

# Ubiquitination default as a familial non- medullary thyroid cancer marker

Cláudia Marques Pereira Fernandes

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## Supervisor

Ana Paula Soares Dias Ferreira

Associate professor, Medical Faculty of the University of Porto

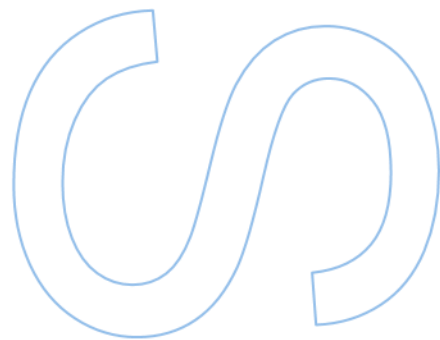
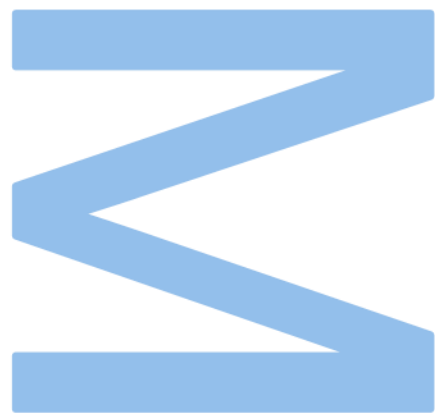
Leader of the Cancer Signalling and Metabolism Group of the  
Institute of Molecular Pathology and Immunology of the University  
of Porto / Institute of Research and Innovation in Health of the  
University of Porto (IPATIMUP/i3S)

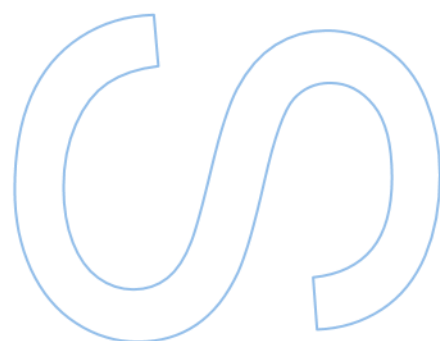
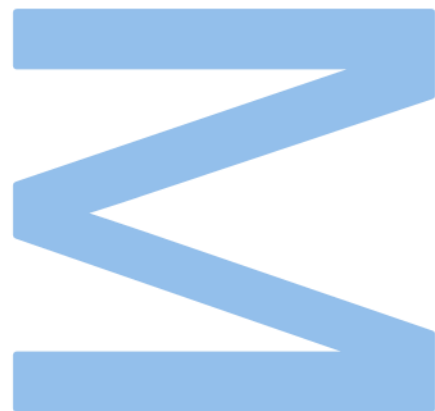
## Co-supervisor

Raquel Maria da Silva Graça Almeida, PhD

Invited assistant professor of the Sciences Faculty of the University  
of Porto

Leader of the Differentiation & Cancer Group of the Institute of  
Molecular Pathology and Immunology of the University of Porto /  
Institute of Research and Innovation in Health of the University of  
Porto (IPATIMUP/i3S)





*"Happiness can be found even in the darkest of times,  
if only one remembers to turn on the light."*

*Albus Dumbledore (J.K. Rowling)*

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# Resumo

O cancro hereditário da tiroide representa cerca de 10% de todos os casos de cancro da tiroide. O cancro familiar não-medular da tiroide (FNMTTC) tem origem nas células foliculares da tiroide e é diagnosticado quando a doença está presente em dois ou mais familiares de primeiro grau, e na ausência de outras síndromes familiares conhecidas. Uma mulher de 36 anos foi diagnosticada com a Variante Oncocítica do Carcinoma Papilar da Tiroide, juntamente com as suas filhas gémeas de 19 anos. Por sequenciação de nova geração (NGS), foi encontrada uma mutação germinativa (p.G486R) no gene da protease específica da ubiquitina 42 (USP42). A USP42 pertence à família das enzimas desubiquitinantes (DUBs), conhecidas por regularem a paragem do ciclo celular, a apoptose e outras funções celulares relevantes. Evidências consistentes têm demonstrado que a desregulação de DUBs pode levar a processos tumorigénicos. Assim, o objetivo desta tese foi validar a alteração da USP42 como uma causa subjacente ao FNMTTC.

