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Study of molecular and cellular mechanisms involved in anaplastic thyroid carcinoma aggressiveness and response to therapy

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Abstract

Thyroid cancer (TC) is the most common endocrine neoplasia. Thyroid carcinomas derived from follicular cells or thyrocytes are designated as nonmedullary thyroid carcinomas (NMTCs). The most undifferentiated subset of NMTC are anaplastic thyroid carcinomas (ATCs) which, although rare, are the most aggressive and lethal type of TC. ATCs are highly invasive, and lost the intrinsic functions of normal thyrocytes, presenting a downregulation of the sodium-iodide symporter (*NIS*), which makes the usual treatments for differentiated thyroid cancer, such as surgery and radioactive iodine, much less effective. Therefore, it is necessary to search for new therapeutic options, such as targeted therapies. The most promising targeted therapy for ATCs is the combination of dabrafenib (BRAF inhibitor) plus trametinib (MEK inhibitor) which, in a clinical trial, showed a significant response rate in ATC patients with the *BRAF* p.V600E mutation. This combination (DT) was recently approved only by the Food and Drug Administration (FDA) for this indication. Since some patients may develop resistance to DT, it is important to investigate the mechanisms involved and also new molecular therapeutic targets, in order to improve their overall survival.

More than 50% of ATC's tumour microenvironment (TME) is comprised by tumour associated macrophages (TAMs), which are associated with ATC aggressiveness, and may represent a possible novel target for ATC treatment. In previous proteomic studies developed by the Molecular Endocrinology group, on ATC-TAM crosstalk, Sprouty RTK Signaling Antagonist 4 (*SPRY4*) was identified as being a potential modulator of these interactions.

The main aim of this work was to investigate the molecular mechanisms involved in ATC aggressiveness and response to DT therapy. To achieve this goal, two major approaches were used: (1) characterization of the molecular profiles of nine ATC *BRAF* p.V600E-positive patients, through the analysis of a multigene panel by Next Generation Sequencing (NGS) and Sanger sequencing, in order to investigate the alterations that could be associated with DT response and resistance, and to define treatment options in these cases; (2) *in vitro* experimental procedures, using 2D transwell cultures of ATC cell lines C3948 and T235, and a monocyte cell line (THP-1), in monocultures (without THP-1) and in co-cultures (with THP-1), to further study the role of *SPRY4* in the ATC-TAM crosstalk.

In the first task, we identified telomerase promotor (*TERTp*) mutations in all patients, which in concomitance with *BRAF* p.V600E, were likely contributing to the excellent initial DT therapy response. In one patient, a *NRAS* activating mutation was identified, representing an acquired off-target resistance mechanism, as previously described in ATCs. *PIK3CA* mutations were also detected in three patients and could be related with the selection of resistant clones to DT, as described in melanoma. A novel likely pathogenic mutation in *MAP2K1*, which encodes for MEK1, (p.E120Q), was identified in one patient, so far responding to DT. A *MTOR* mutation was identified in one case and may represent a novel therapeutic target in ATCs. *TP53* mutations were detected, in five patients and, although known to be related with ATC development, did not appear to be directly associated with DT efficacy. Although ATC is a rare disease, the characterization of additional cases and molecular targets, may further support and extend the present findings.

In the second task, western blot analysis showed that SPRY4 was downregulated in T235 co-cultures and upregulated in C3948 co-cultures, confirming previous proteomic data. Using *SPRY4* siRNA-mediated silencing (> 50% of silencing), it was possible to observe that, in both cells lines, the invasion capabilities significantly increased in co-culture, similar to that observed in T235 using the non-target control (siNT) but contrasting with the decrease observed for C3948 cells with siNT. Also, the analysis of ATC cells' cytoskeletal alterations and morphology, through immunofluorescence with phalloidin to detect actin filaments, confirmed the spreading behavior under si*SPRY4* conditions in both cell lines, consistent with invasion assays results.

Taken together, these results unveiled a tumour suppressor gene role for *SPRY4* in ATC, in the presence of TAMs, with an influence in ATC-TAMs interactions and ATC aggressiveness. Additional studies are warranted to further elucidate *SPRY4*-related signaling mechanisms in this context.
In conclusion, this study contributed to improve the present knowledge on the cellular and molecular mechanisms involved ATC response to therapy, and TME/TAM interactions, that are associated with ATC aggressiveness, which may eventually lead to new therapeutic approaches capable of improving the prognosis and overall survival of ATC patients.

Keywords: Anaplastic thyroid carcinoma; Targeted therapies; Drug Resistance; Tumour microenvironment; Tumour associated macrophages.

Resumo

O cancro da tiróide (TC) é a neoplasia endócrina mais comum. Os carcinomas da tiróide derivados de células foliculares ou tirócitos são denominados como carcinomas não medulares da tiróide (NMTCs). O subconjunto mais indiferenciado de NMTC são os carcinomas anaplásicos da tiróide (ATCs) que, embora raros, são o tipo mais agressivo e letal de TC. Os ATCs são extremamente invasivos e perderam as funções intrínsecas dos tirócitos normais, apresentando uma subexpressão do simporte de sódio/iodo (*NIS*), o que torna os tratamentos convencionais para carcinomas diferenciados da tiroide, como a cirurgia e o uso de iodo radioativo, menos eficientes. Por conseguinte, é necessário procurar novas opções terapêuticas, tais como terapêuticas dirigidas. A terapêutica dirigida mais promissora para os ATCs é a combinação de dabrafenib (inibidor de BRAF) e trametinib (inibidor de MEK) que, num ensaio clínico, mostrou uma taxa de resposta significativa em doentes com ATC com a mutação de *BRAF* p.V600E. Esta combinação (DT) foi recentemente aprovada apenas pela Food and Drug Administration (FDA) para este tipo de cancro. Dado que alguns doentes podem desenvolver resistência à DT, é importante investigar os mecanismos envolvidos e também novos alvos terapêuticos moleculares, a fim de melhorar a sua sobrevivência global.

Mais de 50% do microambiente tumoral (TME) dos ATCs é composto por macrófagos associados a tumor (TAM), que estão relacionados com sua a agressividade, e podem representar um possível novo alvo para o tratamento destes tumores. Em estudos de proteómica anteriormente desenvolvidos pelo grupo de Endocrinologia Molecular, sobre as interações ATC-TAM, o gene *Sprouty RTK Signaling Antagonist 4* (*SPRY4*) foi identificado como sendo um potencial modulador destas interações.

O principal objectivo deste trabalho foi investigar os mecanismos moleculares envolvidos na agressividade e resposta à terapêutica DT pelos ATCs. Para atingir este objectivo, foram utilizadas duas abordagens principais: (1) caracterização dos perfis moleculares de nove ATCs *BRAF* p.V600E-positivos, através da análise de um painel multigénico por sequenciação de nova geração (NGS) e sequenciação de Sanger, a fim de investigar as alterações que poderiam ser associadas à resposta e resistência a DT, e definir opções de tratamento nestes casos; (2) procedimentos experimentais *in vitro*, utilizando culturas *transwell* 2D com linhas celulares de ATC, C3948 e T235, e uma linha de monócitos (THP-1), em monoculturas (sem THP-1) e em co-culturas (com THP-1), para estudar o papel do *SPRY4* nas interações ATC-TAM.

Na primeira abordagem, identificámos, em todos os doentes, mutações no promotor da telomerase (*TERTp*) que, em concomitância com *BRAF* p.V600E, estariam possivelmente a contribuir para uma excelente resposta inicial a DT. Num doente foi identificado uma mutação ativadora de *NRAS*, que possivelmente representa um mecanismo de resistência adquirido *off-target*, como já descrito em ATCs. Mutações *PIK3CA* também foram detetadas em três doentes e poderão estar relacionadas com a seleção de clones resistentes a DT, tal como descrito em melanoma. Uma nova mutação provavelmente patogénica no gene *MAP2K1*, que codifica a proteína MEK1, (p.E120Q), foi identificada num doente, que presentemente continua a responder ao DT. Uma mutação *MTOR* foi identificada num caso e poderá representar um novo alvo terapêutico em ATCs. Foram detetadas mutações no gene *TP53*, em cinco doentes e, embora se saiba estarem relacionadas com o desenvolvimento de ATC, não pareceram estar diretamente associadas à eficácia de DT. Embora o ATC seja uma doença rara, a caracterização de casos adicionais e de mais alvos moleculares, poderá confirmar e ampliar os achados deste estudo.

Na segunda abordagem, a análise de *western blot* mostrou que a proteína SPRY4 se apresentava subexpressa nas co-culturas de T235 e sobrexpressa nas co-culturas de C3948, confirmando dados de proteómica. Usando o silenciamento mediado por siRNA de *SPRY4* (> 50% de silenciamento), foi possível observar que, em ambas as linhas celulares, as capacidades de invasão aumentaram significativamente em co-cultura, à semelhança do que foi observado nas células T235 usando o controlo não direcionado ao alvo (siNT), mas contrastando com a diminuição observada para as C3948 com

siNT. De acordo com estes resultados, a análise das alterações morfológicas e no citoesqueleto das células de ATC, através da imunofluorescência com faloidina para detetar filamentos de actina, confirmou o comportamento de disseminação sob condições de si*SPRY4* em ambas as linhas celulares.
No seu conjunto, estes resultados apoiam um papel de supressor tumoral para o *SPRY4* em ATCs na presença de TAMs. Estudos adicionais serão necessários para elucidar melhor os mecanismos de sinalização relacionados com o *SPRY4* neste contexto.
Em conclusão, este estudo contribuiu para aumentar os atuais conhecimentos sobre os mecanismos celulares e moleculares envolvidos na resposta dos ATCs à terapêutica, e interações TME/TAM que estão associadas à agressividade dos ATCs, que podem eventualmente conduzir a novas abordagens terapêuticas, capazes de melhorar o prognóstico e a sobrevivência global dos doentes com ATC.

Palavras-chave: Carcinoma anaplásico da tiróide; Terapêuticas dirigidas; Resistência a fármacos; Microambiente tumoral; Macrófagos associados ao tumor.

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List of acronyms, abbreviations and symbols

%KD -	Percentage of knockdown
A -	Amperes
AKT -	Protein kinase B gene
Ala -	Alanine
ALK -	Anaplastic lymphoma kinase
ANGPT1 -	Angiopoietin 2
APS -	Ammonium persulfate
Arg -	Arginine
Asn -	Asparagine
Asp -	Aspartic acid
ATC -	Anaplastic thyroid carcinoma
ATP -	Adenosine triphosphate
Bad -	BCL2-associated agonist of cell death
BAM -	Binary Alignment Map
BCL2 -	B-cell lymphoma 2
bp -	Base pairs
BRAF -	b-Rapidly Accelerated Fibrosarcoma
CCAT1 -	Colon cancer associated transcript-1
CCDC6 -	Coiled-coil domain containing 6
CCL -	C-C motif chemokine ligand
CCR -	C-C motif chemokine receptor

CD - Cluster of differentiation

CDK4/6 - Cyclin-dependent kinase 4 and 6 gene

CDKI - Cyclin dependent kinase inhibitor gene

CDKN2A - Cyclin dependent kinase inhibitor 2A gene

Chr - Chromosome

CNV - Copy number variation

COSMIC - Catalogue of Somatic Mutations in Cancer

CRD - Cysteine rich domain

CSF1 - Colony-stimulating factor 1

CSFR - Colony-stimulating factor receptor

Ct - Threshold cycle

Cys - Cysteine

DAPI - 4',6'-diamino-2-fenil-indol

DBD - DNA binding domain

dbSNP - Single nucleotide polymorphism database

DIS3L2 - DIS3 Like 3'-5' Exoribonuclease 2

DNA - Deoxyribonucleic acid

dNTP - Deoxyribonucleotide triphosphate

DT - Dabrafenib plus Trametinib

DTC - Differentiated thyroid carcinoma

DTT - Dithiothreitol

EGFR - Epidermal growth factor receptor gene

EIF1AX - Eukaryotic translation initiation factor 1A X-linked

EMT - Epithelial-to-mesenchymal transition

ERK - Extracellular signal-regulated kinase

ETS-TF - E26 transformation-specific transcription factors

Exo I - Exonuclease I

FastAP - Thermosensitive Alkaline Phosphatase

FATHMM - Functional Analysis through Hidden Markov Models

FBS - Fetal bovine serum

FC - Fold change

FDA - Food and Drug administration

FFPE - Formalin-fixed paraffin-embedded

FNMTC - Familial nonmedullary thyroid carcinoma

FOXE1 - Forkhead box protein E1 / thyroid transcription factor 2

FOXO - Forkhead O family of transcription factors

Fs - Frameshift

FTC - Follicular thyroid carcinoma

GDP - Guanosine diphosphate

Gln - Glutamine

Glu - Glutamic acid

Gly - Glycine

GM-CSF - Granulocyte-macrophage colony stimulating factor

GSK3α/β - Glycogen synthetase kinase-3α/β

GTP - Guanosine triphosphate

GTPases - GTP phosphatases

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HHEX - Haematopoietically-expressed homeobox protein

His - Histidine

HL - Hayflick limit

HPRT1 - Hypoxanthine phosphoribosyltransferase 1

HRAS - Harvey Rat Sarcoma Viral Oncogene Homolog

HRP - Horseradish peroxidase

IFN - Interferon

IGF2BP1 - Insulin-like growth factor 2 mRNA binding protein 1

IL - Interleukin

Indels - Insertions/deletions

iNOS - Inducible nitric oxide synthase

IPOLFG - Instituto Português de Oncologia de Lisboa Francisco Gentil

KRAS - Kirsten Rat Sarcoma Viral Oncogene Homolog

Leu - Leucine

Lys - Lysine

MAGEA3 - Melanoma-associated antigen 3

MAP2K1 - Mitogen-activated protein kinase gene

MAPK - Mitogen-activated protein kinase signaling pathway

M-CSF - Macrophage colony-stimulating factor

MEK - Mitogen-activated protein kinase

min - minutes

mRNA - Messenger RNA

MTC - Medullary thyroid carcinoma

MTOR - Mechanistic target of rapamycin gene

mTORC - Mechanistic target of rapamycin complex

NCO4 - Nuclear receptor coactivator 4

NF1 - Neurofibromatosis type 1

NGS - Next Generation Sequencing

NIS - Sodium/iodide symporter gene

NKX-2.1 - Homeobox protein 2.1 / thyroid transcription factor 1

NMTC - Nonmedullary thyroid carcinoma

NRAS - Neuroblastoma Rat Sarcoma Viral Oncogene Homolog

NSCLC - Non-small cell lung cancer

OG - Oncogene

OMIM - Online Mendelian Inheritance in Man database

p110α - p110 alpha subunit

PAX8 - Paired box protein PAX8

PBS - Phosphate Buffered Saline

PCR - Polymerase Chain reaction

PD-1 - Programmed cell death protein 1

PD-L1/2 - Programmed cell death ligand 1 and 2

PDRK1 - 3-phosphoinositide-dependent protein kinase-1

PDTC - Poorly differentiated thyroid carcinoma

Pen/strep - Penicillin/streptomycin solution

PFA - Paraformaldehyde

PGE2 - Prostaglandin E2

PH - Pleckstrin homology

Phe - Phenylalanine

PI3K - Phosphoinositide 3 kinase

PIK3CA - Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha

PIP_2 - Phosphatidylinositol-4,5-biphosphate

PIP_3 - Phosphatidylinositol-3,4-5-trisphosphate

PLK1 - Polo-like kinase 1 gene

PMA - Phorbol 12-myristate 13-acetate

PolyPhen - Polymorphism Phenotyping

PPARγ - Peroxisome proliferator-activated receptor gamma

Pro - Proline

PTC - Papillary thyroid carcinoma

PTEN - Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase

PVDF - Polyvinylidene difluoride

qRT-PCR - Quantitative Real-time reverse transcription polymerase chain reaction

RAC1 - RAS-related C3 botulinum toxin substrate 1

RAF - Rapidly Accelerated Fibrosarcoma

RAS - Rapidly Accelerated sarcoma

RasGEF - RAS-Guanine nucleotide exchange factor

RB1 -	Retinoblastoma 1
Ref.Seq -	Reference sequence
RET -	Rearranged during Transfection / Proto-oncogene tyrosine-protein kinase receptor Ret
RNA -	Ribonucleic acid
RNaseOUT -	Recombinant Ribonuclease Inhibitor
RPMI-1640 -	Roswell Park Memorial medium 1640
rSNP -	Regulatory Single nucleotide polymorphism
RTK -	Receptor tyrosine kinase
SAM -	Sequence Alignment Map
SD -	Standard deviation
SDS -	Sodium dodecyl sulfate
Ser -	Serine
SIFT -	Sorting Intolerant From Tolerant
siRNA -	Short interference RNA
SKT11 -	Serine/threonine protein kinase 11 gene
SNP -	Single nucleotide polymorphism
SNV -	Single nucleotide variant
SPRY4 -	Sprouty RTK Signaling Antagonist 4
TAMs -	Tumour associated macrophages
TC -	Thyroid cancer
TEMED -	Tetramethylethylenediamine
TERT -	Telomerase reverse transcriptase
TERTp -	Telomerase reverse transcriptase promotor
TGCT -	Testicular germ cell tumours
TGF-β -	Transforming growth factor beta
Tie -	Angiopoietin-1 tyrosine kinase receptor
TKI -	Tyrosine kinase inhibitor

TME -	Tumour microenvironment
TP53 -	Tumour protein 53
TPM -	*TERTp* mutations
TRH -	Thyroid releasing hormone
TRK -	Tropomyosin-related protein
Trp -	Tryptophan
TSG -	Tumour suppressor gene
TSH -	Thyroid stimulating hormone
TSHR -	Thyroid stimulating hormone receptor
TTF -	Thyroid transcription factors
UIPM -	Unidade de Investigação em Patobiologia Molecular
UV -	Ultraviolet
V -	Volts
VAF -	Variant allele frequency
Val -	Valine
VEGF -	Vascular endothelial growth factor
VEGFR -	Vascular endothelial growth factor receptor gene
WNT -	Wingless-related integration site

1. Introduction

1.1. Tumourigenesis and cancer

Cancer is a global term to describe a large number of diseases which are characterized by a malignant neoplasia. In other words, these diseases are characterized by the development of abnormal cells that divide uncontrollably having the ability to infiltrate and destroy normal neighbor tissues and spread to other organs. Cancer has many anatomic and molecular subtypes, this way being a heterogeneous disease, and can affect almost any part of the body (Fior, 2019). Every subtype requires specific treatment and management strategies to diminish the mortality. According to the World Health Organization, cancer is the second leading cause of death globally, with 9.6 million deaths in 2018 (World Health Organization, Cancer, 2018). Knowing this, oncology research has been developing better ways to prevent and treat cancer, improving the overall survival and quality of life of patients.

1.2. Hallmarks of cancer

Nowadays, it is consensual that the vast majority of cancers share some general features, known as hallmarks of cancer, in spite of being a very heterogeneous and complex group of diseases (Hanahan *et al.*, 2000; Hanahan *et al.*, 2011). The hallmarks of cancer are an attempt to rationalize the biological capabilities required for a normal cell to progress into a neoplastic state. In 2000, six major hallmarks of cancer were described by Hanahan and Weinberg: sustaining proliferative signaling, evasion of growth suppressors, resistance to cell death, replicative immortality, induction of angiogenesis and activation of cell invasion and metastasis (Hanahan *et al.*, 2000). These were pathophysiological changes observed during the development of the malignant tumours. Subsequently, two additional hallmarks were added: the genomic instability and tumour promoting inflammation. It is widely known that cancer progression depends in a sequential accumulation of driver mutations within cells and tissues, that lead to the transformation of normal cells in malignant cells (Balani *et al.*, 2017), increasing cell proliferation, decreasing cell death and inducing immortality. This suggests that that genomic instability provides a variety of genetic alterations that drive tumourigenesis (Andor *et al.*, 2017). However, cancer behavior does not only depend on the intrinsic characteristics of cancer cells, because there are different cell types within the tumour microenvironment (TME) (Arneth *et al.*, 2019). TME is considered a complex cellular ecosystem, formed by multiple types of cells, and also proteins and molecules secreted by the TME cells, which modulate the tumour characteristics (Yang *et al.*, 2020; Lv *et al.*, 2021). Inflammatory conditions in the TME contribute to proliferation, survival of the tumour cells, metabolic dysregulation, angiogenesis, metastasis, hijacking of the immune system and reduced response to chemotherapy agents (Murata *et al.*, 2018). The increased knowledge about cancer metabolism and TME led to a review of the main hallmarks of cancer introducing two novel hallmarks: evasion and hijacking of the immune system and deregulation of the cellular metabolism (Hanahan *et al.*, 2011).

