studies with DTC patients for this parameter.

In the analysis of the genetic susceptibility for thyroid cancer according to the patient gender, the subdivision into two groups was made into male patients and female patients.

Since the thyroid carcinoma is more prevalent in women compared with men, in a proportion of 3:1 to 5.5:1, the patient group represented this tendency, where the number of women were far superior to men. Performing this comparison, the *Fok*I polymorphism showed no statistical significant results. The remaining three *VDR* polymorphisms also revealed no statistical significant results in exception for the *Bsm*I polymorphism GA genotype ($p = 0.044$). However, when the Bonferroni's correction was applied to this analysis, the GA genotype for *Bsm*I revealed no significant differences. The common haplotypes to both patient groups also showed no statistical significance.

For the thyroid cancer size, comparisons between two different patient groups were made. The two distinct groups created were separated with a criteria of carcinomas ≤10 mm (microcarcinomas) and >10mm. The patient group that presented a thyroid carcinoma with a size >10 mm accounted twice the number of individuals with carcinomas ≤ 10 mm. In this comparison the four *VDR* polymorphisms revealed no statistical significant results. Despite the relevance of this analysis, there are no previous studies with DTC patients to compare these results.

The risk of lymph node metastasis and distant metastasis was also assessed. Two distinct groups were made from the patients group, dividing those with metastasis and those without metastasis. The patients without metastasis were in a greater number compared to the other ones. Analysing the results given, there were some statistical significance. The genotype AA and alleles G and A for *Bsm*I, and the genotype CC of *Taq*I polymorphism showed statistical significance. The remaining results for genotypic, allelic and haplotype frequencies weren't significant. Even though the Bonferroni's correction were made, the genotype AA ($p = 0.004$) of the *Bsm*I polymorphism presented a p-value < 0.005. No other studies have reported similar findings. However, the *Bsm*I genotype AA could be implicated with the presence of lymph node metastasis in patients with DTC.

For the multicentricity analysis of thyroid cancer, two patients groups were considered. These groups were divided according to the presence of multicentric patterns or not. The patient group that presented multicentricity had a lower number of individuals compared to the patient group without multicentricity. Analysing the results by comparing both groups, all the genotypes, alleles and haplotypes distributions presented no statistical significance, with the exception of the genotype AA of *Bsm*I, and genotype CC of *Taq*I polymorphism, with a p-value < 0.05. However, performing the multiple comparisons correction, the results showed no statistical significant differences.

The final parameter to be assessed was the thyroid carcinoma stage. From the stages considered I, II, III, and IV, two groups from the patients were created. One group represented the patients with cancer stages I and II. The other group represented the patients with stages III and IV. Moreover, the patients group that present the I-II stages were in superior number to the other ones. For this parameter, the *Fok*I polymorphism showed no

significant results. The same occurred with the *Bsm*I and *Taq*I polymorphisms. However, the *Apa*I polymorphism GT and TT genotypes and the alleles G and T showed statistical significant results with a p-value < 0.05. Performing the Bonferroni's correction, the genotype TT (p = 0.004) of *Apa*I appears to have statistical significance. No other studies have reported similar findings, yet, this genotype could be implicated with a more aggressive cancer stage.

After the analysis of all these different parameters in the thyroid cancer genetic susceptibility, we can say that the statistical significant results points towards a direction that should be considered in further studies of the *VDR* gene. However, we should be careful in the analysis of these findings, because the small population analysed in this study could influence the final results. Therefore, for more accurate conclusions, the population studied in both groups should be increased in order to dissipate some doubts in some significant results. In addition, some extrinsic factors (e.g. environment factors) could influence the outcome values. Furthermore, the Bonferroni's correction was used since there were many parameters assessed for the thyroid cancer genetic susceptibility analysis. Thus, with this statistical correction which performs an adjustment for multiple comparisons, the statistical significant p-value was lowered to 0.005, reducing possible results that could be false positive results.

In conclusion, and considering only the results with a statistical significant p-value < 0.005, according to Bonferroni's correction, the results suggests that *Bsm*I polymorphism genotype AA may influence the presence of lymph node metastasis in patients with DTC. Moreover, the TT genotype of *Apa*I polymorphism may increase the predisposition for more aggressive phenotypes of DTC, since it is overrepresented in patients with more advanced cancer stages (III-IV).

Since association studies of *VDR* and genetic susceptibility for thyroid cancer are few, these findings could not be explained or compared to other studies. However, we could compare the data showed in this study to other results already showed for *VDR* and genetic susceptibility in other cancers. In prostate cancer, Taylor et al. (86) showed the relationship between the *Taq*I polymorphism and an increased risk of prostate cancer (51). Other studies showed that haplotypes with the alleles G of *Bsm*I, G of *Apa*I and C of *Taq*I were associated as biomarkers for prostate cancer (71). In other studies, *Bsm*I heterozygous genotypes were associated with a reduced risk for this cancer (67, 87). For breast cancer, some studies correlate an association between *Taq*I polymorphisms and metastasis (51). In addition, Lowe et al. (88) demonstrated that genotype AA of *Bsm*I was associated to an increased risk of developing breast tumours. In other meta-analysis studies, breast cancer risk for carriers of the TT genotype of *Fok*I polymorphism was increased significantly (67). For colon cancer, there are studies which associated *Bsm*I polymorphism with an increased risk for tumour

development (51). Furthermore, the CC genotype of *Fok*I polymorphism was also associated with an increased risk for tumorigenesis (71).

All these studies associated the *VDR* polymorphisms with the development of other types of tumours. Some of the results of these studies for the *VDR* polymorphisms and the genetic susceptibility for different types of cancer are in conflict. However, these contradictory results could be explained because different populations were assessed, along with the different environmental factors that affect these individuals.

Summarizing, the *VDR* gene polymorphisms may be useful risk markers for DTC, as they are for other type of cancers. However, conclusions from these findings can not be taken since further studies are needed to understand *VDR* cellular and molecular actions, that remain unclear. For that purpose, functional genomic studies need to be done, in order to verify how these polymorphisms can influence the genetic susceptibility to thyroid cancer.

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