Para validar a hipótese, foram efetuadas análises *in silico* recorrendo a bases de dados de NGS disponíveis online. Foram efetuados ensaios *in vitro* utilizando a linha celular Nthy-Ori 3-1. Foram utilizados siRNAs para silenciar o gene USP42 e, ao mesmo tempo, foi utilizada a metodologia CRISPR-RNP para silenciar o gene USP42. Efetuaram-se ainda ensaios morfológicos e funcionais nos clones celulares transformados.

Através de 2 bases de dados NGS, inferiu-se que a USP42 tem um aumento na variação do número de cópias (CNV) e que podem ocorrer mutações esporádicas em tumores de tiroide. O tecido da tiroide humana expressa normalmente o gene USP42. Através de siRNAs, silenciou-se com sucesso este gene em Nthy-Ori 3-1, como verificado através da análise de RNA, por RT-qPCR; a expressão das proteínas USP42, p53 e Ciclina D1, analisada pela técnica de Western blot, mostrou uma diminuição às 72h; e os ensaios funcionais celulares pareceram indicar taxas apoptóticas mais elevadas com o silenciamento de USP42.

Os resultados mostram que alterações da USP42 têm impacto no comportamento da tiroide, podendo ter um papel como potencial gene de suscetibilidade em FNMTTC.

**Palavras-chave:** Cancro hereditário da tiroide, Cancro familiar não-medular da tiroide, mutação germinativa, USP42, oncologia molecular.

# Abstract

Hereditary thyroid cancer accounts for approximately 10% of all thyroid cancer cases. Familial Non-Medullary Thyroid Cancer (FNMTc) arises from thyroid follicular cells and is diagnosed when the disease is present in two or more first-degree relatives in the absence of other known familial syndromes. A 36-year-old woman was diagnosed with Oncocytic Variant of Papillary Thyroid Carcinoma, along with her twin 19-year-old daughters. By Next Generation Sequencing (NGS), a germline mutation (p.G486R) in ubiquitin-specific protease 42 gene (USP42) was found. USP42 belongs to the family of deubiquitinating enzymes, known for regulating cell cycle arrest, apoptosis, and other relevant cellular functions. Consistent evidence has shown that deregulation of DUBs may lead to tumorigenic processes. Therefore, the aim of this thesis was to validate USP42 alteration as an underlying cause of FNMTc.

To validate the hypothesis, *in silico* analysis resorting to online available NGS databases were conducted. *In vitro* assays using Nthy-Ori 3-1 cell line were performed. siRNAs were used to silence the USP42 gene while, at the same time, CRISPR-RNP methodology was used to knockout the USP42 gene. Morphologic and functional assays were performed in the transformed cell clones.

Through 2 NGS databases, it was inferred that USP42 has an increase in copy number variation (CNV) and sporadic mutations can occur. Human thyroid tissue normally expresses the USP42 gene. By siRNAs, this gene was successfully silenced in Nthy-Ori 3-1, as verified by RT-qPCR RNA analysis; USP42, p53 and Cyclin D1 protein expression, analysed by Western blot technique, showed a decrease at the 72h timepoint; and cellular functional assays appeared to indicate higher apoptotic rates with the silencing of USP42.

The results obtained show that changes in USP42 has an impact on the behaviour of thyroid cells, and may have a role as a potential susceptibility gene for FNMTc.

**Keywords:** Hereditary thyroid cancer, Familial Non-Medullary Thyroid Carcinoma, germline mutation, USP42, molecular oncology.