1.3. Thyroid Gland

The thyroid is an endocrine gland, located at the base of the throat, anterior to the trachea, which comprises two wing-shaped lobes connected by the isthmus. This gland has a crucial role in the human body because it synthetizes, with the use of iodine, two hormones, thyroxine (T4) and triiodothyronine (T3), essential for the regulation of vital functions, such as heart rate, blood pressure, body temperature, and basal metabolic rate (Nitsch *et al.*, 2010; Nguyen *et al.*, 2015). It is composed by multiple types of cells, that are formed from all the germ layers, however, there are two cell types that are functionally more relevant: the follicular cells (thyrocytes), that synthesize and secrete T3 and T4 to the bloodstream,

and the parafollicular cells (C cells), that secrete calcitonin, a hormone responsible for calcium regulation (Brent *et al.*, 2012; Grasberger *et al.*, 2017). The production of these hormones is regulated by the hypothalamus-hypophysis-thyroid axis. The hypothalamus secretes the thyroid releasing hormone (TRH), which stimulates the anterior pituitary to release the thyroid stimulating hormone (TSH) to the bloodstream. TSH binds its receptor (TSHR) (Calebiro *et al.*, 2010) expressed by the thyrocytes and stimulates these cells to synthetize T4 (80%) and/or T3 (20%). Also, when there are high levels of T3 and/or T4 in the bloodstream, there is a negative feedback that T4 and T3 exert in TSH and TRH release. Additionally, there are four thyroid transcription factors (TTF), that are crucial for the survival and differentiation of the precursors of the thyroid cells, during embryogenesis (Fagman *et al.*, 2011), and the maintenance of the normal thyroid morphology, differentiation and function. These transcription factors are homeobox protein NKX-2.1 (NKX-2.1; TTF1) (Santisteban *et al.*, 2005), forkhead box protein E1 (FOXE1; TTF2) (Pereira *et al.*, 2015), paired box protein PAX8 (PAX8) (Plachov *et al.*, 1990) and haematopoietically-expressed homeobox protein (HHEX) (Fernández *et al.*, 2015).

1.4. Thyroid cancer

Thyroid cancer (TC) is one of the most common endocrine neoplasia, accounting for 1-3% of all malignancies in humans (van der Zwan *et al.*, 2012). The frequency of TC is 3-fold higher in women than in men (Siegel *et al.*, 2017). This could be explained by the higher levels of estrogen in women, that promotes the proliferation of thyroid cells (Derwahl *et al.*, 2014), and also by the expression of its receptor in thyroid gland cells (Lu *et al.*, 2016), which is more evident in women than in men in certain age groups (20-49 years old). The incidence of TC has increased over the past 30 years due to improvement of diagnostic methods and increased diagnostic activity, which allow the detection and treatment at earlier stages (La Vecchia *et al.*, 2014). In Portugal, thyroid cancer is the third most frequent cancer among women, with an incidence rate of 20.9/100,000 in women and 4.7/100,000 in men, and a mortality rate of 1.2/100,000 in women and 0.7/100,000 in men (Raposo *et al.*, 2017). The development of thyroid carcinomas can be influenced by environmental, hormonal or genetic factors, and by the interaction between them. The most well known risk factor for TC is the exposure to ionizing radiation. (Lloyd *et al.*, 2017). Differences in dietary iodine intake have also been reported to be a risk (Fiore *et al.*, 2020; Li *et al.*, 2016).

1.4.1. Clinical and molecular aspects of nonmedullary thyroid carcinoma

Thyroid cancer can be divided in two major subsets of neoplasia, depending on the thyroid cells in which these carcinomas originate: medullary thyroid carcinomas (MTC) (Trimboli *et al.*, 2015; Acquaviva *et al.*, 2017), arising from the parafollicular cells, and nonmedullary thyroid carcinomas (NMTC) (Pstrąg *et al.*, 2018), arising from follicular cells. NMTCs represent 90 to 95% of all TCs, being divided according to the degree of cell differentiation in: differentiated thyroid carcinoma (DTC), poorly differentiated thyroid carcinoma (PDTC) and anaplastic thyroid carcinoma (ATC) (Pstrąg *et al.*, 2018). DTC can be subdivided in papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), Hurthle cell carcinoma, and other DTC variants. Benign tumours such as follicular adenomas, may also derive from follicular cells (Muro-Cacho *et al.*, 2000) and could have the potential to be precursors of FTC (Nikiforov *et al.*, 2011). Furthermore, thyroid tumours can occur in association with other benign lesions, such as thyroiditis and nodular hyperplasia (Xing *et al.*, 2013). The most common mutations in PTCs include *BRAF* (b-Rapidly Accelerated Fibrosarcoma) mutations and *RET* (Rearranged during Transfection)/*PTC* fusions with different partner genes [*e.g. CCDC6* (coiled-coil domain containing 6) (Nikiforov *et al.*, 2011) and *NCO4* (nuclear receptor coactivator 4) (Xing *et al.*, 2013)], and in FTCs the most common alterations are *RAS* (Rapidly Accelerated Sarcoma) mutations (Gil *et al.*, 2020) and

PAX8-PPARγ (Peroxisome proliferator-activated receptor gamma) rearrangements (Chmielik *et al.*, 2018; Strickland *et al.*, 2018). Also, *ALK* (Anaplastic lymphoma kinase) rearrangements, *EIF1AX* (Eukaryotic translation initiation factor 1A X-linked) and *TERT* (Telomerase reverse transcriptase) promoter (*TERT*p) mutations are additional drivers (Shen *et al.*, 2017). PDTC and ATC are the least frequent NMTC. PDTC is a neoplasia hard to categorize and represents an intermediate state between differentiated and undifferentiated TC (Muro-Cacho *et al.*, 2000). The most common mutations in PDTCs occur in *BRAF*, *RAS*, *TP53* (Tumour protein 53), *CDKI* (cyclin dependent kinase inhibitor) and *TERTp* (Landa *et al.*, 2016; Pita *et al.*, 2014). ATCs are very aggressive undifferentiated tumors, having one of the worst prognosis and higher mortality among all human cancers, as they are extremely fast growing, invasive and metastatic (Simões-Pereira *et al.*, 2019; Yang *et al.*, 2020a). The most frequent mutations in ATC are *BRAF* (p.Val600Glu - p.V600E), *TP53*, *TERTp* and *RAS* mutations. The clinical and molecular characteristics of ATC will be described further in the introduction, in section 1.4.2.

1.4.1.1. MAPK and PI3K/AKT/mTOR signaling pathways

The transformation of follicular thyroid cells into NMTC frequently involves point mutations, epigenetic alterations and chromosomal rearrangements in effectors of two receptor tyrosine kinase (RTK) signaling pathways: the mitogen-activated protein kinase (MAPK) pathway and the PI3K/AKT/mTOR (PI3K- phosphoinositide 3 kinase; AKT- protein kinase B; mTOR- mechanistic target of rapamycin) pathway (Nikiforov *et al.*, 2011; Xing *et al.*, 2013; Stern *et al.*, 2018; Haglund *et al.*, 2007; Zolotov *et al.*, 2016) which appear to be crucial in thyroid cancer initiation. The MAPK signaling pathway is a kinase signaling phosphorylation cascade involved in homeostasis of the cells and in physiological processes such as survival, differentiation and proliferation (Sun *et al.*, 2015) in the response to extracellular signals (*e.g.* insulin, growth factors, chemokines, cytokines) (Zhang *et al.*, 2002). The *RAS* genes encode for guanosine triphosphate (GTP) phosphatases (GTPases), a major component of the MAPK pathway, located upstream of the pathway at the inner surface of the cell membrane, which interact with the RTK and provide signaling to both MAPK and PI3K/AKT/mTOR pathways (Wang *et al.*, 2008; Nikiforov *et al.*, 2011). *RAS* has three human isoforms: *HRAS* (Harvey Rat Sarcoma Viral Oncogene Homolog), *KRAS* (Kirsten Rat Sarcoma Viral Oncogene Homolog) and *NRAS* (Neuroblastoma Rat Sarcoma Viral Oncogene Homolog). Gain-of-function point missense mutations in *RAS* genes are found in ~25% of human cancers, usually affecting codons 12, 13 and 61 (Hobbs *et al.*, 2016). After the activation of RAS, by the association with GTP, it is possible to recruit serine/threonine-protein kinases RAF (Rapidly Accelerated Fibrosarcoma) to the cell membrane, where they become active by RAS mediated phosphorylation in the amino acid residues of serine and/or threonine (Fior, 2019). One of the most important and potent members of the RAF kinase family is BRAF, being p.V600E the most frequent mutation in *BRAF* gene in TC (Cabanillas *et al.*, 2020; Gil *et al.*, 2020). Activated BRAF phosphorylates and activates dual specific proteins: the mitogen-activated protein kinase 1 and 2 (MEK1 and MEK2), which, posteriorly to the activation, phosphorylate and activate downstream effectors, the extracellular signal-regulated kinase 1 and 2 (ERK1 and ERK2) (Fior, 2019). The PIK3/AKT/mTOR signaling pathway is also frequently dysregulated in most human cancers (Fruman *et al.*, 2014; Janku *et al.*, 2017). This pathway is involved in the regulation of essential functions for cell survival, such as metabolism, motility, growth, and proliferation. Upon activation, by growth factors and chemokines, PI3K catalytic domain catalysis the conversion of phosphatidylinositol-4,5-biphosphate (PIP_2) into a lipid second messenger phosphatidylinositol-3,4-5-trisphosphate (PIP_3) (Fior, 2019; Song *et al.*, 2005; Cantley *et al.*, 2002). The presence of PIP_2 triggers the recruitment of protein containing a pleckstrin homology (PH) domain to the membrane proximity. These proteins with the PH domain, include the major downstream effectors of this signaling pathway, the AKT, 3-phosphoinositide-dependent protein kinase-1 (PDRK1) and the mechanistic target of rapamycin complex 2 (mTORC2). The co-localization of these three proteins, elicits the AKT phosphorylation by

PDRK1 and mTORC2, and subsequent AKT activation. The activated AKT is able to activate or repress, through phosphorylation, several downstream protein effectors. In this way, activated AKT1 can: inhibit forkhead O family of transcription factors (FOXO), which promote the transcription of pro-apoptotic genes and cell cycle inhibitors; inhibit the glycogen synthetase kinase-3α/β (GSK3α/β) thus, promoting cell proliferation and viability; inhibit Bad (B-cell lymphoma 2 (BCL2)-associated agonist of cell death) and caspase-2, both involved in directly in apoptosis; and lead to the activation of mTORC1 complex, crucial for cell growth and metabolism (Fior, 2019; Barata *et al*., 2005; Song *et al*., 2005; Cantley *et al*., 2002). This pathway can be constitutively activated by multiple mechanisms, including genetic alteration in Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (*PIK3CA*), Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase (*PTEN*), *AKT*, Serine/threonine protein kinase 11 gene (*STK11*), *MTOR*, and other oncogenes and tumour suppressor genes (Noorolyai *et al*., 2019). The knowledge that constitutive activation of certain signaling pathways is causal in cancer progression, and is associated with resistance to conventional chemotherapy, makes these pathways an attractive target for anti-cancer therapies.

1.4.2. Anaplastic thyroid carcinoma clinical and molecular features

ATC is the rarest type of TC, accounting for 1-2% of the NMTC cases. As referred above, it is one of the most lethal malignancies, with a median of overall survival of the patients around 3-6 months, after diagnosis, and with a mortality rate of almost 100% (Simões-Pereira *et al*., 2019). ATC undifferentiation promotes a high proliferation rate and aggressiveness (Shah *et al*., 2015). Due to the fast proliferation, great invasive nature, and predisposition to spread through metastasis to other organs, such as the lungs, liver, bones, and brain, only about 10% of the patients with ATC have cancer confined to the thyroid (Ranganath *et al*., 2015). The large tumour size and invasion of local vital anatomical structures makes surgical removal of the tumour extremely difficult (Masui *et al*., 2021). ATC cells lost the intrinsic characteristics and functions of normal thyrocytes, such as the morphology and the ability to uptake iodine due to downregulation of the TTFs (Fernández *et al*., 2015) and the sodium/iodide symporter gene (*NIS*) (Yang *et al*., 2020a), respectively. The presence of genomic instability, one of the hallmarks of cancer (Hanahan *et al*., 2011), has a big influence on ATC development (Salvatore *et al*., 2007) and response to therapy, leading to a more aggressive phenotype (Pstrąg *et al*., 2018) due to the acquisition of a high number of mutations (Gil *et al*., 2020). Several studies in ATC reported that *TP53* was mutated in 60 to 65% of the ATC (Pita *et al*., 2014; Latteyer *et al*., 2016; Landa *et al*., 2016), *TERTp* in 33% (Melo *et al*., 2014), *BRAF* p.V600E in 41 to 45% and *RAS* in 10 to 27% (Pita *et al*., 2014; Cabanillas *et al*., 2020; Xing *et al*., 2016; Latteyer *et al*., 2016). There are other genes, not so frequently mutated, like retinoblastoma 1 (*RB1*) (Gil *et al*., 2020), PIK3/AKT/mTOR signaling pathway genes (Lai *et al*., 2020), *EIF1AX* (Simões-Pereira *et al*., 2018), Cyclin dependent kinase inhibitor 2A *(CDKN2A)*, Cyclin-dependent kinase 4 and 6 (*CDK4/6*) (Lopes-Ventura *et al*., 2018; Pita *et al*., 2014) and also Polo-like kinase 1 (*PLK1*) (Salvatore *et al*., 2007). Recent studies by Haase *et al.* provided evidence that Insulin-like growth factor 2 mRNA binding protein 1 (*IGF2BP1*) and the Melanoma-associated antigen 3 (*MAGEA3*) are upregulated in ATCs, with a distinct expression when comparing with PDTCs and other TC subsets (Haase *et al*., 2021), having *IGF2BP1* a *de novo* expression in ATCs. These two genes are the first two robust biomarkers for ATC, which will likely facilitate the diagnosis, guide the surgical decision, therapy choice and monitoring strategies. ATC's histological features are characterized by a marked pleomorphism, high proliferation rates, invasive and infiltrative growth, spindle cells, pleomorphic giant cells, epithelioid and/or squamous cells (Yang *et al*., 2020a; Xing *et al*., 2021). Also, ATCs possess a high number of tumour associated macrophages (TAMs), constituting 50 to 70% of the tumour mass (Muro-Cacho *et al*., 2000), and exhibit a dense ramified interconnected network of TAMs, that are in direct contact with tumour cells (Molinaro *et al*., 2017). In the advanced stage of ATC, the increased density of TAMs is associated with a high tumour invasiveness and infiltration, and high

aggressiveness, and with a decrease in overall survival, probably due to the pro-tumorigenic behavior of the M2 macrophages (Yin *et al.*, 2020; Lv *et al.*, 2021).

1.4.2.1. Targeted therapeutics in ATC

Curative surgery is the major therapeutic treatment, however due to the fast proliferation of ATC cells and the frequent invasion of anatomical vital structures in the throat area, surgery may not be possible to be successful (Masui *et al.*, 2021; Wächter *et al.*, 2020). Furthermore, since ATC cells do not uptake iodine due to the downregulation of *NIS*, radioactive iodine treatment is ineffective in these patients (Simões-Pereira *et al.*, 2019).

In a study developed by Subbiah *et al*. (Subbiah *et al.*, 2018) in patients with locally advanced or metastatic ATC *BRAF* p.V600E-positive, a robust clinical activity was demonstrated by combination of two tyrosine kinase inhibitor (TKI) therapies targeting the MAPK pathway: dabrafenib (BRAF inhibitor) (Kainthla *et al.*, 2014) and trametinib (MEK inhibitor) (Zeiser *et al.*, 2018). This combinatory therapy of dabrafenib plus trametinib (DT) was approved for *BRAF* p.V600E-positive ATC patients by food and drug administration (FDA) in 2018 and is presently being used in these cases in IPO Lisboa. DT has shown to be successful in promoting the apoptosis of cancer cells, increasing the median overall survival, with less toxicity, and decreasing the tumour mass size (Wang *et al*., 2019; Silva *et al.*, 2021). However, ATC cells can develop resistance to TKIs during the intra-tumoral evolution (McGranahan *et al.*, 2017), acquiring resistant cell clones (Lim *et al.*, 2020; Iyer *et al.*, 2018; Capdevila *et al*., 2020; Ferrari *et al*., 2020; De Leo et al., 2020). In stage IVC ATC patients with high PD-L1/2 (programmed cell death ligand 1 and 2) expression or high genomic instability, checkpoint inhibitors can be considered in the absence of other targetable alterations or in association with DT (Kwok et al., 2016; Bible *et al.*, 2021). These inhibitors block the association between PD-L1/L2 and its receptor, PD-1 (programmed cell death protein 1) (Kwok et al., 2016; Seliger *et al*., 2019; Somasundaram *et al*., 2021). Nevertheless preliminary results as single agents have not been promising. Since for ATCs, most of the conventional therapies for DTCs are not effective, and some patients are *BRAF* negative, and others may develop resistance to DT, it is important to investigate new molecular targets, and identify novel targeted therapies, in order to improve the overall survival of the patients.

1.5. Tumour Microenvironment

TME is a complex cellular ecosystem in which the tumour cells are comprised (Arneth *et al.*, 2019; Balkwill *et al.*, 2012). The TME is composed by the surrounding immune cells, blood vessels, endothelial cells, fibroblasts, extracellular matrix components, lymphocytes, monocytes, macrophages, and molecules, like cytokines, growth factors, metalloproteinases, that influence tumour progression, modulating cancer cell gene expression, tumour growth, invasion and metastasis, and therapeutic efficacy (Yang *et al.*, 2020), due to a crosstalk between the stroma cells and the tumour cells (Wu *et al.*, 2017). The major immune component of the ATC's TME are macrophages (Arneth *et al.*, 2019). Macrophages in TME promote the evasion of the tumour cells into the circulatory system and suppress immune anti-tumour mechanisms and responses (Stewart *et al*., 2008). The identification of a multitude of cells in the TME has brought a novel concept of anticancer drug development, focused not only on targeting cancer cells, but directed against the pro-tumorigenic interactions between cancer cells and other components of the TME (Pitt *et al.*, 2016).