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# List of Abbreviations

|                          |                                                 |
|--------------------------|-------------------------------------------------|
| <b>2<sup>-ΔΔCt</sup></b> | Fold-change                                     |
| <b>AMPK</b>              | 5'-AMP-Activated Protein Kinase                 |
| <b>APC</b>               | Adenomatous Polyposis Coli                      |
| <b>ATC</b>               | Anaplastic Thyroid Carcinoma                    |
| <b>ATP</b>               | Adenosine Triphosphate                          |
| <b>BMI</b>               | Body Mass Index                                 |
| <b>BRAF</b>              | V-raf murine sarcoma viral oncogenes B1         |
| <b>CNV</b>               | Copy Number Variation                           |
| <b>CRAVAT</b>            | Cancer-Related Analysis of Variants Toolkit     |
| <b>CS</b>                | Carney Syndrome                                 |
| <b>CT</b>                | Computed Tomography                             |
| <b>D2</b>                | Hormone-activating type II desiodinase          |
| <b>dDNA</b>              | Donor DNA                                       |
| <b>dH<sub>2</sub>O</b>   | Distilled water                                 |
| <b>DNA</b>               | Desoxyribonucleic Acid                          |
| <b>DUB</b>               | Deubiquitinating Enzyme                         |
| <b>EDCs</b>              | Endocrine Disrupting Chemicals                  |
| <b>EMT</b>               | Endothelial-Mesenchymal Transition              |
| <b>EvC</b>               | Ellis-van Creveld                               |
| <b>F</b>                 | Forward                                         |
| <b>FAP</b>               | Familial Adenomatous Polyposis                  |
| <b>FBS</b>               | Foetal Bovine Serum                             |
| <b>FCUP</b>              | Faculty of Sciences of the University of Porto  |
| <b>FFPE</b>              | Formalin Fixed Paraffin Embedded                |
| <b>FMNG</b>              | Familial Multinodular Goitre                    |
| <b>FMTc</b>              | Familial Medullary Thyroid Carcinoma            |
| <b>FNMTc</b>             | Familial Non-Medullary Thyroid Carcinoma        |
| <b>FNMTc1</b>            | Familial Non-Medullary Thyroid Carcinoma Type 1 |
| <b>FOXE1</b>             | Forkhead Box E1                                 |
| <b>FOXM1</b>             | Forkhead Box Protein M1                         |
| <b>FOXO3</b>             | Forkhead Box O3                                 |
| <b>fPTC</b>              | Familial Papillary Thyroid Carcinoma            |
| <b>FTC</b>               | Follicular Thyroid Carcinoma                    |
| <b>GATK</b>              | Genome Analysis Tool Kit                        |

|                         |                                                               |
|-------------------------|---------------------------------------------------------------|
| <b>GWA</b>              | Genome-Wide Association                                       |
| <b>HABP2</b>            | Hyaluronan Binding Protein 2                                  |
| <b>HECT</b>             | Homologous to the E6AP Carboxyl Terminus                      |
| <b>IFN</b>              | Interferons                                                   |
| <b>IGS</b>              | Integrative Genomics Viewer                                   |
| <b>JNK</b>              | c-Jun N-terminus Kinase                                       |
| <b>KO</b>               | Knockout                                                      |
| <b>MAN</b>              | Multiple Adenomatous Nodules                                  |
| <b>MAP2K5</b>           | Mitogen-Activated Protein Kinase Kinase 5                     |
| <b>MAPK</b>             | Mitogen-Activated Protein Kinase                              |
| <b>MCPIP</b>            | Monocyte Chemotactic Protein-Induced Proteins                 |
| <b>MEN</b>              | Multiple Endocrine Neoplasia                                  |
| <b>Mg<sup>2+</sup></b>  | Magnesium                                                     |
| <b>MINDY</b>            | Motif Interacting with ubiquitin-containing novel DUB         |
| <b>MJD</b>              | Machado-Joseph Disease protein domain proteases               |
| <b>NF-κB</b>            | Nuclear Factor kappa B                                        |
| <b>NGS</b>              | Next Generation Sequencing                                    |
| <b>NIS</b>              | Sodium-Iodine Symporter                                       |
| <b>NSCLC</b>            | Non-Small Cell Lung Carcinoma                                 |
| <b>NTRK</b>             | Neurotrophic Tyrosine Receptor Kinase                         |
| <b>OTU</b>              | Ovarian Tumours Proteases                                     |
| <b>OVPTC</b>            | Oncocytic Variant of Papillary Thyroid Carcinoma              |
| <b>PAX8</b>             | Paired Box 8                                                  |
| <b>PCR</b>              | Polymerase Chain Reaction                                     |
| <b>PI3K/AKT</b>         | Phosphoinositide 3 Kinase/V-akt murine thymoma viral oncogene |
| <b>PPAR<sub>γ</sub></b> | Peroxisome Proliferator-Activated Receptors-gamma             |
| <b>PRKAR1A</b>          | Protein Kinase cAMP-Dependent Type I Regulatory Subunit Alpha |
| <b>PTC</b>              | Papillary Thyroid Carcinoma                                   |
| <b>PTEN</b>             | Phosphate and Tensin Homolog                                  |
| <b>R</b>                | Reverse                                                       |
| <b>RAS</b>              | Rat sarcoma viral oncogenes homolog                           |
| <b>RET/PTC</b>          | Rearranged during transfection/Papillary Thyroid Carcinoma    |
| <b>RING-IBR-RING</b>    | RING-in between RINGs-RING                                    |
| <b>RIPA</b>             | Radioimmunoprecipitation assay                                |