1.5.1. Tumour associated Macrophages

TAMs, as the name implies, are macrophages that are associated with tumour cells comprised in TME. In the cancer context, TAMs are originated from circulating monocytes and/or derived from tissue-resident macrophages, that are recruited to the TME through a set of chemotactic axis such as C-C motif

chemokine ligand (CCL) 2 and its receptors C-C motif chemokine receptor (CCR) 2 and CCR5 (CCL2/CCR2, CCL2/CCR5), interleukin (IL) 1β/IL-1R, vascular endothelial growth factor A (VEGFA) and the receptors VEGFR1/2, colony-stimulating factor 1 and its receptor, (CSF1/CSFR) and Angiopoietin-1 tyrosine kinase receptor (Tie)/Angiopoietin 2 (ANGPT1) (Beltraminelli *et al.*, 2020; Zhou *et al.*, 2020; Lee *et al.*, 2013), constitutively expressed by cancer cells, stromal cells of the TME, and the monocytes (Quail *et al.*, 2013; Yang *et al.*, 2018). Several tumour associated cell types, have been reported to express CCL2, both in primary tumour sites and pre-metastatic/secondary tumour sites (Linde *et al.*, 2018). This CCL2-CCR2 axis seems to be important to mobilize pro-tumoural monocytes and macrophages into different cancer types, especially in metastasis context (Kitamura *et al.*, 2015; Laviron *et al.*, 2019). Furthermore, the association between CCL2 to CCR2 prompts the recruited monocytes to secrete CCL3, which functions in an autocrine manner promoting the differentiation and retention of metastasis-promoting macrophages (Kitamura *et al.*, 2015; Beltraminelli *et al.*, 2020). Tumour cells also produce and secrete other soluble factors that stimulate the proliferation of bone marrow myeloid progenitor cells, increasing the number of monocytes and macrophages, such as the granulocyte-macrophage colony stimulating factor (GM-CSF) (Spitler *et al.*, 2017). Once infiltrated in the TME, the monocytes differentiate into macrophages, through the influence of macrophage colony stimulating factor (M-CSF) secreted by the tumour cells (Fridman *et al.*, 2012). Also, it has been described that metabolism affects TAM polarization, such as the presence of cancer-derived succinate (Wu *et al.*, 2020). In terms of macrophage classification, TAMs are classified as M1 and M2 macrophages depending on the secretory phenotype and expressed biomarkers. M1-TAMs have an anti-tumoural activity, promoting endogenous inflammation by secreting pro-inflammatory cytokines and other mediators, being characterized by the expression of inducible nitric oxide synthase (iNOS), IL-12, IL-23, cluster of differentiation (CD) 16, 32, 64, 80 and 86 (Mantovani *et al.*, 2013). M2-TAM have an important role in tumourigenesis (protumoural activity) by suppressing the immune system responses, remodeling the extracellular matrix and in the stimulation of angiogenesis (Marques *et al.*, 2020; Mantovani *et al.*, 2013; Sica *et al.*, 2006). M2-macrophages are characterized by the expression of CD14, arginase, CD23 and CD163 and 203, and by mechanisms of immunosuppression which include the expression of PD-L1, prostaglandin E2 (PGE2), transforming growth factor beta (TGF-β), IL10, CCL2, and by the blocking of anti-tumour T cells and interferon (IFN) type I responses (Woo *et al.*, 2015). M2-TAM actions are key players in tumour aggressiveness, correlating with poor clinical outcomes and overall survival (Liang *et al.*, 2020; Zhukova *et al.*, 2020; Aras *et al.*, 2017; Williams *et al.*, 2016). Indeed, several reports demonstrated that the density of TAMs correlates with the proliferation of the cells in several types of tumours, like breast, thyroid, endometrial and renal cancers (Heusinkveld *et al.*, 2011; Sawa-Wejksza *et al.*, 2018), and is associated with poor prognosis (Liang *et al.*, 2020). Since TAMs influence various aspects of cancer progression, they could serve as a target for specific therapies.

1.5.2. The influence of TAMs in anaplastic thyroid carcinoma

As mentioned in section 1.4.2, ATCs are characterized by a high infiltration of tumour associated macrophages, in which its density comprises approximately 50-70% of the tumour mass (Muro-Cacho *et al.*, 2000), forming an interconnected cellular network in ATC, supporting cancer cells and providing a direct contact between ATC cells and TME (Caillou *et al.*, 2011; Molinaro *et al.*, 2017). This density of TAMs is associated with aggressiveness and poor overall survival of the ATC patients due to the protumorigenic behavior of the M2-TAMs (Yin *et al.*, 2020; Ryder *et al.*, 2008). Some reports showed that the density of TAMs was associated with tumour progression in advanced TC, not only on ATCs. For example, Quig *et al.* reported that the overall density of TAMs was higher in PTC than in benign thyroid lesions and was positively associated with lymph node metastasis and TNM stage (Quig *et al.*, 2012). The interconnected cellular network in which the ATC cells are in direct contact with the TAMs, allows the ATC cells to be more resistant to several types of therapies, such as chemo- and radiotherapies, when

comparing with other tumours that do not have this interconnected structure (Caillou *et al.*, 2011; Molinaro *et al.*, 2017). This characteristic seems to be exclusive to ATC, among TCs, and reinforces the role of pro-tumoural macrophages in ATC progression. All these observations provide evidence that TAMs might be a potential therapeutic target for cancer treatment in ATC. Several different experimental therapies have been conducted for targeting TAMs, such as: (1) inducing TAM apoptosis, using clodronate loaded liposome (Piaggio *et al.*, 2016), as an adjuvant therapy, reducing metastasis and angiogenesis, or zoledronic acid (Rogers *et al.*, 2011; Veltman *et al.*, 2010); (2) inhibiting monocyte differentiation into M2-TAMs using M-CSF receptor inhibitor and other drugs that block or reprogram the macrophages (Conway *et al.*, 2005; Spitler *et al.*, 2017); and (3) stimulation the polarization of M2 macrophages into M1 macrophages using pantoprazole (Vishvakarma *et al.*, 2010) or IL-12 (Watkins *et al.*, 2007; Fior, 2019). This crosstalk between TAMs and ATC happens in a way that both cells take advantage of it. TAMs contribute to build a pro-tumoural microenvironment and secrete factors, such as metalloproteinases, cathepsins, that degrade the extracellular matrix, enabling an easier cell invasion and migration (Mott *et al.*, 2004). ATC contribute to secrete M2-TAM inducing factors and metabolites which promote TAM proliferation and the secretion of pro-tumoural factors (Yin *et al.*, 2020). This could explain the high predisposition of ATC cells to metastasize, and that only 10% of the patients with ATC have cancer confined to the thyroid (Ranganath *et al.*, 2015).

Based on these ATC and macrophage features, our group (Molecular Endocrinology Group, UIPM, IPOLFG) has been conducting studies on TAM-ATC crosstalk. 2D transwell *in vitro* cultures, using a human acute monocytic leukemia cell line, THP-1 (Tsuchiya *et al.*, 1980), as a TAM model, and two ATC cells lines C3948 (Pinto *et al.*, 2019) and T235 (Rodrigues *et al.*, 2007), in monocultures (no contact with TAMs) and co-cultures (in contact with TAMs) were undertaken. An increase of invasion and migration of the T235 ATC cells, when co-cultured with THP-1 (pro-tumoural activity), and a decrease in the case of the C3948 co-cultures (anti-tumoural activity) was observed (Supplementary Figure 2; Mol. End. Group's unpublished data). Subsequently, proteomic analysis showed that there was an upregulation of SPRY4 protein (encoded by *SPRY4* - Sprouty RTK Signaling Antagonist 4, OMIM * 607984) in co-cultures of C3948, but there was a downregulation in the case of the co-cultures of T235, when comparing with the respective monocultures (Supplementary Figure 1; Eduardo *et al.*, 2020 - manuscript in preparation). These results suggested that *SPRY4* could be a tumour suppressor gene (TSG) in ATC with an influence in ATC-TAM crosstalk, however, additional studies are needed to further understand these mechanisms associated with ATC progression and aggressiveness.

2. Aims and thesis hypothesis

The main aim of this study is to further understand the cellular and molecular mechanisms involved in anaplastic thyroid carcinoma aggressiveness and differential response to therapy. The specific aims of this project are:

1. To characterize the molecular mechanisms involved in ATC response to DT combination therapy, through the analysis of a multigene panel by Next generation sequencing (NGS) and Sanger Sequencing. This characterization will be useful to understand the response and resistance to the DT therapy and to define treatment options in these cases.

2. To further study the role of *SPRY4* gene in TAMs interaction with ATC, and the cellular mechanisms involved, that might be associated with ATC aggressiveness. These studies may unravel the mechanisms involved in TAMs-ATC crosstalk, eventually leading to new therapeutic approaches capable of improving the prognosis of ATC patients.

3. Materials and methods

3.1. Cases and biological samples

This study was approved by the Institution Ethical Committee from IPOLFG.
A total of nine patients with *BRAF* p.V600E-positive (p.Val600Glu | rs121913377) ATCs, treated with the DT combination therapy, were selected to this study. ATC samples, pre-DT (#9) and post-DT (#2) therapy, were collected, after the informed consent of the patient, by surgery and biopsy, and were preserved in paraffin blocks (formalin-fixed paraffin-embedded - FFPE). Sections of the FFPE tumours were supplied by the Pathology Department (IPOLFG, Lisbon, Portugal) to the Molecular Endocrinology group (IPOLFG, Lisbon, Portugal) to be analyzed. Also, sections of five FFPE normal thyroids were provided to be used as a baseline for copy number variation (CNV) analysis. DNA and RNA were extracted from ATC sections to be molecularly characterized through Sanger sequencing and Next Generation Sequencing (NGS), with the AmpliSeq™ for Illumina Focus Panel (Illumina, California, USA) for somatic alterations [single nucleotide variants (SNVs), insertions/deletions (indels), CNVs, fusions], as detailed below.

3.2. DNA and RNA extraction from formalin-fixed paraffin-embedded (FFPE) samples

Nucleic acid extraction from FFPE tumour and normal thyroid samples was performed by a semi-automatic procedure, using the Maxwell® RSC DNA FFPE kit and the Maxwell® RSC RNA FFPE kit (Promega Corporation, Wisconsin, USA), according to the manufacturer's protocol. Then, DNA and RNA were quantified using UV spectrophotometry (NanoDrop ND-1000, Thermo Fisher Scientific, Wilmington, DE, USA). For more accurate quantification, a Qubit 2.0 fluorometer (Invitrogen, California, USA), the Qubit™ dsDNA Broad range kit (Invitrogen, California, USA), and Qubit™ RNA High sensitivity kit (Invitrogen, California, USA), were used according to the manufacturer's protocol. DNA stocks were stored at -20°C and the RNA stocks were stored at -80°C to preserve the nucleic acids until further procedures.

3.3. Polymerase Chain reaction (PCR)

This method was used to amplify the DNA sequences for subsequent Sanger sequencing. Primers were designed using Primer3web version 4.0.0 tool. The PCRs were performed using a Platinum Taq DNA polymerase High Fidelity (Invitrogen, California, USA) protocol. The reactions were prepared for a final volume of 12.5 µL with the following components: DNA template (20 ng/µL), forward primer (10 pmol/µL), reverse primer (10 pmol/µL), 10x High Fidelity PCR buffer, dNTP's mix (2.5 mM), $MgSO_4$ (50 mM), Platinum Taq DNA polymerase High Fidelity (5 U/µL), and the remaining volume was made up with bi-distilled water. PCR tubes were placed in a thermocycler (Biometra T3 thermocycler, Germany). Primers and PCR conditions are summarized in supplementary table 1.

3.4. Agarose gel electrophoresis of the PCR products

PCR products were analyzed by agarose gel electrophoresis. For this procedure, 2% (w/v) agarose gels were used. The agarose gels were loaded with 3 µL of PCR products and 1 µL of Gene ruler 50 bp DNA ladder (Thermo Fisher Scientific™, Massachusetts, USA), that allowed the determination of the molecular weight of the amplified products. Prior to the loading in the gel, the 3 µL of PCR products and the 1 µL of Gene ruler 50 bp DNA ladder were mixed with 1 µL of loading dye (6x TriTrack DNA dye; Thermo Fisher Scientific™, Massachusetts, USA) separately. The electrophoresis was performed in a horizontal gel system (Cleaver Scientific, Waswickshire, UK), at 120 volts (V), for 30 minutes (min).

For the visualization and image capture of the gel bands, a transilluminator, coupled to a video camera and to an informatics system (BioDocAnalyze, Biometra, Germany), was used to expose the agarose gel to UV light radiation.

3.5. Automated Sanger sequencing

Sanger sequencing was used to analyse mutations in the patient's samples. This sequencing methodology includes the purification of the PCR products, PCR sequencing reaction, precipitation of the sequencing products, and capillary electrophoresis, as follows: after PCR amplification, PCR products were purified using two enzymes: Exo I (20 U/μL; Exonuclease I; Thermo Fisher Scientific™, Massachusetts, USA) and FastAP (2 U/μL; Thermosensitive Alkaline Phosphatase; Thermo Fisher Scientific™, Massachusetts, USA). Sequencing products were obtained using BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, California, USA), according to the manufacturer's protocol, and then precipitated using Ethanol/EDTA/Sodium Acetate precipitation protocol. Final products were analysed in a Genetic analyzer 3500 (Applied Biosystems, California, USA) automated sequencer and subjected to a capillary electrophoresis to provide electropherograms, using a Sequencing Analysis Software 6 (Applied Biosystems, California, USA). Posteriorly, the electropherograms were analyzed using the Variant Reporter v3.0 software (Applied Biosystems, California, USA).

3.6. Next generation sequencing and Bioinformatic analysis

NGS was performed using the AmpliSeq™ for Illumina Focus Panel (Illumina, California, USA), a 52 genes panel (Supplementary Table 2) with known relevance in solid tumours, which allows the analysis of SNVs, indels, CNVs, and gene fusions, and an Illumina MiSeq™ (Illumina, California, USA), according with the manufacturer's protocol. FastQ files provided by the NGS were loaded to the BaseSpace sequence hub by Illumina. The reads were aligned against the human reference genome version GRCh37, using the BaseSpace sequence hub software. Aligned reads were converted (SAM to BAM). Specific filters were applied in the bioinformatic analysis such as: Somatic variant frequency threshold = 0.99; Variant Caller Depth Filter = 10; Minimum Depth = 10. Then Vcf.gz type files were analysed using the Variant Reporter software, also provided by Illumina. Annotation was done using BaseSpace Annotation Engine 3.6.2.0. Filters were also applied to select the relevant variants: Population filters GnomADExome frequency < 0.01 and GnomAD frequency < 0.01, to exclude polymorphisms, and Small Variant QC Metrics with Allele depth > 5 and Variant read frequency > 0.01. To predict the potential functional consequences of the candidate variants, *in silico* analysis was done using PolyPhen, SIFT and FATHMM prediction tools.

3.7. ATC and THP-1 cell culture and expansion

Two anaplastic thyroid carcinoma cell lines, established in IPOLFG, from two different ATC patients, were used: T235 (*BRAF* p.Val600Glu; *TERTp* C228T; *TP53* p.Leu194Arg) (Rodrigues *et al*., 2007) and C3948 (*TP53* p.Glu346Ter; *STK11* p.Ser216Phe; *DIS3L2* p.Trp361Leu) cells (Pinto *et al*., 2019). Also, THP-1, a human acute monocytic leukemia cell line (Tsuchiya *et al*., 1980), gently provided by Doctor Raquel Gonçalves from Instituto Nacional de Engenharia Biomédica (INEB/i3S), was used in the experimental procedures. The cells were kept cryopreserved in liquid/vapor phase nitrogen tanks. They were thawed by submerging the cryovials in a 37°C water bath. ATC cells were cultured in Roswell Park Memorial medium 1640 (RPMI-1640; LonzaTM, Verviers, Belgium) with 25 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) supplemented with 10% (v/v) Fetal bovine serum (FBS) (Pan-biotech, Aidenbach, Germany), 1% (v/v) L-glutamine (Gibco® Life Technologies, Paisley, UK) and 1% (v/v) penicillin/streptomycin solution (pen/strep; Gibco®, Life Technologies, Paisley,

UK). ATC cell lines grow by attachment, so cell sub-cultures were done using the trypsin method. THP-1 cells grow in suspension; thus, the cells were expanded by diluting the medium, always maintaining exhausted culture medium because these cells secrete growth factors and aggregation inducing factors (Lyons, 2017). Expansions and sub-cultures were done according to the confluence and to the total cell number. The determination of total cell number [**Equation 3.1 - Total cell number = Average Number viable cells * 10^4 * dilution factor**] was estimated by the Trypan Blue dye (Gibco® Life Technologies, Paisley, UK) exclusion method [0.4% (v/v)], using a hemocytometer (0.0025 mm^2, Neubauer Improved, Erlangen, Germany). All reagents stored at 4°C were always preheated at 37°C to avoid cell stress caused by the differences in temperature. All cell observations were performed in an inverted microscope Axiovert 135 (Zeiss, Germany). Cell cultures were maintained in an incubator (MCO-170AIC-PE IncuSafe CO2 Incubator; Panasonic, Etten-Leur, Netherlands), at 37°C, in a 5% CO_2 humidified atmosphere. All the procedures and manipulations were done in a sterile environment in a vertical laminar flow chamber to avoid contaminations.

3.8. 2D transwell cultures for western blot SPRY4 analysis

2D transwell cultures were performed to investigate the role of SPRY4 protein expression in TAM and ATC crosstalk. C3948, T235, and THP-1 cell lines were used for this procedure. This assay starts with THP-1 ($6*10^5$ cells/insert) plating in the top of transwell inserts (0.4 µm pores and 23 mm diameter, #353090, Corning, New York, USA) placed in 6-well plates. The THP-1 cells were plated with RPMI-1640 with HEPES supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine and 1% (v/v) penicillin/streptomycin. This culture medium was supplemented with 200 nM of PMA (Phorbol 12-myristate 13-acetate; #P8139, Sigma-Aldrich, Missouri, USA), in order to differentiate THP-1 monocytes into macrophages, for 24 h, followed by a 48 h resting phase without PMA. After this resting phase, ATC cancer cells ($2*10^5$ cells/well) were plated in the bottom of the wells, for co-culture and monoculture, with RPMI-1640, with HEPES, supplemented 2% (v/v) FBS, instead of the 10% (v/v) FBS previously used.

3.8.1. SPRY4 western blot analysis

Twenty-four hours after ATC plating, the cells were lysed using RIPA buffer (#89900, ThermoFisher Scientific, Massachusetts, USA), supplemented with 1x Sigma protease and phosphatase inhibitor cocktail (#P8340, Sigma-Aldrich, Missouri, USA). Total protein quantification was performed using the Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Illinois, USA), according to the manufacturer's protocol. Standards provided by the Pierce™ BCA protein assay kit were used for the calibration curve. The assay was performed in a clear 96-well plate and the absorbance was measured at 595 nm on Microplate Absorbance Reader (iMARK™; Bio-Rad Laboratories, California, USA). Proteins were prepared in 5x Laemmli Buffer (Sigma-Aldrich, Missouri, USA) and β-mercaptoethanol (Sigma-Aldrich, Missouri, USA), in a dilution of 1:20, and then denatured at 100°C for 5 min in a Techne Dri-block DB.2A® (Jencons Scientific, Leighton Buzzard, UK).
For western blotting, 12% and 15% polyacrylamide gels were prepared using ddH_20, 30% acrylamide mix (Bio-Rad Laboratories, California, USA), 1.5 M Tris (pH 8.8; Bio-Rad Laboratories, California, USA), 10% Sodium dodecyl sulfate (SDS; AppliChem, Darmstadt, Germany), 10% ammonium persulfate (APS; Sigma-Aldrich, Missouri, USA) and TEMED (Tetramethylethylenediamine; AppliChem, Darmstadt, Germany). Protein loading was done using 10 and 30 µg, for the 12% and 15% gels, respectively, and placed in a Multi-PROTEIN® Tetra System (Bio-Rad Laboratories, California, USA) with 1x Running Buffer (#161-0772, Bio-Rad Laboratories, California, USA). BenchMark™ pre-stained protein ladder (Life Technologies, California, USA) was used as a molecular marker. Protein transfer was done using polyvinylidene difluoride (PVDF) membranes (#1704157, Bio-Rad

Laboratories, California, USA), reservoir stacks (Bio-Rad Laboratories, California, USA), and 1x (v/v) Transfer Buffer (#1610734, Bio-Rad Laboratories, California, USA), and placed in a Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories, California, USA) for 30 min, at 25 V and 1 amperes (A). The PVDF membranes of the 12% gels, were incubated, overnight, with anti-SPRY4 rabbit monoclonal antibody (clone EPR12127, #ab176337, Abcam, Cambridge, UK) diluted 1:5000 with the antibody solution. Secondary antibody incubation for SPRY4 was done using goat polyclonal anti-rabbit conjugated with horseradish peroxidase (HRP) diluted 1:10,000 (#31460, ThermoFisher Scientific, Massachusetts, USA). Beta-actin was used as an endogenous control gene. Primary antibody incubation was done using the anti-β-actin mouse monoclonal antibody (clone AC-15, #A5441, Sigma-Aldrich, Missouri, USA) diluted 1:5000 and secondary antibody incubation was done using the secondary antibody goat polyclonal anti-mouse conjugated with HRP (#31430, ThermoFisher Scientific, Massachusetts, USA) diluted 1:10,000. In the case of the membranes of the 15% gels, the procedure was the same, although using an anti-SPRY4 rabbit polyclonal antibody (AB_2458875, Invitrogen, California, USA) diluted 1:500 in antibody solution. Alfa-tubulin was used as the endogenous gene, so the antibody incubation was done with an anti-α-tubulin mouse monoclonal antibody diluted 1:4000 (clone B-5-1-2; #T5168, Sigma-Aldrich, Missouri, USA) and a secondary incubation using the goat polyclonal anti-mouse conjugated with HRP (#31430, ThermoFisher Scientific, Massachusetts, USA) diluted 1:10,000. Revelation procedure was performed with a Clarity™ Western ECL Blotting Substrate (Bio-Rad Laboratories, California, USA), that enhances the chemiluminescence, and reveled using the Molecular imager® ChemiDoc™ XRS+ (Bio-Rad Laboratories, California, USA).