|                |                                                              |
|----------------|--------------------------------------------------------------|
| <b>RNP</b>     | Ribonucleoprotein                                            |
| <b>ROS</b>     | Reactive Oxygen Species                                      |
| <b>RPS27A</b>  | Ribosomal Protein S27A                                       |
| <b>RT-qPCR</b> | Reverse Transcriptase-quantitative Polymerase Chain Reaction |
| <b>SNHG5</b>   | Small Nuclear RNA Host Gene 5                                |
| <b>SNP</b>     | Single Nucleotide Polymorphisms                              |
| <b>SRGAP1</b>  | SLIT-ROBO Rho GTPase Activating Protein 1                    |
| <b>T3</b>      | Triiodothyrodine                                             |
| <b>T4</b>      | Thyroxine                                                    |
| <b>TBP</b>     | TATA Binding Protein                                         |
| <b>TBS-T</b>   | TBS-Tween                                                    |
| <b>TC</b>      | Thyroid Cancer                                               |
| <b>TCGA</b>    | The Cancer Genome Atlas                                      |
| <b>TERT</b>    | Telomerase Reverse Transcriptase                             |
| <b>TH</b>      | Thyroid Hormone                                              |
| <b>TITF-1</b>  | Thyroid Transcription Factor-1                               |
| <b>TNM</b>     | Tumour, Node and Metastasis                                  |
| <b>TRH</b>     | Thyrotropin-Releasing Hormone                                |
| <b>TSH</b>     | Thyroid Stimulating Hormone                                  |
| <b>TβRII</b>   | TGF-β receptor II                                            |
| <b>UBA52</b>   | Ubiquitin A-52 Residue Ribosomal Protein Fusion Product 1    |
| <b>UBB</b>     | Ubiquitin B                                                  |
| <b>UBC</b>     | Ubiquitin C                                                  |
| <b>UCHs</b>    | Ubiquitin C-terminal Hydrolases                              |
| <b>UP</b>      | University of Porto                                          |
| <b>USP</b>     | Ubiquitin-Specific Proteases                                 |
| <b>VCF</b>     | Variant Call Format                                          |
| <b>vgKCN</b>   | Voltage-gated Potassium Channels                             |
| <b>WES</b>     | Whole-Exome Sequencing                                       |
| <b>WHO</b>     | World Health Organisation                                    |
| <b>WRN</b>     | Werner Syndrome ATP-dependent helicase                       |
| <b>WS</b>      | Werner Syndrome                                              |
| <b>ZUFSP</b>   | Zn finger and UFSP domain protein                            |

# Chapter 1. State of the art

## 1.1. Thyroid gland: embryology, anatomy, and function

The thyroid gland is an endocrine organ characterised by having two elongated lobes (left and right) connected by the isthmus. This organ can be found anteriorly in the lower part of the neck, is well vascularized and, in adults, weights around 20-25 grams (**Figure 1**) [1].

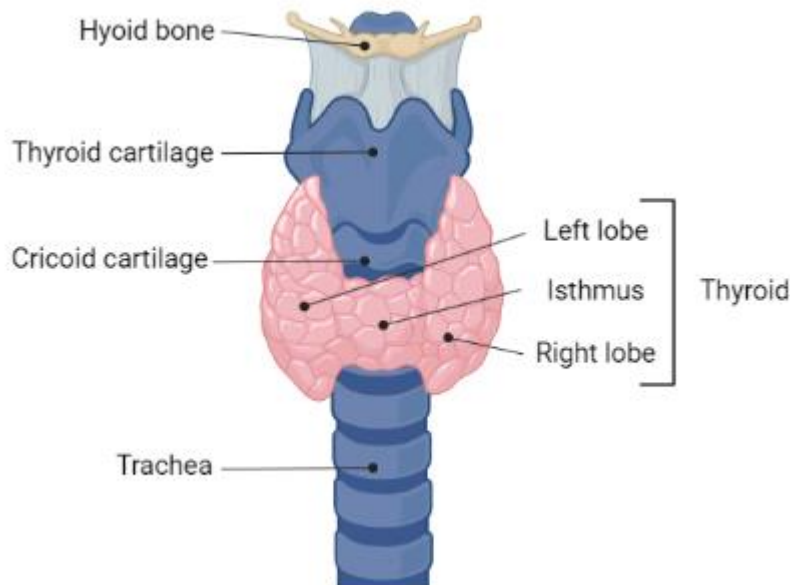


**Figure 1. Thyroid anatomy.** The thyroid is divided in left and right lobes connected by the isthmus. The thyroid is located in front of the trachea and the cricoid cartilage. Adapted from Netter (978-1-4160-5951-6) and created with Biorender.

The thyroid is supplied by the superior and the inferior thyroid arteries, and venous drainage occurs through superior, middle, and inferior thyroid veins. The superior thyroid artery divides in anterior and posterior branches the first supplies the anterior surface of the gland, and the latter supplies the lateral and medial surfaces. The inferior thyroid artery divides into ascending and descending branches; and supplies the inferior and posterior surfaces of the thyroid. The ascending branch also supplies the parathyroid glands. The superior thyroid vein drains the upper part of the thyroid, the middle thyroid vein drains the isthmus region and a bit of its surroundings, and the inferior vein drains the lower part of the thyroid [1].

Embryonically, the thyroid gland originates from the embryonic endoderm at the 3<sup>rd</sup> week of gestation, being the first endocrine structure to appear, and is already identifiable by the 4<sup>th</sup> week. It emerges from the thyroid diverticulum, which is in the primitive pharyngeal floor, and is connected to it by the thyroglossal duct. During several weeks, the embryonic thyroid suffers morphological changes, such as enlargement,

bifurcation and lobulation; and in the 7<sup>th</sup> week occurs its detachment from the first site, beginning to descend. In the 11<sup>th</sup> week of gestation, the embryonic thyroid is already set in its final position, and thyroid hormones may start being detected in foetal circulation [2, 3].

In terms of histology, the thyroid is surrounded by a thin layer of connective tissue and is made of thousands of thyroid follicles. These follicles measure between 0,02 and 0,09 millimetres and present a spheric-like aspect. They possess a cavity filled with colloid that is surrounded by a monolayer of follicular cells. The colloid is almost entirely made of a glycoprotein – thyroglobulin, that is enriched in tyrosine molecules. Thyroid hormones are stored in the colloid connected to thyroglobulin (**Figure 2**) [1, 4, 5].



**Figure 2. Thyroid histology.** The thyroid is mainly composed from thyroid follicles, blood vessels and parafollicular cells. Thyroid follicles are filled with colloid, surrounded by follicular cells. Adapted from Basic Histology (978-85-277-2311-4) and modified from a Biorender template.

The thyroid is composed by 2 types of hormone-producing cells: follicular cells and parafollicular cells (also named C cells). Parafollicular cells are less abundant and produce the parathyroid hormone. Follicular cells constitute the large majority of thyroid gland cells and synthesise thyroid hormones (THs), thyroxine (T4 – it carries four iodine atoms) and triiodothyronine (T3 – it carries three iodine atoms). The synthesis and accumulation of these hormones happen sequentially, as firstly iodide ions enter the follicular cell by active transport using sodium-iodine symporter (NIS). Then shortly after the entry, iodide ions are oxidised to form iodine, that connects to newly formed thyroglobulin. In a correct thyroid function, T4 and T3 are produced in a ratio of 90% and 10%, respectively. The hypothalamic-pituitary-thyroid axis acts as a regulator of THs synthesis. Thyrotropin-releasing hormone (TRH) is produced in the hypothalamus and controls, by stimulation (depending on its circulating concentration), the production of thyroid-stimulating hormone (TSH), a hormone produced by the anterior pituitary gland. TSH, in its turn, stimulates the production of THs (**Figure 3**). When there is a high amount

of THs in circulation, by negative feedback, the hypothalamus and the pituitary gland diminish the production of TRH and TSH, respectively [3, 4, 6, 7].