3.9. 2D transwell cultures of ATC cell lines silenced for *SPRY4* with siRNA

In order to study the role of *SPRY4* in TAM and ATC crosstalk, 2D transwell cultures were performed using ATC cell lines, C3948 and T235, silenced for *SPRY4* with short interfering RNA (siRNA). Optimizations were undertaken in order to determine the best siRNA concentrations and timepoints to achieve acceptable levels of silencing, using concentrations between 12.5 nM and 50 nM of siRNA and timepoints of 48h and 72h. ATC cells were plated ($1.5*10^5$ cells/well) in 12-well plates' bottom wells. For the endogenous *SPRY4* gene silencing, C3948 and T235 were transfected ON-TARGETplus Human SPRY4 siRNA-SMARTpool (Dharmacon, Lafayette, US), using the DharmaFECT$_{TM}$ (Dharmacon, Lafayette, US) as a transfection reagent. The ON-TARGETplus Non-targeting pool siRNA (Dharmacon, Lafayette, US) was used as a transfection control. The culture medium of the bottom wells was carefully removed and then 800 µL of RPMI-1640 with HEPES supplemented 10% FBS, 1% L-glutamine, pen/strep-free medium and 200 µL of the mix between siRNA + DharmaFECT were added. Then, total RNA and protein were extracted at 48h and 72h post-transfection. Protein extraction and quantification was done as described in sections 3.8.1. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according with the manufacturer's protocol, and then quantified by UV spectrophotometry (NanoDrop ND-1000, Thermo Fisher Scientific, Wilmington, DE, USA).

3.9.1. cDNA synthesis and quantification of *SPRY4* gene expression by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

cDNA synthesis was done using 1 µg of total RNA resuspended in RNase-free water. The protocol involved the use of two mixes: first, a "mini-mix" was prepared for each sample, which included 0.4 µL of dNTP mix (2.5 mM) and 0.1 µL of random primers p(dN)6 (3 µg/µL) (Roche Diagnostics Corporation, Indianapolis, USA). The mix was added to the previously prepared 1 µg of total RNA, and then placed in a thermocycler (Biometra T3 thermocycler, Germany) in order to denature RNA, at 65ºC, for 10 min. After that, a "Master mix" was prepared with 4 µL of 5x First Strand Buffer [250 mM Tris-

HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$; Invitrogen™], 2 µL of 0.1 M Dithiothreitol (DTT; Invitrogen™), 0.5 µL of 40 U/µL RNaseOUT™ (Recombinant Ribonuclease Inhibitor; Invitrogen™), 0.5 µL of H_2O DEPC treated water, and 0.5 µL of 200 U/µL SuperScript II Reverse Transcriptase (Invitrogen™), for each sample. Finally, 7.5 µL of this mix were added to the "mini-mix" plus RNA, and placed again in the thermocycler, using a program with the following steps: primer annealing at 25ºC for 10 min, cDNA synthesis at 42ºC for 50 min, and enzyme inactivation at 70ºC for 15 min. All the components described above, and the cDNA samples were stored at -20ºC.

For the qRT-PCR, a reaction mixture was prepared composed by: 10 µL SYBR Green (Applied Biosystems, California, USA), 0.5 µL of primers for *SPRY4* (Forward: 5'-TCCGTACAGTCCAGGACCTC-3', Reverse: 5'-GGCTGGACCATGACTGAGTT-3') or for the control *HPRT1* (Forward: 5'-TGACACTGGCAAAACAATGCA-3', Reverse: 5'-GGTCCTTTTCACCAGCAAGCT-3'), 4 µL H_2O DEPC and 5 µL cDNA, to a final volume of 20 µL. The qRT-PCR was performed in a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems, California, USA), using the following program: a holding stage (95°C, 10 min), followed by 40 amplification cycles (95°C, 15 seconds, and 60°C, 1 min). For data analysis, the relative gene expression was calculated by the ΔΔCt method [(**ΔΔCt = (Ct target gene - Ct reference gene) treatment sample - (Ct target gene - Ct reference gene) control sample**); Ct - threshold cycle]. This was done by a normalization of the target gene (*SPRY4*) with the endogenous control gene (Hypoxanthine phosphoribosyltransferase 1; *HPRT1*), whose expression remains unchanged. The fold change [**FC = 2 – ΔΔCt**] and the percentage of knockdown [$\mathbf{\%KD = (1 - FC) * 100}$] of the target gene in the treated cells, were also calculated.

3.9.2. Viability assays

The effect of *SPRY4* silencing in ATC cells' viability was assessed in monocultures using Trypan blue dye (Gibco® Life Technologies, Paisley, UK) exclusion method [0.4% (v/v)]. ATC cells were plated in 12-well plates ($1.5*10^5$ cells/well) with RPMI-1640 with HEPES supplemented with 2% (v/v) FBS, 1% (v/v) L-glutamine and 1% (v/v) penicillin/streptomycin. Then the cells were silenced for *SPRY4* and non-target control, as mentioned before in section 3.9. After 72h of silencing, cells were harvested by detachment using 0.05% (v/v) Trypsin-EDTA and viable cells were counted using 0.4% Trypan blue in a hemocytometer (0.100 mm; Neubauer Improved, Erlangen, Germany). Equation 3.1 was used to calculate the total number of viable cells.

3.9.3. 2D Transwell invasion assays

In order to evaluate the influence of macrophages and *SPRY4* silencing on the invasion ability of ATC cells, 2D transwell invasion assays were performed, using matrigel based inserts with 8.0 µm pores (Corning, Manassas, USA) in 24-well plates (Corning, Manassas, USA). ATC cells were plated in 12-well plate's bottom wells ($1.5*10^5$ cells/well) for pre-invasion assay *SPRY4* silencing. THP-1 cells were plated, on the bottom wells of a 24-well plate ($2*10^5$ cells/well) and differentiated into macrophages as previously described. In the same day, *SPRY4* was silenced in ATC cells, as described above. After the 48h of resting period, THP-1 culture medium was discarded and fresh RPMI-1640 with HEPES supplemented with 10% FBS (v/v) 1% (v/v) L-glutamine and 1% (v/v) pen/strep, was added to the wells containing THP-1 differentiated macrophages. After 72h of *SPRY4* silencing, ATC cells were plated in the invasion inserts of the 24-well plates ($7.5*10^4$ cells/insert), in RPMI-1640 with HEPES supplemented with 2% FBS (v/v) to create a chemoattractive gradient to ATC cells. Finally, after 24h, the invasion was stopped using ice-cold methanol and strained using Vectashield + 4',6'-diamino-2-fenil-indol

(DAPI) (Vector laboratories, CA, USA). Ten images for each membrane were captured using an Olympus IX53 fluorescence microscope and the Cell Sens Standard program (Olympus, Tokyo, Japan). The cell numbers were obtained using Fiji image-J software (imagej.nih.gov/ij/).

3.9.4. 2D Transwell morphology assays

To investigate if TAMs promoted alterations in ATC's morphology, 2D transwell cultures were performed in using transwell inserts (0.4 µm pores and 10.5 mm diameter, #353180, Corning, New York, USA) placed in 12-well plates. ATCs were plated in 12-well plate ($1.5*10^5$ cells/well) for a pre-*SPRY4* silencing. THP-1 cells were plated in 12-well plate's inserts ($1.7*10^5$ cells/insert), for macrophage differentiation, as mentioned before. After 72h of *SPRY4* silencing, glass slides were added to the bottom wells of a 12-well plate and covered with a solution of 0.2% (w/v) gelatin from porcine skin (G-1890, Sigma-Aldrich, Missouri, USA) and incubated at 37°C for 15 min. Then, silenced ATCs were plated (C3948 - $3*10^4$ cells/well; T235 - $4*10^4$ cells/well) on the gelatin-covered glass slides, in RPMI-1640 with HEPES supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine and 1% (v/v) pen/strep. After 48h of co-culture, the cells were washed with 1x phosphate buffered saline (PBS) and fixed with 4% (w/v) PFA (paraformaldehyde; Sigma-Aldrich, Missouri, USA) for 20 min. Then, cells were stained with phalloidin (P5282; Sigma-Aldrich, Missouri, USA) in a 1:500 dilution, for 1h 30 min, and then strained using Vectashield + DAPI (Vector laboratories, CA, USA). Images were captured using an Olympus IX53 fluorescence microscope and the Cell Sens Standard program (Olympus, Tokyo, Japan).

3.10. Statistical analysis

The analysis of SPRY4 protein expression by western blot was performed in three independent assays. In this case, the statistical analysis was done using a parametric unpaired T test. The analysis of *SPRY4* mRNA expression by qRT-PCR and the viability assays were performed in four independent assays and data was analysed using the parametric unpaired T test. Invasion analysis was performed in four independent assays and data was analysed using a One-way ANOVA (Tukey's test) for total cell number, and a parametric unpaired T test for fold change. All the analyses were expressed as the mean ± standard deviation (SD) and plots were done using Graphpad prism software (version 8.0.1; www.graphpad.com). All p-values < 0.05 were considered statistically significant.

4. Results and Discussion

This project aims to contribute a better understanding of the molecular and cellular mechanisms involved in anaplastic thyroid carcinoma aggressiveness and differential response to therapies, since these mechanisms are still poorly clarified. This main task included two specific aims:

1. The first objective consisted in the characterization of the molecular mechanisms involved in ATC response to DT combination therapies. In this context, Sanger sequencing and NGS were performed to investigate which genes and molecular gene alterations were involved DT efficacy: which of those were associated with a favorable response or resistance mechanisms.
2. The second objective consisted in the study of the role of *SPRY4* gene in TAMs interaction with ATC. In this regard, 2D transwell cultures of ATC and TAM cell lines, with and without *SPRY4* siRNA mediated silencing, were carried out to investigate the role of this gene, together with TAMs, in ATC cell morphology, viability, invasion, and underlying signaling pathways - TAM crosstalk.

Therefore, the results and discussion for each topic will be presented in two sections (4.1 and 4.2), followed by a specific topic discussion.

4.1. Molecular characterization of patients with *BRAF* p.V600E-positive ATC under Dabrafenib and Trametinib treatment

4.1.1. Results

4.1.1.1. Patient's clinical information

Clinical data of the patients was provided by the Co-Supervisor of this Master thesis, Dr. Tiago Nunes da Silva, from Serviço de Endocrinologia of IPO Lisboa, and it is summarized in table 4.1. According to the present data, nine patients had an excellent initial response, and subsequently, two (P3 and P4) presented ATC progression due to DT resistance. Six patients are still alive (follow up from 2.4 to 28 months). In this specific cohort, the median age of diagnosis was 77 years. DT therapy is increasing both the overall survival and progression free survival (Silva *et al.*, 2021) with 410 and 270 days respectively. These data corroborate the results of Subbiah *et al.*, in the absence of resistance mutations, such as the case of patient P1, P2, P5, P6, P7, and P9, who are still responding to DT therapy. Cases P3, P4, and P8 had disease progression, which reflects in their present state (deceased). For cases of P3 and P4, samples were obtained under clinical progression, unfortunately due to quick progression and death no sample was available from P8. Nevertheless, they showed a clear trend towards increased overall survival *versus* the historical IPOLFG cohort [between 3 to 13 months *vs* 3 months (Simões-Pereira *et al.*, 2019)].

Table 4.1. Clinical features from *BRAF* p.V600E-positive patients under DT therapy

Patient number	Age at diagnosis	Sex	DT treatment				Present state
			Response duration (months)	Disease progression	Disease status before DT	Disease status after DT	
P1	59	M	28	No	Pulmonary metastasis after ATC surgery	Disappearance of metastasis (**complete response**)	Alive
P2	77	M	21	No	Local recurrence (thyroid bed) after ATC surgery	Disappearance local recurrence (**complete response**)	Alive
P3	58	F	3	Yes	Used neoadjuvancy in thyroid and bone metastasis	First recurrence thyroid bed, resolution of bone metastasis. Second recurrence with brain metastasis	Deceased
P4	59	M	1	Yes	Used neoadjuvancy in thyroid	Local recurrence (thyroid and lymph nodes)	Deceased
P5	77	F	17	No	Used neoadjuvancy in thyroid	Total disappearance of thyroid ATC. Stabilization of pulmonary metastasis that appear after stopping DT for surgery and RT	Alive
P6	61	F	6	Yes	Used neoadjuvancy in thyroid	Reduction of ATC in thyroid (**partial response**)	Alive
P7	81	F	4.5	No	Used neoadjuvancy in thyroid	Reduction of ATC in thyroid (**partial response**)	Alive
P8	83	F	2.6	Yes	Used neoadjuvancy in thyroid	Fast recurrence after 2 months of response	Deceased
P9	56	M	2.4	No	Several mediastinal, pulmonary, and pleural metastasis after ATC surgery	Reduction of mediastinal, pulmonary, and pleural metastasis	Alive

4.1.1.2. *TERTp* Sanger sequencing analysis

The genetic duet of *BRAF* p.V600E and *TERTp* mutations has been identified in several human cancers, including thyroid cancer, to synergistically promote aggressive clinicopathological outcomes, but, on the other hand, was also found, in *in vitro* and *in vivo* studies, to improve therapeutic sensitivity to BRAF/MEK inhibitors (Tan *et al.*, 2020). *TERTp* mutations were not detected by the multigene panel analysed by NGS. Therefore, mutations in this region were assessed by Sanger sequencing analysis in

#9 pre-DT and #2 post-DT ATC samples. In the nine ATC patients, it was observed that all patients, except P7, had the presence of the C228T mutation in the *TERTp*, as it is shown in Figure 4.1A. Patient P7 presented the C250T mutation (Figure 4.1B).

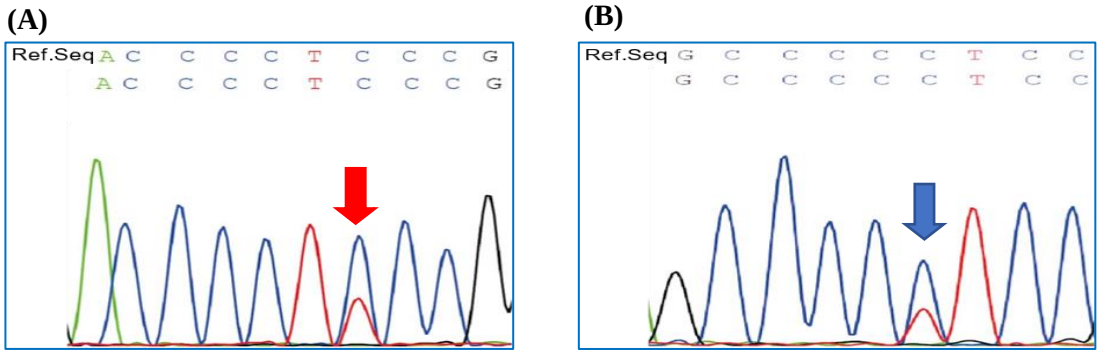

Figure 4.1. Detection of *TERTp* mutations C228T (c.1-124C>T) and C250T (c.1-146C>T). The upper sequence represents the reference sequence (Ref.Seq). (A) The figure represents the sequence of an ATC with the C228T mutation (red arrow). (B) The figure represents the sequence of an ATC with the C250T mutation (blue arrow).

A germline polymorphism c.1-245T>C (rs2853669), a regulatory SNP (rSNP) positioned 245 base pairs (bp) upstream of the transcription start site of *TERT*, was also present in some patients (P2, P4, P6, P7, P8, P9). In the present ATC series, when analyzing the presence of rs2853669 genotypes (Figure 4.2) and patient's clinical data, it was possible to observe that ATCs from patients P1 and P5, who had the TT genotype (Figure 4.2A), are still responding well to DT therapy, contrasting with ATC patients P4 and P6 (Figure 4.2B), who had the CC genotype, and did not show this positive response to therapy. P2 and P7, carrying the polymorphism in heterozygosity (TC) presented a good response (Figure 4.2C), P8 had a good initial response, although having the CC genotype (Figure 4.2B) and P3, carrying the TT genotype, had a good initial response to DT (Figure 4.2A).

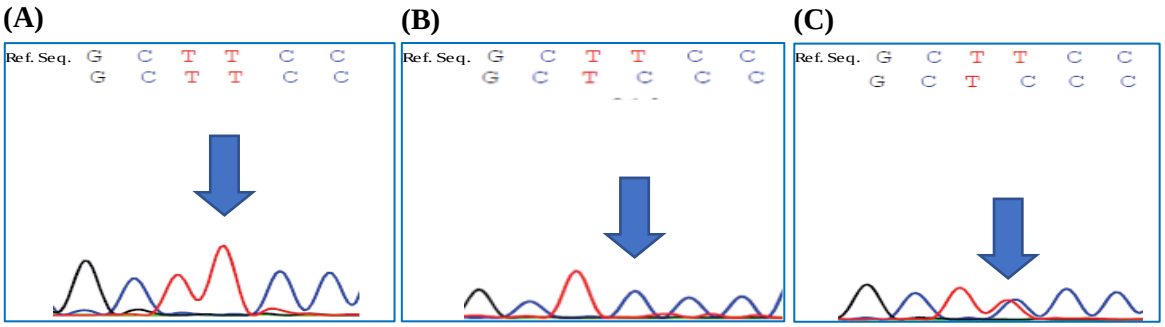

Figure 4.2. Detection of the c.1-245T>C (rs2853669) polymorphism in *TERT* promotor. The upper sequence represents the reference sequence (Ref.Seq). (A) represents the sequence of a patient with the TT genotype in homozygosity. (B) represents the sequence of a patient with the CC genotype in homozygosity. (C) represents the sequence of a patient with the TC genotype in heterozygosity.

4.1.1.3. NGS mutation profiling of ATC patients

NGS analysis of a multigene panel was performed in #9 pre-DT and #2 post-DT ATC samples. The results are summarized in table 4.2. In brief, all pre-DT ATC samples presented *BRAF* p.V600E and *TERTp* mutations. In 5/9 pre-DT ATCs, *TP53* mutations were identified; in 4/9, mutations in genes related with the PI3K/AKT/mTOR signaling pathway were verified, which may have a role on decreasing DT efficacy. Also, in 1/9 cases, a new mutation in *MAP2K1* (MEK1 gene) (p.E120Q) was detected. In one of the two cases that was possible to analyse the post-DT sample, the mutation *NRAS* p.Q61K was identified, and represents an off-target resistance mechanism to DT. Neither CNVs nor fusions were identified.

Table 4.2. Molecular data provided by NGS and Sanger sequencing from *BRAF* p.V600E-positive ATC patients under DT therapy.

Patient number		Cancer	Detected mutations													
			Gene/DNA sequence	Chr.	Transcript	Consequence	HGVSC	HGVSP	Zygosity	SIFT prediction	PolyPhen prediction	FATHMM prediction	COSMIC ID	dbSNP ID	VAF (%)	Total Read Depth
P1 (pre-DT)		ATC	*BRAF*	7	NM_004333.4	Missense	c.1799T>A	p.V600E	Heterozygous	Deleterious	Probably damaging	Pathogenic	COSV56056643	rs113488022	35.9	1159
			*TERTp**	5	NM_198253.2	Regulatory region variant	c.1-124C>T	-	Heterozygous	-	-	Pathogenic	-	rs124253815	-	-
P2 (pre-DT)		ATC	*BRAF*	7	NM_004333.4	Missense	c.1799T>A	p.V600E	Heterozygous	Deleterious	Probably damaging	Pathogenic	COSV56056643	rs113488022	23.3	2170
			*TERTp**	5	NM_198253.2	Regulatory region variant	c.1-124C>T	-	Heterozygous	-	-	Pathogenic	-	rs124253815	-	-
			PIK3CA	3	NM_006218.3	Missense	c.1633G>A	p.E545K	Heterozygous	Deleterious	Probably damaging	Pathogenic	COSV55873239	rs104886003	22.5	3208
			TP53	17	NM_000546.5	Stop gained variant	c.853G>T	p.E285ter	Heterozygous	-	-	Pathogenic	COSV52670664	rs786201059	30	1510
			MAP2K1	15	NM_002755.3	Missense	c.358G>C	p.E120Q	Heterozygous	Deleterious	Possibly damaging	Pathogenic	-	-	25.3	3894
P3	a (pre-DT)	ATC	*BRAF*	7	NM_004333.4	Missense	c.1799T>A	p.V600E	Heterozygous	Deleterious	Probably damaging	Pathogenic	COSV56056643	rs113488022	20.6	3802
			*TERTp**	5	NM_198253.2	Regulatory region variant	c.1-124C>T	-	Heterozygous	-	-	Pathogenic	-	rs124253815	-	-
	b (post-DT)		*BRAF*	7	NM_004333.4	Missense	c.1799T>A	p.V600E	Heterozygous	Deleterious	Probably damaging	Pathogenic	COSV56056643	rs113488022	58	3546
			*TERTp**	5	NM_198253.2	Regulatory region variant	c.1-124C>T	-	Heterozygous	-	-	Pathogenic	-	rs124253815	-	-
			NRAS	1	NM_002524.4	Missense	c.181C>A	p.Q61K	Heterozygous	Deleterious	Probably damaging	Pathogenic	COSV54736310	rs121913254	21	5174
P4	a (pre-DT)	ATC	*BRAF*	7	NM_004333.4	Missense	c.1799T>A	p.V600E	Heterozygous	Deleterious	Probably damaging	Pathogenic	COSV56056643	rs113488022	19.4	5519
			*TERTp**	5	NM_198253.2	Regulatory region variant	c.1-124C>T	-	Heterozygous	-	-	Pathogenic	-	rs124253815	-	-
			PIK3CA	3	NM_006218.3	Missense	c.1633G>A	p.E545K	Heterozygous	Deleterious	Probably damaging	Pathogenic	COSV55873239	rs104886003	23.2	7848
			TP53	17	NM_000546.5	Missense	c.396G>C	p.K132N	Heterozygous	Deleterious	Probably damaging	Pathogenic	COSV52688381	rs866775781	20.8	6220
	b (post-DT)		*BRAF*	7	NM_004333.4	Missense	c.1799T>A	p.V600E	Heterozygous	Deleterious	Probably damaging	Pathogenic	COSV56056643	rs113488022	24.4	3660
			*TERTp**	5	NM_198253.2	Regulatory region variant	c.1-124C>T	-	Heterozygous	-	-	Pathogenic	-	rs124253815	-	-

			PIK3CA	3	NM_006218.3	Missense	c.1633G>A	p.E545K	Heterozygous	Deleterious	Probably damaging	Pathogenic	COSV55873239	rs104886003	29.6	6149
			TP53	17	NM_000546.5	Missense	c.396G>C	p.K132N	Heterozygous	Deleterious	Probably damaging	Pathogenic	COSV52688381	rs866775781	33.2	3374
P5 (pre-DT)		ATC	*BRAF*	7	NM_004333.4	Missense	c.1799T>A	p.V600E	Heterozygous	Deleterious	Probably damaging	Pathogenic	COSV56056643	rs113488022	33.5	8053
			*TERTp**	5	NM_198253.2	Regulatory region variant	c.1-124C>T	-	Heterozygous	-	-	Pathogenic	-	rs124253815	-	-
			TP53	17	NM_000546.5	Missense	c.524G>A	p.R175H	Heterozygous	Deleterious	Probably damaging	Pathogenic	COSV52661038	rs28934578	39.5	3338
P6 (pre-DT)		ATC	*BRAF*	7	NM_004333.4	Missense	c.1799T>A	p.V600E	Heterozygous	Deleterious	Probably damaging	Pathogenic	COSV56056643	rs113488022	4.62	4304
			*TERTp**	5	NM_198253.2	Regulatory region variant	c.1-124C>T	-	Heterozygous	-	-	Pathogenic	-	rs124253815	-	-
			PIK3CA	3	NM_006218.3	Missense	c.3140A>G	p.H1047R	Heterozygous	Deleterious	Probably damaging	Pathogenic	COSV55873195	rs121913279	4.05	5282
P7 (pre-DT)		ATC	*BRAF*	7	M_004333.4	Missense	c.1799T>A	p.V600E	Heterozygous	Deleterious	Probably damaging	Pathogenic	COSV56056643	rs113488022	19	1681
			*TERTp**	5	NM_198253.2	Regulatory region variant	c.1-146C>T	-	Heterozygous	-	-	Pathogenic	-	rs156121 5364	-	-
P8 (Pre-DT)		ATC	*BRAF*	7	NM_004333.4	Missense	c.1799T>A	p.V600E	Heterozygous	Deleterious	Probably damaging	Pathogenic	COSV56056643	rs113488022	50.9	3393
			*TERTp**	5	NM_198253.2	Regulatory region variant	c.1-124C>T	-	Heterozygous	-	-	Pathogenic	-	rs124253815	-	-
			TP53	17	NM_000546.5	Insertion-frameshift variant	c.876dupA	p.G293RfsTer13	Heterozygous	-	-	Pathogenic	COSV52686603	-	71.9	1506
P9 (pre-DT)		ATC	*BRAF*	7	NM_004333.4	Missense	c.1799T>A	p.V600E	Heterozygous	Deleterious	Probably damaging	Pathogenic	COSV56056643	rs113488022	26.7	3799
			*TERTp**	5	NM_198253.2	Regulatory region variant	c.1-124C>T	-	Heterozygous	-	-	Pathogenic	-	rs124253815	-	-
			MTOR	1	NM_004958.4	Missense	c.4376C>A	p.A1459D	Heterozygous	Deleterious	Benign	Pathogenic	COSV63868170	-	15.1	3007
			TP53	17	NM_000546.5	Missense	c.722C>G	p.S241C	Heterozygous	Deleterious	Probably damaging	Pathogenic	COSV52662386	rs28934573	18	4117

*Sanger Sequencing; Chr – chromosome; HGVSC – DNA change; HGSVP – Protein change; VAF – variant allele frequency

4.1.2. Discussion

Subbiah *et al*. described for the first time in 2018 that, in a clinical trial with ATC patients, harboring *BRAF* p.V600E mutation, DT therapy increased the overall survival of the patients. According to this study, dabrafenib plus trametinib (DT) demonstrated a high response rate, a prolonged duration of response and a prolonged survival, with manageable drug toxicity, in *BRAF* p.V600E-positive ATCs (Subbiah *et al.*, 2018). Despite these encouraging results, being a TKI targeted therapy, DT is susceptible to resistance mechanisms. There are two main types of resistance: primary/intrinsic resistance, in which no clinical benefit is achieved during therapy, due to the presence of resistant clones, harboring alterations such as RAS-related C3 botulinum toxin substrate 1 *(RAC1*) mutations, loss of *PTEN* and mutations on neurofibromatosis type 1 (*NF1*); and secondary/acquired resistance, which derive from the selective pressure of the targeted therapy (Manzano *et al.*, 2016), as described in melanoma. Resistance mechanisms can also be divided in two subsets: on-target and off-target. On-target resistance mechanisms include mutations and genomic alterations on *BRAF* or *MAP2K1/2* genes, in protein regions that encode for the kinase domains, and affect the binding of the inhibitors to their specific targets, as described in DTC, ATC and melanoma (Porter *et al.*, 2021; Emery *et al.*, 2009; Manzano *et al.*, 2016). Kinase domain mutations occur in the following major protein regions: solvent front, gatekeeper residue, G-loop, A-loop (xDFG motif). In melanoma, Proietti *et al.* found that *BRAF* splice variants and amplifications could also be possible on-target resistance mechanisms (Proietti *et al.*, 2020). Also, BRAF dimerization, epigenetic or overexpression of the targeted protein have been considered on-target resistance mechanisms (Barouch-Bentov *et al.*, 2011). *MEK1/2* genes mutations are the most known source of MEK inhibitors resistance (Wu *et al.*, 2015; Emery *et al.*, 2009). Off-target resistance mechanisms include mutations and other genomic alterations, such as epigenetic, in other RTKs or downstream mediators of MAPK and PI3K/AKT/mTOR signaling pathways, decreasing the BRAF signaling dependency (Drilon *et al.*, 2019; Khaliq *et al.*, 2019). Cabanillas *et al*. described multiple mechanisms involving BRAF off-target resistance in *BRAF* p.V600E-positive ATCs, in which *RAS* mutations and *RAS* amplifications play a critical role (Cabanillas *et al.*, 2020). *PIK3CA* mutations could also be involved, as described in melanoma (Irvine *et al.*, 2018). Khaliq *et al.* discovered that overexpression of RTK due to methylation in melanoma, provided BRAF inhibitor resistance. There are other types of genomic alterations that could induce DT resistance (Khaliq *et al.*, 2019). Therefore, further studies are needed, in order to identify targets and therapeutics to overcome resistance mechanisms. Sanger sequencing and NGS analysis were performed in #11 tumour samples, from nine ATC cases, including #2 from patients with macroscopic disease progression under DT, to investigate which genes and molecular alterations were involved in response/resistance to DT.

4.1.2.1. Influence of *BRAF* p.V600E and *TERTp* in DT efficacy

BRAF (OMIM ***** 164757) encodes for a serine/threonine kinase, that is present upstream in the MAPK signaling pathway, which is involved in the regulation of cell division, proliferation and differentiation (Nikiforov *et al.*, 2011). BRAF is the predominant RAF protein that is altered in many types of cancers, such as TC (60%), melanoma (60%), colorectal cancer (15%), and non-small cell lung cancer (NSCLC) (5-8%) (Leicht *et al.*, 2007). In ATCs, as mentioned before, it is altered in 41 to 45% of the cases (Cabanillas *et al.*, 2020; Landa *et al.*, 2016). *BRAF* p.V600E is the most common mutation in many cancers, thus, targeting this alteration became one of the most important therapeutic approaches, as referred above (Zaman *et al.*, 2019).
In the present study, all the patients (#9) had a *BRAF* p.V600E mutation in #9 pre-DT samples, and in the #2 post-DT samples that were available to study (P3 and P4).

The telomerase is a ribonucleoprotein enzyme complex that synthesizes telomeric DNA to counter telomere shortening, inhibiting the hayflick limit (HL), allowing the maintenance of cell proliferation, being considered an attractive target of cancer therapy (Guterres *et al*., 2020). To inhibit the HL, 90% of cancers activate the expression of *TERT* (OMIM ***** 187270), which encodes for telomerase reverse transcriptase. This is achieved because of *TERTp* mutations (TPM) that facilitate the binding of specific E26 transformation-specific transcription factors (ETS-TF) to the promotor region (Sizemore *et al*., 2017; Shi *et al*., 2015). Reactivation of *TERT* is linked to several cancer hallmarks such as the resistance to anti-growth signals, angiogenesis, resistance to apoptosis, metastasis, reprogramming of the energy metabolism and genomic instability (Liu *et al*., 2016; Hanahan *et al*., 2011). The most common TPMs in human cancers are C228T (c.1-124C>T) and C250T (c.1-146C>T) (Huang *et al*., 2015; Melo *et al*., 2014), corresponding to the positions 124 and 146 bp, respectively, upstream of the transcription start site (Liu *et al*., 2018). The association between C228T/C250T and *BRAF* p.V600E has been considered high risk factor for ATC and other TC, promoting high aggressiveness, metastasis, advanced tumour stage, and high mortality (Shi *et al*., 2015). However, this association also provides an "Achilles heel" to the DT therapy (Tan *et al*., 2020): *in vitro* and *in vivo* studies showed that, when there is this genetic "duet", DT induces apoptosis of the ATC cells, and decreases the tumour size, compared with ATCs that only have *BRAF* p.V600E (Tan *et al*., 2020). The nine ATC patients from the present study, all had the presence of the C228T or C250T mutation in the *TERTp* and *BRAF* p.V600E. Thus, these results confirm that the coexistence of *BRAF* and TPM correlates with ATC aggressive phenotype, and with a good initial response to DT. In addition, *TERT* could be considered a possible target for anti-telomerase therapies, such as *TERT* peptide vaccines, adoptive cell transfer, oncolytic virotherapy, direct telomerase inhibitors and indirect telomerase inhibitors (Guterres *et al*., 2020). A germline polymorphism c.1-245T>C (rs2853669), a regulatory SNP, positioned in the 245 bp position upstream of the transcription start site of *TERT*, was also detected in P2, P4, P6, P7, P8 and P9 patients. Reports about rs2853669 put forward that this polymorphism (C allele) combined with TPMs was a novel risk factor for poor prognosis in glioblastoma, FTC and cervical cancer (Batista *et al*., 2016; Hirokawa *et al*., 2020; Vinothkumar *et al*., 2020). In both cervical cancer and hepatocellular cancer, Vinothkumar *et al*. and Ko *et al*., respectively, reported that the association between TPMs and rs2853669, promotes a higher expression of *TERT*, in which rs2853669 blocks the association of *TERT* inhibitory transcription factors (Vinothkumar *et al*., 2020; Ko *et al*., 2016) and lead to a more aggressive phenotype, which also could justify the higher aggressiveness of ATC. Although, since it increases *TERT* expression, it could also be postulated that it should increase DT efficacy. Conversely, a meta-analysis done by Shen *et al*. verified that, in melanoma, patients carrying the wild type T allele had a worst prognosis, when compared with patients carrying the polymorphism (Shen *et al*., 2017a). These reports suggest that there is not a unique correlation between rs2853669 and the prognosis for all cancers. However, the present ATC series is too small to investigate a potential correlation between this polymorphism and ATC patients prognosis.

4.1.2.2. *MAP2K1*

In patient 2 (P2), a novel likely pathogenic missense variant in the gene *MAP2K1*, p.E120Q, was detected but it has not been described nor in COSMIC nor in Ensembl genome browser databases (Table 4.2). *MAP2K1* (OMIM ***** 176872), encodes for the serine/threonine kinases, MEK1, present downstream in the MAPK signaling pathway (Nikiforov *et al*., 2011). Although *MAP2K1* is rarely mutated in cancer, there are specific mutations that are sufficient to induce carcinogenesis, which demonstrates pivotal roles of this gene in malignant transformation, being considered a possible target for targeted therapeutics, such as trametinib (Wu *et al*., 2015). MEK1 has unique characteristics that improve development of therapeutics based on MEK1/2 inhibition. First, this protein has a narrow substrate specificity, thus this inhibition shuts off ERK1/2 signaling without affecting other signaling pathways

(Wu *et al.*, 2015). Then, because it has a unique structural protein conformation, the use of selective adenosine triphosphate (ATP)-noncompetitive inhibitors, such as trametinib, changes the conformation of the catalytic domain, inactivating the protein (Fischmann *et al.*, 2009). Multiple mutations in this gene have been reported, and some are related with MEK inhibitor resistance. Indeed, Zhu *et al.* demonstrated that *MAP2K1* mutations, p.P124S (p.Pro124Ser | rs1057519732) and p.E203K (p.Glu203Lys | rs1057519733), located in the protein kinase domain, may attenuate MEK inhibitor responses. On the contrary, mutations not located in this domain, such as p.A52V (p.Ala52Val | rs147489724), do not appear to affect MEK inhibitor responses (Zhu *et al.*, 2020). It could be speculated that p.E120Q mutation could also decrease trametinib efficacy, since codon 120 is also located in the protein kinase domain (Hu *et al.*, 2021). However, it is noteworthy that this mutation has not been reported, and patient 2 is, until the present time, responding well to DT.

4.1.2.3. *NRAS* p.Q61K as a DT resistance mechanism

In patient 3 (P3), the *NRAS* p.Q61K (p.Gln61Lys | rs121913254) missense mutation was detected only in the post-DT tumour sample (P3b), and it is likely associated with disease progression and DT resistance (Table 4.2). This mutation is a hotspot mutation, which lies within the GTP-binding region, conferring a gain-of-function to the NRAS protein (Li *et al.*, 2012). *NRAS* (OMIM * 164790) encodes for a GTPase, present upstream in the MAPK signaling pathway. Until recently, mutations in *RAS* and *BRAF* were reported as being mutually exclusive, in TC and melanoma (Sensi *et al.*, 2006; Cisowski *et al.*, 2017; Danysh *et al.*, 2016), but other reports found concomitant mutations in these two genes in advanced stages of these diseases (Raaijmakers *et al.*, 2016). These double mutational events are due to the heterogeneity of the tumours and emergence of specific clones, that present an acquired resistance to drug therapy. Raaijmakers *et al.* demonstrated that *NRAS* mutations can co-occur with *BRAF* mutations in melanoma cells that are being treated with Vemurafenib, a BRAF inhibitor (Raaijmakers *et al.*, 2016). Therefore, it appears that the selective pressure provided by the drugs will favor the emergence of more aggressive tumour cell sub-clones, through the acquisition of new specific *RAS* mutations, that lead to off-target events of resistance, such as a reactivation of ERK pathway, a constitutive activation of RTK, activation of the PI3K/AKT signaling pathway, and/or overexpression of epidermal growth factor receptor (*EGFR)* and/or *VEGFR* (Tian *et al.*, 2020; Cabanillas *et al.*, 2020; Porter *et al.*, 2021). In the specific case of patient 3, it appears that *NRAS* p.Q61K mutation is an acquired mutation, since it was not detected in the pre-DT sample (P3a), likely associated with an acquired mechanism of off-target resistance to BRAF inhibitors, therefore, reducing DT therapy response. It is thus crucial to identify alternative therapies for these cases of resistance to DT. It was found in PanDrugs database and in Genomics of Drug Sensitivity in Cancer database, that for the mutational profile of this patient (*BRAF* p.V600E + *NRAS* p.Q61K), lenvatinib, a multikinase inhibitor, is, so far, the only drug approved by the FDA in thyroid cancer that could be potentially used as treatment (Figure 4.3). Since this mutation promotes the overexpression of *EGFR* and *VEGFR*, and activation of PI3K/AKT/mTOR, the use of specific inhibitors for each case could be a possibility (Porter *et al.*, 2021).