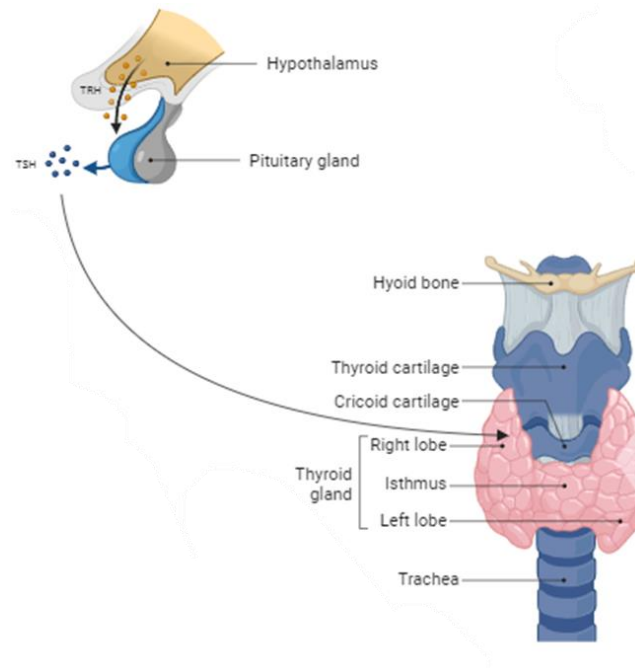


**Figure 3. Hypothalamic-Pituitary-Thyroid axis.** This axis acts as a regulator of THs synthesis. TRH is produced in the hypothalamus and controls, by stimulation, the production of TSH, a hormone produced by the anterior pituitary gland. TSH stimulates the production of THs. When there is a high amount of THs in circulation, by negative feedback, the hypothalamus and the pituitary gland diminish the production of TRH and TSH, respectively. Created on Biorender.

## 1.2. Thyroid cancer

### 1.2.1. Epidemiology

Thyroid cancer (TC) is the most common malignancy found in endocrine organs. TC is more commonly found in women, having a ratio of incidence female-to-male of 3:1 [8-10].

Worldwide, TC is the 10<sup>th</sup> most common diagnosed cancer with an age standardised incidence of 6,6/100.000 inhabitants (**Figure 4A**), however, the same cancer in the worldwide female population ranks 5<sup>th</sup>, with an age standardised incidence of 10,1/100.000 inhabitants (**Figure 4B**). In Portugal, TC ranks 5<sup>th</sup> most common with an age standardised incidence of 11,7/100.000 inhabitants (**Figure 4C**), while in the female population ranks 3<sup>rd</sup> with an age standardised incidence rate of 18,6/100.000 inhabitants (**Figure 4D**) [11, 12].