4.1.2.4. *PIK3CA* mutations in DT resistance

In patients 2, (P2), 4 (P4) and 6 (P6) were detected two different *PIK3CA* missense mutations: in P2 and P4 was detected the *PIK3CA* p.E545K (p.Glu545Lys | rs104886003); and in P6 was detected the *PIK3CA* p.H1047R (p.His1047Arg | rs121913279) (Table 4.2). *PIK3CA* (OMIM * 171834) encodes for the p110 alpha subunit (p110α) of PI3K (Samuels *et al.*, 2004; Baker *et al.*, 2012). Like mentioned in the introduction, PI3K signaling is important for proliferation, migration, transport of materials within cells, and cell survival (Janku *et al.*, 2017). This pathway could be activated directly by RTK or by RAS

phosphorylation (Fior, 2019). *In vivo* mice studies done by Charles *et al*. demonstrated that *PIK3CA* mutations and *BRAF* p.V600E concomitance, promoted ATC carcinogenesis with a more aggressive pattern (Charles *et al*., 2014). *In vitro* and *in vivo* studies developed by Irvine *et al*. showed that PI3K/AKT signaling, through *PIK3CA* and *AKT* mutants, enables the survival of a dormant population of MAPK-inhibited melanoma cells, which are later associated with MAPK reactivation, and may lead to the evolution of tumour subclones highly resistant to MAPK inhibitors (Irvine *et al*., 2018). Also, some studies verified that *PIK3CA* p.E545K provides a resistance against MEK inhibitors in colorectal cancer cells (Tsubaki *et al*., 2019).

In line with that study, in patients P4 (P4a and P4b) and P6, it appears that *PIK3CA* mutations could be selecting cell clones, that are resistant to BRAF inhibitors. However, P2 also had a *PIK3CA* mutation, but did not present so far resistance to DT. Loss of *PTEN* in *PIK3CA* mutated melanoma, appears to be correlated with complete or partial responses to BRAF inhibitors, that could justify the differences between P2, P4 and P6 (Catalanotti *et al*., 2017). Nonetheless, in cases with this possible clone selection resistance mechanism, it is necessary to search for alternative therapeutic approaches. For these patients' mutations profiles, P2/P4 with *BRAF* p.V600E + *PIK3CA* p.E545K, and P6 with *BRAF* p.V600E + *PIK3CA* p.H1047R, everolimus would be a possible drug to treat ATC, although it is still not approved for TC (Figure 4.3). Everolimus, a rapamycin analogue, is an allosteric inhibitor of mTOR, which binds to the mTOR complex 1 inhibiting its activation by AKT-mediated phosphorylation (Harris *et al*., 2019). It demonstrated a manageable toxicity profile in phase II clinical trials and may be a suitable treatment for these specific cases (Hanna *et al*., 2018). Three phase II clinical trials with everolimus, included fewer than 10 patients with ATC (Bible *et al*., 2021) and none had more than one responder. However, in two patients, from two of these trials, had a dramatic response to everolimus. In a clinical trial with 2 ATC patients, Hanna *et al.* observed that everolimus increased the median progression of free survival in 2 months (from 15.2 to 17.9 months) in one patient, and the other had stable disease for 26 months (Hanna *et al*., 2018). It appears that everolimus has a significant antitumour activity in ATC harboring PI3K/AKT/mTOR pathway mutations. Although, according with the 2021 American Thyroid Association Guidelines for Management of Patients with Anaplastic Thyroid Cancer, a larger trial with more selected ATC patients is required to further investigate the antitumour and safety effects of everolimus in these cases (Bible *et al*., 2021).

4.1.2.5. *MTOR* mutations in DT efficacy

Mutation profiling of P9 revealed a *MTOR* missense mutation: *MTOR* p.A1459D (p.Ala1459Asp | rs28934573) (Table 4.2). *MTOR* (OMIM * 601231) encodes for a downstream protein kinase (mTOR) of PI3K/AKT/mTOR pathway. This protein is usually assembled into several complexes, such as mTORC1/2, in cooperation with raptor, rictor, and other mTORC1/2's key components. mTOR catalyzes the phosphorylation of multiple downstream targets such as the eukaryotic translation initiation factor 4E binding protein, and type-I insulin like growth factor receptor (Yin *et al*., 2016). Many mTOR inhibitors have been developed for targeting mTOR in order to decrease the activation of PI3K pathway targets. Mutations in *MTOR* have been reported to lead to an acquired off-target resistance to EGFR kinase inhibitors, in *EGFR*-mutant lung cancers (Yu *et al*., 2018). In the case of TC, specifically in ATCs, there are no reports regarding DT efficacy in the context of *MTOR* mutations, however, these mutations are known to be associated with the activation of PI3K pathway, decreasing cell apoptosis and increasing cell proliferation (Nikiforov *et al*., 2011). In PI3K/AKT/mTOR pathway, mTOR is a preferential target to overcome BRAF inhibitor acquired resistance derived from mutations in PI3K/AKT/mTOR pathway (Caporali *et al*., 2016). When analyzing our results, it does not appear that *MTOR* p.A1459D is affecting DT response since P9, until the present time, has been showing an excellent response to this combined therapy. However, drug prescription for this patient's mutation

profile, according with PanDrugs and Genomics of Drug Sensitivity in Cancer databases, consists in the use of Everolimus (Figure 4.3).

4.1.2.6. *TP53* mutations in DT efficacy

In patients 2 (P2), 4 (P4), 5 (P5), 8 (P8) and 9 (P9) five different *TP53* missense mutations were detected: in P2, the stop gained variant/mutation *TP53* p.E285* (p.Glu285ter); in P4, the missense *TP53* p.K132N (p.Lys132Asn | rs866775781); in P6, the hotspot missense *TP53* p.R175H (p.Arg175His | rs28934578); in P8, an insertion mutation that causes a frameshift (fs), *TP53* p.G293Rfs*13 (p.Gly293ArgfsTer13); and in P9, the missense *TP53* p.S241C (p.Ser241Cys | rs28934573) (Table 4.2). *TP53* (OMIM * 191170) encodes for p53 protein. This gene is associated with multiple anti-tumour mechanisms, and it acts on both transcriptional dependent and independent pathways, playing a critical role in cell signaling, cell cycle arrest, cell senescence, apoptosis, double strand break and single strand break DNA repair, and cell differentiation (Levine *et al.*, 2009; Gupta *et al.*, 2019). Normal function of p53 is altered in more than 50% of the cancers, being inactive by specific mutations, specifically by those that are present in the DNA binding domain (DBD) or lead to the deletion of the carboxy-terminal domain (Soussi *et al.*, 2007). Mutations on *TP53* are classified according with their effects in p53 functionality: loss-of-function, partial function, gain-of-function, and dominant negative effect (Monti *et al.*, 2020). Mutations such as the p.K132N, often lead to the expression of a protein with a dominant negative effect that prevails over the expression of wild type p53. Other mutations lead to a gain- (p.R175H) or a loss-of-function (p.E285*; p.G293Rfs*13; p.S241C) (Jordan *et al.*, 2010; Monti *et al.*, 2020). These mutations promote the ability of p53 to activate or repress the transcription of multiple types of genes or influence the binding to new proteins, that may dysregulate several signaling pathways (Muller *et al.*, 2013). Moreover, since *TP53* is associated with DNA repair, mutations on this gene lead to an increase of the genomic instability, leading to an accumulation of mutations (Muller *et al.*, 2013). In P2 and P5's ATCs, this mutation does not appear to be associated with the response to therapy, because these patients are responding well to DT. In P4, DT efficacy appears to be associated with mutated *PIK3CA* and not with *TP53*. Although, reports on this mutation verified that this one could be a driver for the transformation of FTC and PTC into ATC cells (Manzella *et al.*, 2017) being associated with ATC transformation and progression. Also, since genomic instability is very reflected in ATC cells, these mutations could also be associated with this characteristic (Salvatore *et al.*, 2007). However, this number of cases is still insufficient to put forward any hypothesis regarding *TP53* mutations and therapy response.

4.1.2.7. Overview of the targeted therapies in ATC

Figure 4.3 provides a better visualization of the specific drugs mentioned in this section, and their specific target proteins. In the present study, mutation profiling of ATC patients has provided data that will contribute to further understand the molecular mechanisms involved in DT response and resistance. Overall, *RAS* activation was confirmed as mechanism of resistance. In addition, the detection of *PIK3CA* mutations, in two cases with DT resistance, unveils PI3K/AKT/mTOR activation as another possible resistance mechanism in thyroid, as previously shown in melanoma. Besides the genes that are described in the previous sections, there are other genes and mutations that could affect BRAF inhibitor response. One example is the reported acquired mutation P34R (p.Pro34Arg) in *RAC1*, in PTC, that promotes resistance to dabrafenib (Bagheri-Yarmand *et al.*, 2021). In the cases that developed resistance to DT, it will be mandatory to search for new therapeutic approaches, to further improve overall survival of the patients with *BRAF* p.V600E-positive ATCs. The major limitation of this study was the small sample size, which is explained by ATC rarity and the recent use of DT in these cases. In addition, there was only access to post-DT samples in 2/3 cases of resistance due to the other patients not presenting disease

progression (P1, P2, P5, P6, P7, P9) or the inaccessibility of the post-DT sample (P8). Also, our NGS panel did not include the analysis of epigenetic modifications, *BRAF* expression, *BRAF* splice variants, *PTEN* losses, and *RAC1* mutations, which are additional resistance mechanisms to DT. Subsequent studies, comprising these possible resistance mechanisms, will be important to further understand the processes involved in DT efficacy.

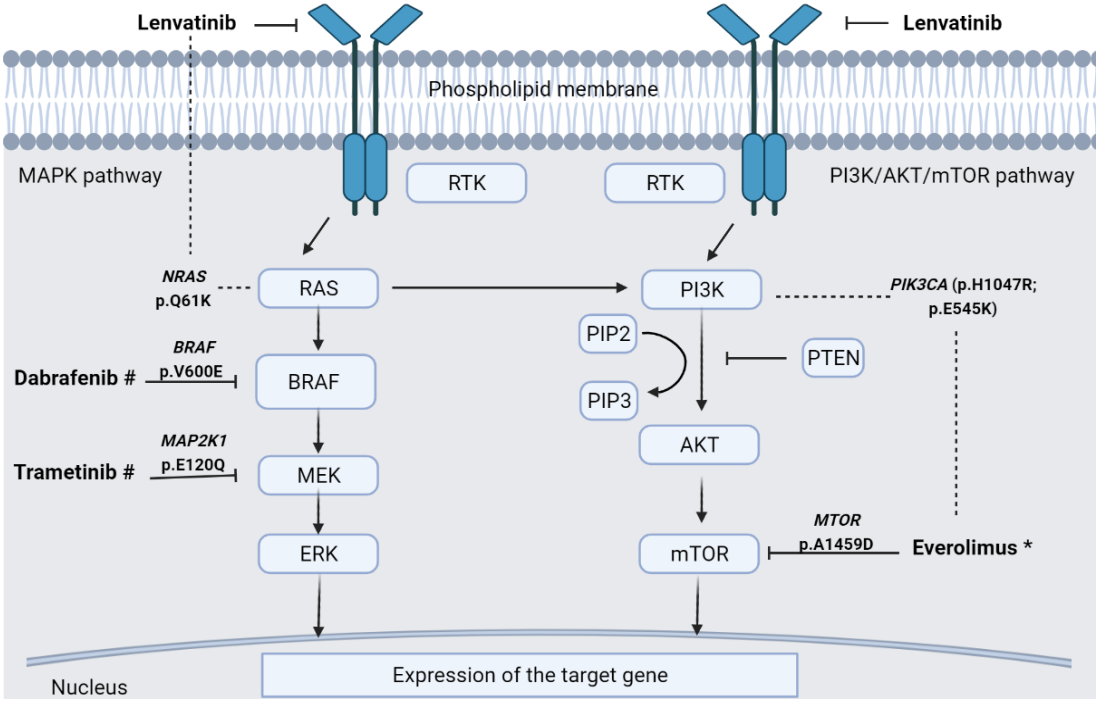

Figure 4.3. MAPK and PI3K/AKT/mTOR pathways' molecular targets for targeted therapies. MAPK and PI3K/AKT/mTOR pathways are modulated by a stimulus that activates a phosphorylation cascade signaling pathway which modulates gene expression. Drugs are used to inhibit (——|) the activation of specific proteins in order to inhibit the expression of cancer related genes. Combinations of drugs could provide a better response and avoid resistance mechanisms. * not approved by the FDA for ATC; # drugs used in combination. Arrows (——>) represent activation. Dot line (·····) means that a targeted therapy can be used as treatment in the presence of those mutations. Scheme created in BioRender.com.

4.2. The role of *SPRY4* in ATC-TAM crosstalk and ATC cells' aggressiveness

4.2.1. Results

4.2.1.1. Assessment of SPRY4 protein levels by western blot in monocultures and co-cultures of ATC

In previous proteomic studies (Supplementary Figure 1) we observed that SPRY4 was upregulated in C3948 co-cultures (in contact with THP-1) and downregulated in T235 co-cultures with comparing with the respective monoculture (without contact with THP-1). In the present study, we performed 2D transwell monoculture and co-culture experiments of T235 and C3948 with THP-1 differentiated macrophages using an anti-SPRY4 monoclonal antibody, in order to validate the previous proteomic results. Semi-quantification verified that there was an increased expression of SPRY4 in the C3948 co-cultures and a significant decrease of SPRY4 expression in the T235 cells ($p < 0.05$), when comparing with respective monoculture (Figure 4.4). In a parallel study, using an anti-SPRY4 polyclonal antibody, it was possible to observe the same trend ($p < 0.01$) in SPRY4 expression, although the relative increase in the expression seemed to be higher in the C3948 co-cultures (Supplementary Figure 3). *SPRY4* has three protein coding transcripts and, consequently, three protein isoforms (5, 33, 35 KDa) (Ensembl

genome browser database). In the C3948 and T235 cultures, using the polyclonal antibody it was possible to detect the two SPRY4 isoforms (33 and 35 kDa). The detection of the two isoforms might be important for subsequent analyses, to further understand their role in ATC-TAM interactions. Taken together, western blot results were consistent with the previous proteomic studies

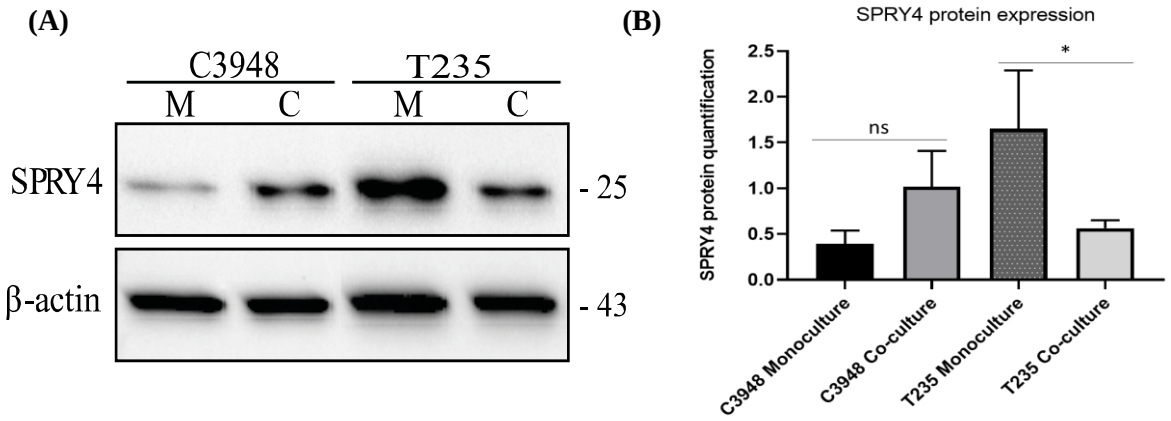

Figure 4.4. Western blot analysis of SPRY4 in ATC cells in monoculture and co-culture with a monoclonal antibody. Ten µg of protein were applied per lane of 12% Bis-Tris gels and transferred to PVDF membranes. SPRY4 was detected by a rabbit monoclonal anti-SPRY4 antibody and β-actin by a mouse monoclonal anti-β-actin antibody. (A) Western blot of representative assays of C3948 and T235. SPRY4 is represented with a molecular weight of 25 kDa, and b-actin with 43 kDa. (B) Semi-quantification of SPRY4 in co-culture and monocultures normalized by β-actin. Data are mean ± SD from three independent experiments. M, monoculture; C, co-culture. Data were analysed using the parametric unpaired T test (* $p < 0.05$, ns - no significance).

4.2.1.2. *SPRY4* siRNA mediated silencing in ATC cell lines

To further elucidate the role of *SPRY4* in ATC, siRNA-mediated gene silencing of *SPRY4* was performed in the two ATC cells lines previously used (T235 and C3948).

Firstly, distinct siRNA concentrations and timepoints were tested, to identify those leading to > 50% *SPRY4* silencing. Optimizations were described in section 3.9.1 being performed using concentrations between 12.5 nM and 50 nM of siRNA and timepoints of 48h and 72h. Non-targeted pool siRNA (siNT) was used in the same concentration as transfection control, in order to evaluate the success of the silencing. Parental cells (without siRNA) were also used. There were similar results between siNT and parental cells, thus only siNT were represented in Figure 4.5. *SPRY4* was silenced more effectively in both cell lines at 72h, having C3948, with 12.5 nM, an average expression reduction of 53% at 48h and 59% at 72h (Figure 4.5A), and T235, with 37.5 nM, a reduction of 59% at 48h and 64% at 72h (Figure 4.5B), assessed by qRT-PCR. Therefore, the 72h timepoint with 12.5 nM for C3948 and 37.5 nM for T235, was used in subsequent *SPRY4* siRNA-mediated silencing experiments.

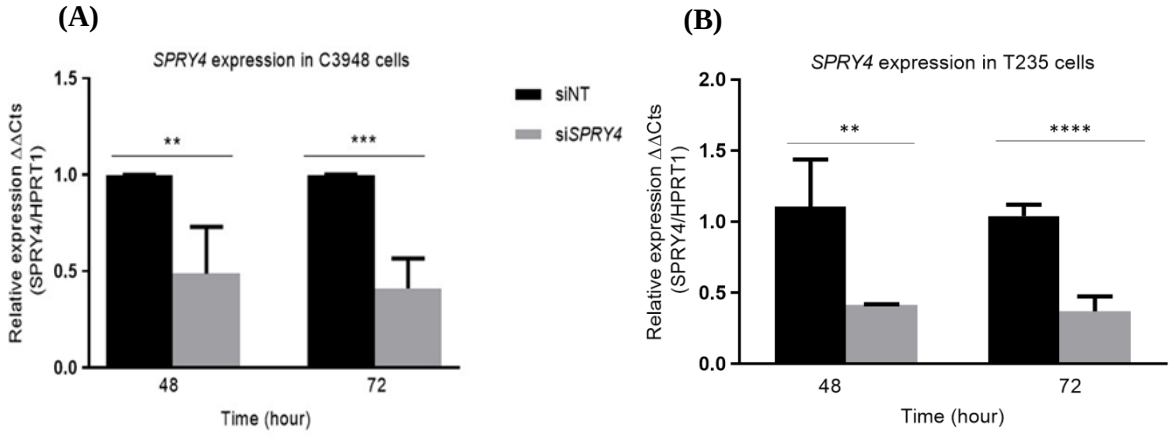

Figure 4.5. Effect of siRNA mediated silencing in *SPRY4* gene expression in ATC cells. (A) The silencing of *SPRY4* in C3948 cell line with 12.5 nM of siRNA resulted in an average reduction of 53% at 48h and 58% at 72h in RNA expression levels (analysed by qRT-PCR). (B) The silencing of *SPRY4* in T235 cell line with 37.5 nM of siRNA resulted in an average reduction of 59% at 48h and 64% at 72h in RNA expression levels (analysed by qRT-PCR). As a transfection control, a non-targeting negative control siRNA (siNT) was used. The bars represent the mean and standard deviation (SD) from four independent assays. Data were analysed using the parametric unpaired T test (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

4.2.1.3. Effects of *SPRY4* silencing in ATC cells' viability

Since the effect of *SPRY4* gene silencing in ATC cell viability has never been reported, and its role as TSG or oncogene has not been elucidated in this context, the silencing of *SPRY4* in T235 and C3948 cells viability was analyzed using siRNA, under the previously established conditions that provided acceptable levels of silencing. Viability was assessed by cell counting, using trypan blue exclusion assay. There were no significant differences in the case of the C3948 cell line (Figure 4.6A), and a significant decrease of T235 cells viability with *SPRY4* silencing ($p < 0.01$) (Figure 4.6B) compared with the control (siNT).

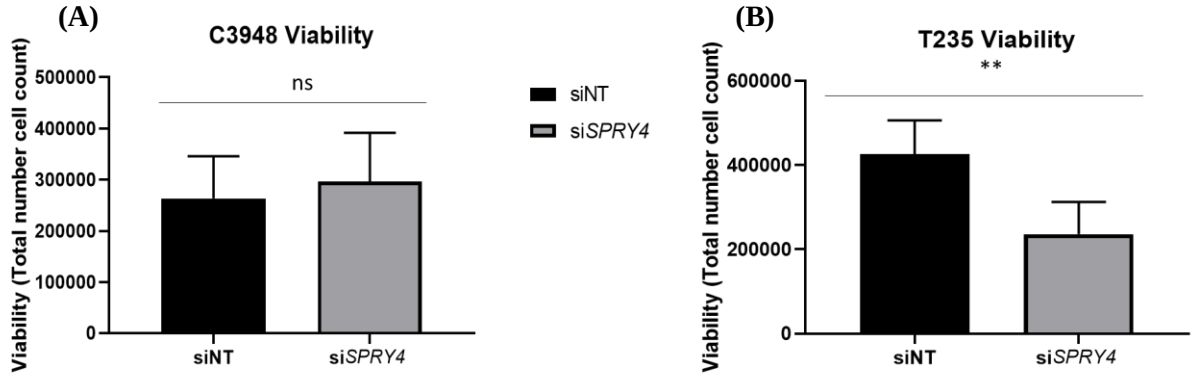

Figure 4.6. Effect of *SPRY4* silencing in ATC cells' viability. (A) Viability of C3948 cell lines post *SPRY4* silencing . (B) Viability of T235 cells post *SPRY4* silencing. As a transfection control, a non-targeting negative control siRNA (siNT) was used. The bars represent the mean and standard deviation (SD) from five independent assays. Data were analysed using the parametric unpaired T test (** $p < 0.01$, ns - no significance).

4.2.1.4. *SPRY4* silencing modulates the invasion capability of ATC cells in monocultures and co-cultures

The effect of silencing *SPRY4* in T235 and C3948 cells invasion capability was analyzed using siRNA, under the conditions that provided acceptable levels of silencing. For the invasion assays, 2D transwell monocultures and co-cultures were performed, using invasion matrigel based inserts. The invasion ability of C3948 cells, in the control conditions (siNT), significantly decreased in co-culture (fold change (FC) $p < 0.01$) (Figure 4.7A, B, E), but the opposite effect was observed in *SPRY4* silenced cells, which significantly increased invasion (FC $p < 0.05$). The invasion ability of T235 cells, in the control conditions (siNT), significantly increased in co-culture (FC $p < 0.001$) (Figure 4.7C, D, F), and this increase was enhanced in the *SPRY4* silenced cells (FC $p < 0.001$). Also, it is noteworthy that in T235 cells (Figure 4.7C) *SPRY4* silencing, promoted a significant increase in the total number of invasive cells in co-culture, compared with all the remaining conditions [(si*SPRY4* co-culture *vs* siNT monoculture – $p < 0.01$), (si*SPRY4* co-culture *vs* siNT co-culture – $p < 0.05$), (si*SPRY4* co-culture *vs* si*SPRY4* monoculture – $p < 0.05$)]. In C3948 cells, *SPRY4* silencing promoted an increase of the total number of invasive cells in monoculture and co-culture when comparing with siNT conditions (Figure 4.7A) although it was only statistically significant in si*SPRY4* co-culture *vs* siNT co-culture ($p < 0.05$).

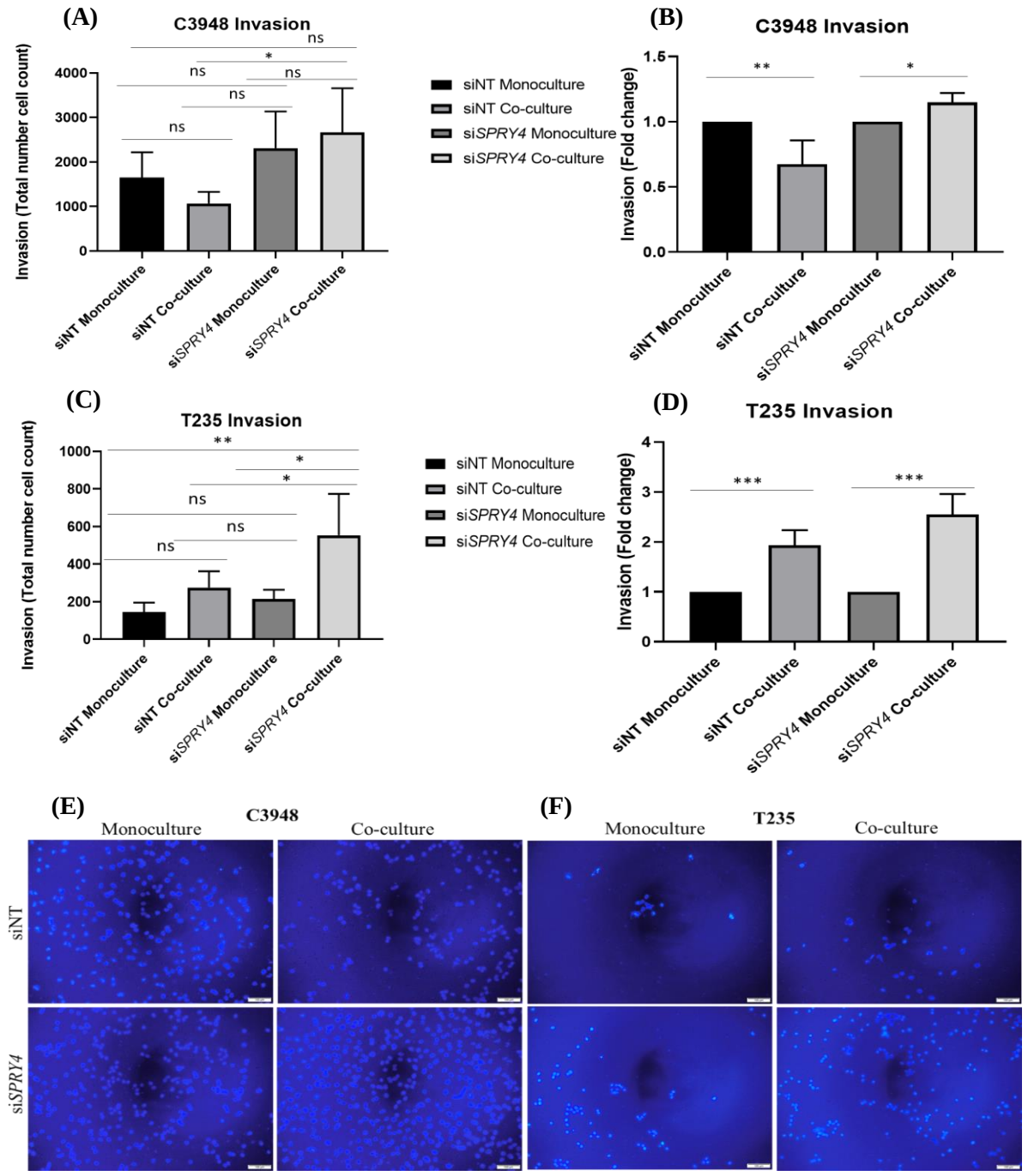

Figure 4.7. Effect of *SPRY4* silencing and THP-1 differentiated macrophages in ATC invasion capability, in monoculture and co-culture, using matrigel based inserts and DAPI staining. Invasion ability of C3948 cells, in mono and co-culture, in control (siNT) and silenced (si*SPRY4*) conditions: total number cell count (A) and fold change (B). Invasion ability of T235 cells, in monocultures and co-culture, in control (siNT) and silenced (si*SPRY4*) conditions: total number cell count (C) and fold change (D). The bars represent the mean and standard deviation (SD) from four independent assays. (E, F) Representative images of monocultures and co-cultures, in siNT and si*SPRY4* conditions, of C3948 and T235 cells, respectively. Image scales at 100 µm at 100x. In A and C, total number cell count data was analysed using One-way ANOVA test. In B and D, invasion fold change data was analysed using parametric unpaired T test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns - no significance).

4.2.1.5. Assessment of the influence of *SPRY4* silencing on cytoskeleton alterations by fluorescence staining

To investigate if *SPRY4* silencing affects cytoskeleton, promoting alterations in this cell compartment, phalloidin immunofluorescence staining was performed in monocultures and co-cultures of C3948 and

T235 with si*SPRY4* and siNT. Previous results obtained by the group (unreported) showed that co-culture with THP-1 derived macrophages promoted cytoskeleton alterations in C3948 and T235 ATC cell lines, and that these alterations were more noticeable in T235 (Supplementary Figure 4; Mol. End. Group's unpublished data), consistent with the less round and more stretched morphology. In the present study, in C3948 cells (Figures 4.8A, C) it was possible to observe that, when comparing monoculture *vs* co-culture, under control conditions (siNT), cells in co-cultures seemed to become more rounded, which was not so evident in the previous single experiment performed for parental cells (Supplementary Figure 4). Interestingly, under silencing conditions (si*SPRY4*), cells in co-culture acquired phenotypic alterations, seeming to be more stretched, consistent with the observed increase in cell invasion. In the case of T235 cells (Figures 4.8B, D), under control conditions (siNT), when comparing monoculture *vs* co-culture, cells in co-culture seemed to become more stretched, some with protrusions, as previously observed in parental cells. When *SPRY4* was silenced (si*SPRY4*), T235 cells in co-culture also acquired cytoskeleton alterations, seeming to be more stretched and with pseudopodia, consistent with the observed increase in cell invasion.

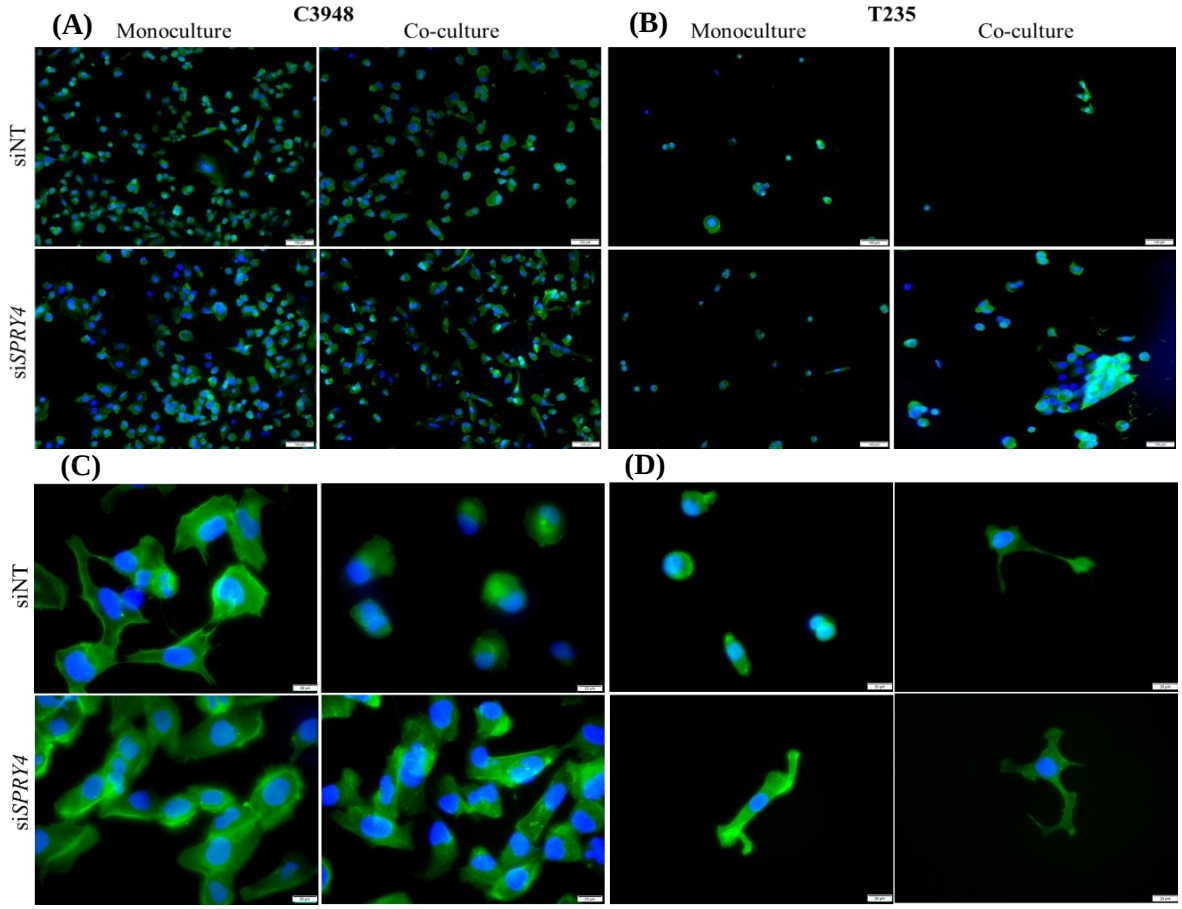

Figure 4.8. The effect of *SPRY4* silencing in cytoskeleton alterations in C3948 and T235 ATC cell lines in mono-cultures and co-cultures with phalloidin (green) and DAPI staining (blue). Images of monocultures and co-cultures of C3948 and T235 in siNT and si*SPRY4* conditions, respectively. (A, B) Image scales at 100 µm at ampliation of 100x. (C, D) Image scales at 20 µm at ampliation of 400x. Three experimental assays were performed.

4.2.2. Discussion

The dynamic interactions of cancer cells with their microenvironment (cells and extracellular matrix components) is crucial to stimulate cancer cell heterogeneity, clonal evolution, and to promote drug resistance, leading to cancer cell progression and metastasis. ATC's TME is composed mostly by TAMs, which modulate ATC behavior. As mentioned in the introduction section, proteomic studies developed

by our group, identified *SPRY4* as having a possible role in ATC-TAM crosstalk. Sprouty proteins are feedback regulators of RTK, which restrain RTK-mediated signaling, thereby having critical roles in the regulation of cell proliferation, survival and differentiation (Yang *et al.*, 2013; Cabrita *et al.*, 2008). *SPRY4* (OMIM * 607984) encodes for an inhibitor of the MAPK signaling pathway. It is positioned upstream of RAS activation and impairs the formation of active GTP-RAS by inhibiting the phosphorylation by RAS-Guanine nucleotide exchange factor (RasGEF), that triggers the release of guanosine diphosphate (GDP) from RAS and the association between RAS and GTP (Fior, 2019; Masoumi-Moghaddam *et al.*, 2014). Can also block ERK activation by associating to the cysteine rich domain (CRD) of RAF1, a RAS independent pathway (Masoumi-Moghaddam *et al.*, 2014). Tennis *et al*. suggested that *SPRY4* could be a downstream target of WNT (Wingless-related integration site) signaling pathway, inhibiting cancer cell growth, migration and invasion in NSCLC (Tennis *et al.*, 2010). These evidence suggests that this gene could be a TSG, although knockdown and upregulation in DTC (Marques *et al.*, 2021), testicular germ cell tumours (TGCT) (Das *et al.*, 2018) and ovary cancers (So *et al.*, 2016) suggested that it behaves as an oncogene (OG). Previous functional *in vitro* studies, developed by our group, of a novel *SPRY4* variant (c.701C>T, p.Thr234Met), detected in a family with familial nonmedullary thyroid carcinoma (FNMTC), showed that it's overexpression, in thyroid follicular normal and tumour cells, induced a significantly higher viability, compared with wild type *SPRY4* cells, and an increased ability to form colonies, through ERK phosphorylation, suggesting that the variant could confer an oncogenic function to *SPRY4*. In addition, *SPRY4* silencing in those cell models, led to a decrease in cell viability, indicating a possible role as an oncogene in DTC. This study led to the identification of *SPRY4* as a novel candidate susceptibility gene for FNMTC (Marques *et al.*, 2021). The oncogenic *vs* tumour suppressor behavior of *SPRY4* is yet to be clarified in ATC cells, although, western blot, invasion, migration and morphology studies, together with proteomic data, obtained in our previous studies, suggest a TSG function. In the present study, western blot results were consistent with the previous proteomic studies, showing that there was an increased expression of SPRY4 protein in the C3948 co-cultures and a significant decrease of SPRY4 protein expression in the T235 cells, when comparing with respective monoculture.

In order to further explore *SPRY4* role in ATC-TAM interaction, *SPRY4* siRNA was used to modulate its expression. Since the effect *SPRY4* gene silencing in ATC cell viability had never been reported, and its role as TSG or oncogene has not been elucidated in this tumour context, we performed viability assays following *SPRY4* silencing in the two ATC cells lines. A significant decrease of T235 cells viability with *SPRY4* silencing, and no significant differences in the case of the C3948 cell line, compared with the control (siNT) were observed. These different effects could be associated with the distinct genetic backgrounds of the cells lines, since T235 is *BRAF* p.V600E-positive (Rodrigues *et al.*, 2007) and C3948 is *BRAF* p.V600E-negative (Pinto *et al.*, 2019), and/or additional alterations in signaling and/or gene regulation not addressed in these studies. Marques *et al*. described a significant decrease in viable cells in *SPRY4* silenced TPC-1 cells (PTC cell line *RET/PTC* positive) (Marques *et al.*, 2021), similarly to what was observed with *BRAF*-positive T235 cells.

One of the most outstanding characteristics of cancer cells, which promotes tumour progression and spreading, is the capacity to invade the neighbor tissues. The invasion process permits neoplastic cells to spread loco-regionally with metastases to lymph nodes, and/or systematically forming secondary tumours in distinct organs. It is thus crucial to elucidate the underlying cellular and molecular mechanisms. To further investigate the effect of silencing *SPRY4* in ATC-TAM crosstalk, T235 and C3948 cells invasion ability was assessed using siRNA, in monocultures and co-cultures. The invasion ability of C3948 cells significantly decreased in co-culture, in control conditions, and increased with *SPRY4* silencing. Particularly, in these cells, *SPRY4* silencing promoted an increase in the total number of invasive cells in co-culture, compared with all the remaining conditions. It appears that a likely anti-tumoural effect of TAMs was abrogated by *SPRY4* silencing, increasing the invasion ability, and that,

in this context, *SPRY4* could be playing a TSG role. The invasion ability of T235 cells significantly increased in co-culture, in control conditions, and this increase was enhanced with *SPRY4* silencing. Thus, in these cells, it seems that a likely pro-tumoural effect of TAMs was enhanced by *SPRY4* silencing, increasing the invasion ability, and that, again, *SPRY4* could be acting as a TSG. It is however intriguing why, when viability was assessed in T235 cells monoculture, *SPRY4* seemed to be playing an oncogene role. It could be hypothesized that, in these cells, *SPRY4* role changed in the presence of TAMs, starting to behave as a TSG. It would be interesting, in future studies, to analyse viability, including also *SPRY4* silenced co-cultures, to compare with the respective monocultures, to investigate TAMs effect in this context.

Increased invasion ability is related with cell morphology alterations, in order to subsequently promote metastasis. Indeed, the first clues of metastasis formation are morphological alterations in the cytoskeleton (Huber *et al.*, 2005). This cell behavior arises from mutations in OGs or TSGs that awake the embryonic development program, the epithelial-to-mesenchymal transition (EMT) first described by Betty Hay in vertebrate embryonic development (Hay, 1968; Das *et al.*, 2019). EMT process is a key step in metastasis and in transdifferentiation process in solid cancer development and progression. There is evidence that suggest that EMT in breast cancer is associated with stemness, metastasis and also drug resistance (Wang *et al.*, 2011; Das *et al.*, 2019). The first processes associated with EMT is based on the elimination of several epithelial structural features, such as cell to cell junctions and apical-basal polarity, downregulation of genes associated with epithelial functions, such as E-cadherin, occludin, type IV collagen, and laminin-1, and the upregulation of genes associated with mesenchymal functions, such as N-cadherin, vimentin, fibronectin, β-catenin and type I and III collagen (Kalluri *et al.*, 2009). All these mesenchymal interactions are correlated with cytoskeleton alterations, since it has been reported that the loss of cellular junctions triggers the reorganization of the actinic network (Beaty *et al.*, 2014), originating spindle-shaped cells (Kalluri *et al.*, 2009). In the present study, alterations in the cytoskeleton were observed in si*SPRY4* condition in both cell lines, which appear to acquire a more stretched morphology when comparing with siNT in both monoculture and co-culture. Furthermore, in co-culture conditions either in siNT and si*SPRY4*, cytoskeleton alterations characterized by a more stretched morphology, are more predominant than in monoculture, consisting with the invasion data. Control siNT in C3948 and T235 corroborated the previous acquired results in parental cell lines. There are several mechanisms of gene regulation, that may intrinsically modulate *SPRY4* expression, and that could explain the observed differences in the effects/roles of *SPRY4* induced silencing in viability and invasion. Zhang *et al*. reported that H3K27 acetylation-activated long noncoding RNA Colon cancer associated transcript-1 (CCAT1) knockdown significantly reduced cell proliferation and migration, by decreasing the methylation of *SPRY4* promotor and thereby increasing *SPRY4* expression, in esophageal squamous cell carcinoma (Zhang *et al.*, 2017). Furthermore, it has been described that Sprouty proteins have a post-transcriptional regulation by polyubiquitination and proteasomal degradation, which could also be affecting our results, by increasing or decreasing SPRY4 expression (Rubin *et al.*, 2003). Also, other Sprouty family members could be affecting viability, such as *SPRY1* and *SPRY2* that, unlike *SPRY4*, have been reported to hinder cell growth in prostate cancer and sarcomas, respectively (Masoumi-Moghaddam *et al.*, 2014). TME is not only constituted by cells, but also by mediators, like cytokines, chemokines, metalloproteinases, secreted by the tumour and stromal cells into the extracellular matrix (Madden *et al.*, 2020), therefore, TAMs could be modulating the behavior of *SPRY4* by secreting specific mediators to the TME. The present studies corroborate a possible role for *SPRY4* as a TSG, in which its silencing promotes alterations in the cytoskeleton, consistent with an increase of cell invasion. Taken together, *SPRY4* gene seems to be modulated in ATC in the presence of TAMs, and thus to have an influence in ATC-TAMs crosstalk.

5. Conclusions and perspectives

This work aimed to further clarify the mechanisms underlying ATC aggressiveness and the response to the new DT combination therapy for ATC. In the molecular characterization of *BRAF* p.V600E-positive ATCs, we identified mutations that affect DT efficacy. The concomitance between *BRAF* p.V600E and *TERTp* mutations appears to be correlated with a good initial response to DT. Acquired *NRAS* mutations appear to decrease DT response, being considered possible off-target resistance mechanisms to DT. PI3K/AKT/mTOR mutations also appear to influence DT response and could be related with the selection of resistant clones to DT, as described in melanoma. In these cases, it is crucial to search for new therapeutic options, in order to overcome refractoriness, and to provide better treatment to patients. Our cohort of study had a low sample size since ATC is a rare disease, therefore, the characterization of additional cases may support and extend the present findings. The NGS panel used in this study did not allow to assess epigenetic changes, *BRAF* overexpression and splice variants, *PTEN* losses, and *RAC1* mutations, which are possible resistance mechanisms to DT.

In the attempt to study the role of *SPRY4* in ATC-TAMs crosstalk, we undertook *in vitro* studies. It was possible to verify that SPRY4 was upregulated in C3948 co-cultures and downregulated in T235 co-cultures, confirming previous proteomic data. The effects of *SPRY4* silencing were investigated in both cell lines, to better understand the function of this gene. Silenced *SPRY4* increased invasion capabilities of both cell lines in co-culture, which was corroborated by the cytoskeleton alterations observed under these conditions. Unexpectedly, viability from T235 decreased in *SPRY4* silenced conditions. Altogether, these data suggested that *SPRY4* could act as a TSG in ATC-TAM interaction, however, more studies are required to further validate these results.

In the follow-up of this project, additional research could improve the present findings and conclusions. In the first task, increasing the number of total ATC and resistant ATC cases will consolidate our present findings, and certainly improve the present knowledge of DT resistance mechanisms. Then, characterization of other "omics", immune biomarkers, *ex vivo* and *in vivo* assays using patients' ATCs with different mutational profiles, and the use of liquid biopsy, could bring more information regarding the mechanisms associated with DT response. Indeed, liquid biopsy, a non-invasive procedure, would be an ideal tool for research and for therapeutic decision in inoperable cases with extensive neck tissue invasion, or in relapse involving vital organ metastasis inaccessible to biopsy, allowing to identify molecular alterations, associated with DT response and/or representing alternative therapeutic targets. In the second task, it will be important to extend the present characterization of *SPRY4* involvement in ATC-TAM crosstalk, through the analysis of the effect of *SPRY4* silencing on proliferation and migration, and the assessment of downstream effectors of MAPK pathway, such as ERK. Furthermore, secretome analysis of culture medium from T235 and C3948 co-cultures with THP-1, in order to study cytokines, growth factors, metalloproteinases, and other TME mediators, would provide more information about *SPRY4* modulation and effects on ATC-TAM crosstalk. Also, correlation of ATC patients' clinicopathological features with tumour *SPRY4* expression, and the presence immune cells, using immunohistochemistry, could further support our results.

In conclusion, the present study improved the knowledge about the molecular and genetic features underlying ATC aggressiveness and response to DT therapy, which will hopefully contribute to improve management and treatment of this lethal and rare subset of thyroid cancer.

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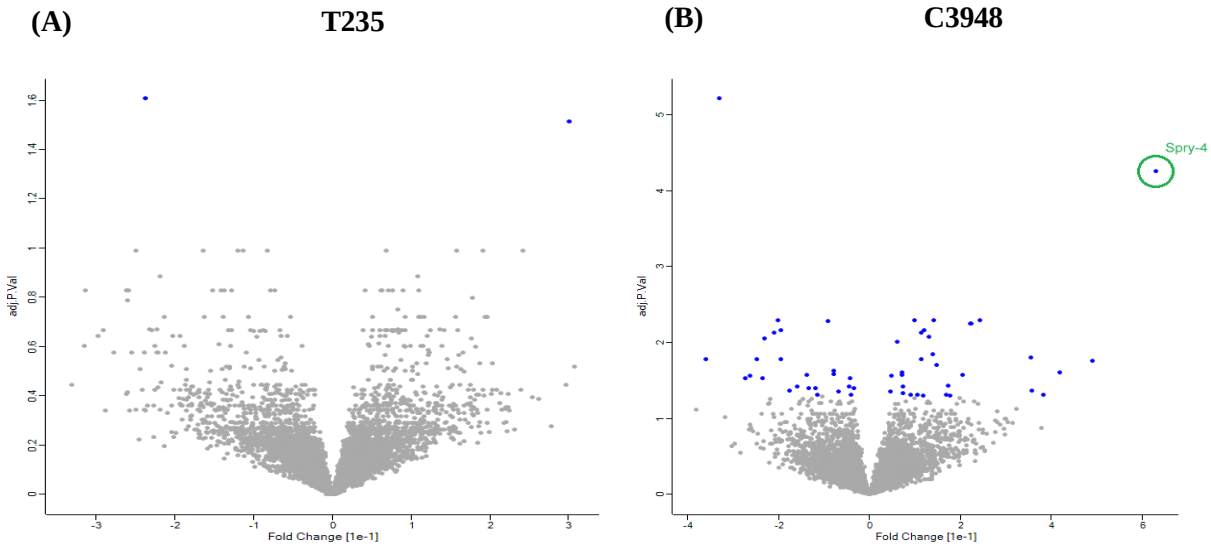
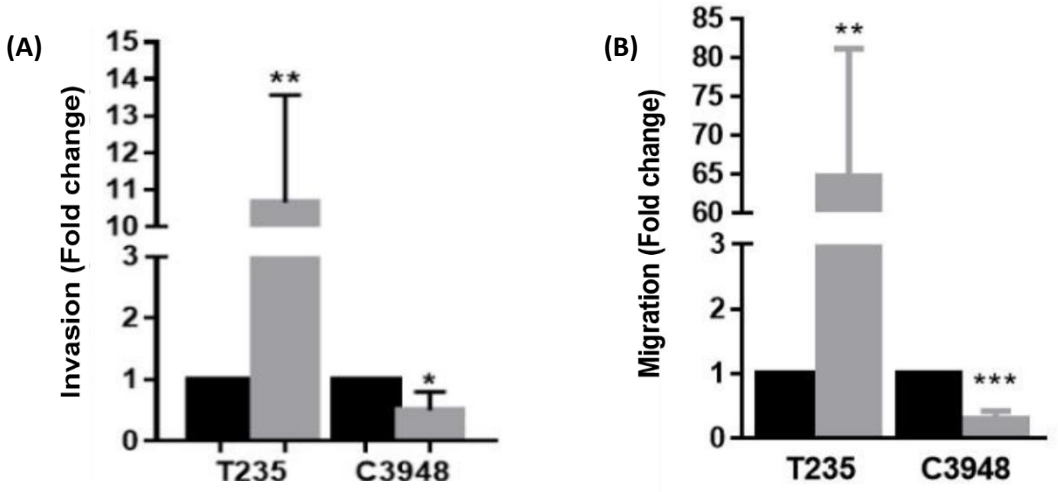
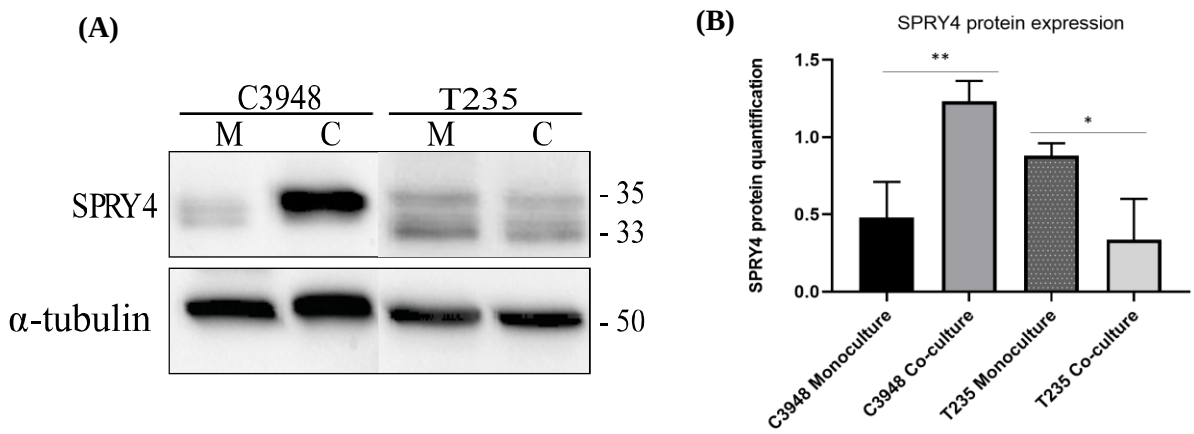
7. Supplementary Material



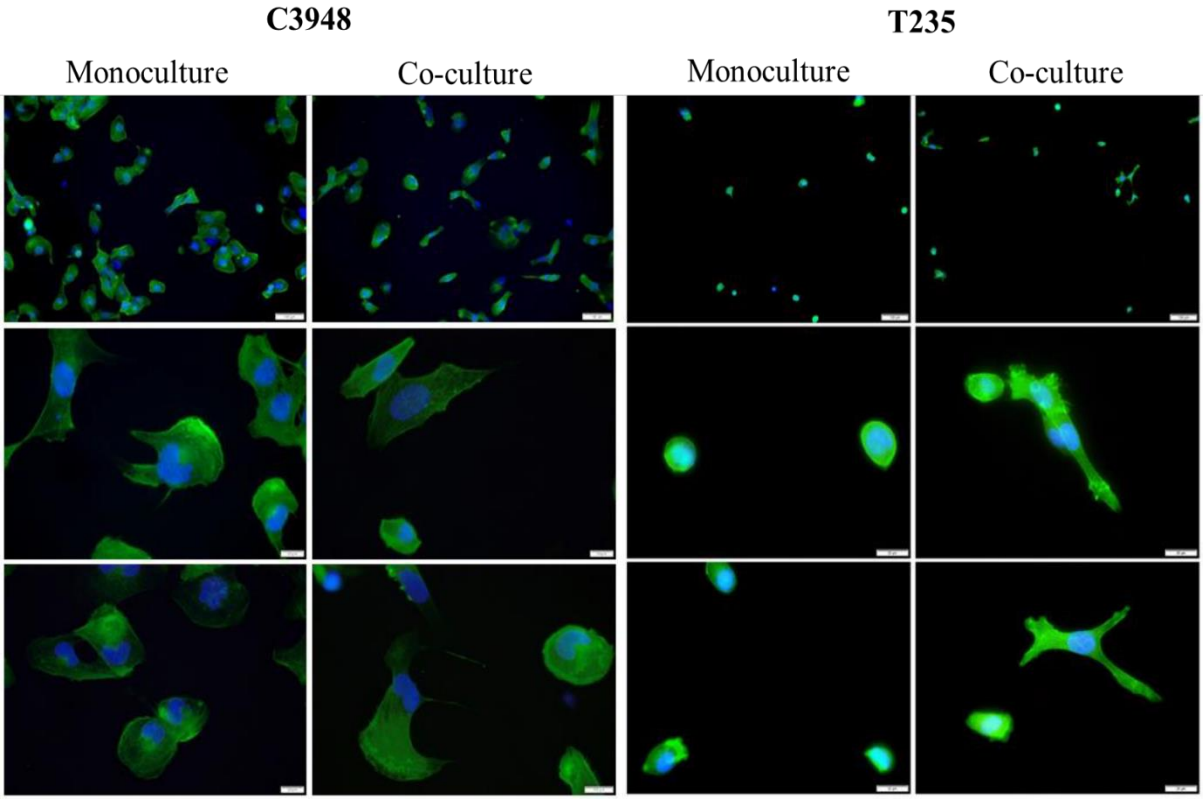
Supplementary Figure 1. Volcano plots of proteomics data. The data for all genes are plotted as log2 fold change of co-culture over monoculture versus the -log10 of the adjusted p-value. Genes selected as significant, with $p < 0.05$, are highlighted as blue circles. Note the prominent position of the SPRY4 in (B) (Scheme adapted from Eduardo *et al.*, 2020).



Supplementary Figure 2. Quantification of the effect of THP-1 differentiated macrophages on the invasion (A) and migration (B) ability of ATC cells using a system of transwell inserts and DAPI staining. Monoculture (black) and co-culture (grey) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) (Mol. End. Group's unpublished data).



Supplementary Figure 3. Western blot analysis of SPRY4 in ATC cells in monoculture and co-culture with a polyclonal antibody. Thirty µg of protein were applied per lane of 12% Bis-Tris gels and transferred to PVDF membranes. SPRY4 was detected by a rabbit monoclonal anti-SPRY4 antibody and α-tubulin by a mouse monoclonal anti-α-tubulin antibody. (A) Western blot of representative assays of C3948 and T235. SPRY4 is represented with a molecular weight of 33 and 35 kDa, and α-tubulin with 50 kDa. (B) Semi-quantification of SPRY4 in co-culture and monocultures normalized by α-tubulin. Data are mean ± SD from three independent experiments. M, monoculture; C, co-culture. Data were analysed using the parametric unpaired T test (* $p < 0.05$, ** $p < 0.01$).



Supplementary Figure 4. Assessment of the cytoskeleton alterations in C3948 and T235 ATC cell lines in monocultures and co-cultures with phalloidin (green) and DAPI staining (blue). The first line of the figure has image scales at 100 µm at ampliation of 100x. The second and third lines of the figure have image scales at 20 µm at ampliation of 400x. One experimental procedure was performed (Mol. End. Group's unpublished data).

Supplementary Table 1. Primer sequences and conditions for PCR amplification

Primer sequences and conditions for PCR amplification

Gene	Primer Name	Sequence (5'→3')	Size (bp)	$MgSO_4$ (mM)	PCR additi-ves	Desnatura-tion (time)	Annealing		Extension (time)	Cycles (number)	Reference
							Time	Temperature			
Genomic DNA from FFPE											
TERTp	*TERTp* F *TERTp* R	GCACAGACGCCCAGGACCGCGCT TTCCCACGTGCGCAGCAGGACGCA	244	2.0	-	30 s	40 s	69.5°C	50 s	35	*
PIK3CA	*PIK3CA-e10 F* *PIK3CA-e10 R*	GCTTTTTCTGTAAATCATCTGTG CATGCTGAGATCAGCCAAATTC	250	4.0	-	30 s	40 s	61°C	50 s	35	Samuels *et al.*, 2004
Pseudo-gene chr22	*Pse- F* *Pse- R*	TCCTCTCTCTGAAATCACTGA ACATGCTGAGATCAGCCAAAT	150	4.0	-	30 s	40 s	58°C	50 s	35	Baker *et al.*, 2012
MAP2K1	*MAP2K1-e3 F* *MAP2K1-e3 R*	GGAGATCAAACCCGCAATCC GGCTGAGAGGGTGTCACATA	157	2.2	-	1 min	50 s	58°C	1 min 10 s	35	*

* Primers designed for this study

Supplementary Table 2. Gene content of the NGS AmpliSeq™ for Illumina Focus Panel

DNA (#35) **Hotspot mutations**		**CNVs** (#19)	**RNA** (#24) **Fusions**	
AKT1	*JAK1*	*ALK*	*ABL1*	*NTRK2*
ALK	*JAK2*	*AR*	*ALK*	*NTRK3*
AR	*JAK3*	*BRAF*	*AKT3*	*PDGFRA*
BRAF	*KIT*	*CCND1*	*AXL*	*RET*
CDK4	*KRAS*	*CDK4*	*PPARG*	*ROS1*
CTNNB1	*MAP2K1*	*CDK6*	*RAF1*	
DDR2	*MAP2K2*	*EGFR*	*ROS1*	
EGFR	*MET*	*ERBB2*	*BRAF*	
ERBB2	*MTOR*	*FGFR1*	*EGFR*	
ERBB3	*NRAS*	*FGFR2*	*ERBB2*	
ERBB4	*PDGFRA*	*FGFR3*	*ERG*	
ESR1	*PIK3CA*	*FGFR4*	*ETV1*	
FGFR2	*RAF1*	*KIT*	*ETV4*	
FGFR3	*RET*	*KRAS*	*ETV5*	
GNA11	*ROS1*	*MET*	*FGFR1*	
GNAQ	*SMO*	*MYC*	*FGFR2*	
HRAS		*MYCN*	*FGFR3*	
IDH1		*PDGFRA*	*MET*	
IDH2		*PIK3CA*	*NTRK1*	
DNA Spike in (#4)				
TP53				
TERT				
TCFL7				
H3F3A				