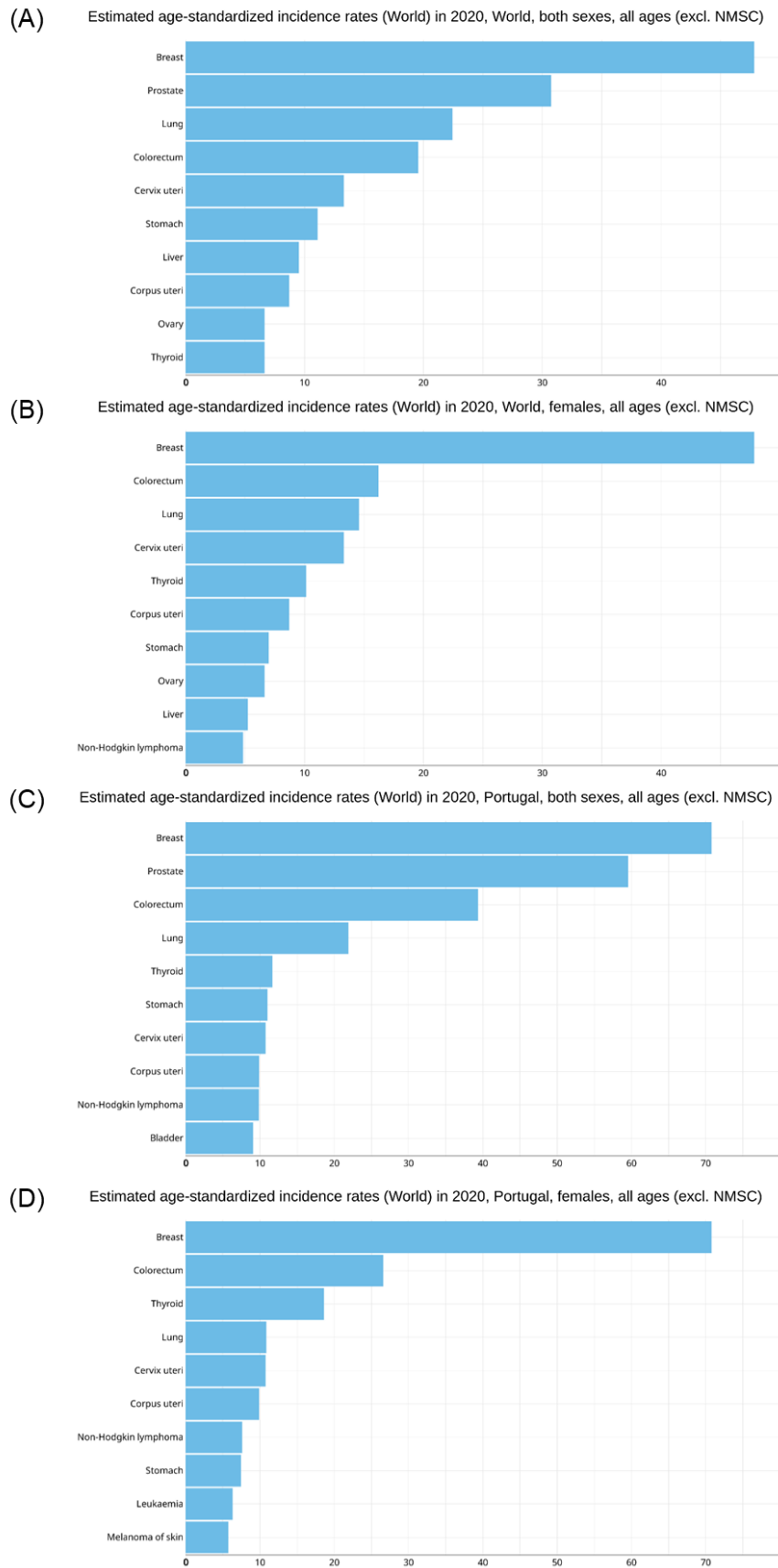


**Figure 4. Thyroid cancer epidemiology.** (A) Age standardised incidence rate for thyroid cancer worldwide. (B) Age standardised incidence rate for thyroid cancer in the female worldwide population. (C) Age standardised incidence rate for thyroid cancer in Portugal. (D) Age standardised incidence rate for thyroid cancer in the female Portuguese population. Data collected from GLOBOCAN.

TC has a higher incidence in 1<sup>st</sup> world countries, such as Canada, USA, Italy, Republic of Korea, amongst others (**Figure 5**). This may be due to higher access to healthcare, which ultimately led to an increased diagnostic rate, but also to environmental factors. There has been a rising incidence of diagnosis in TC, which can be described as overdiagnosis, due to the evolution of imaging techniques and the increasing use of fine needle aspiration [8, 13].

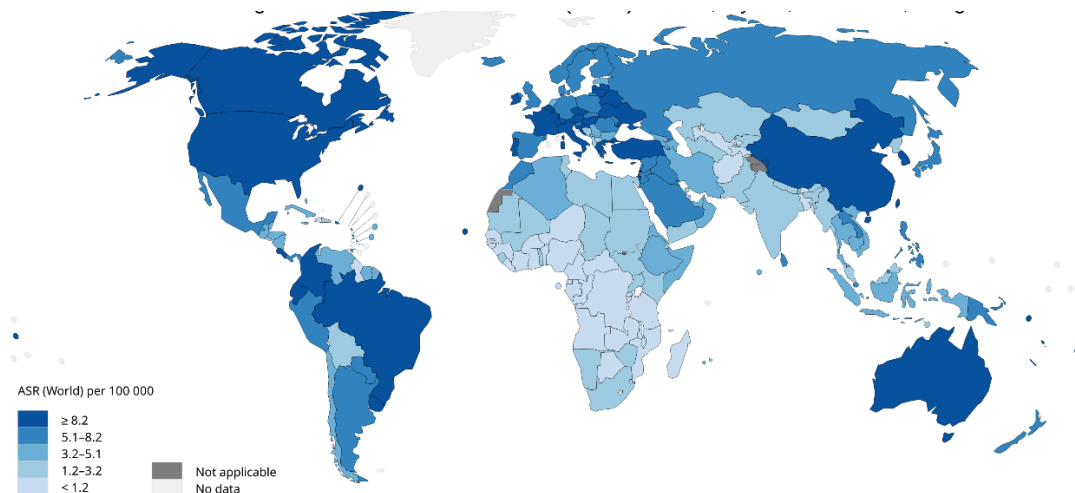


**Figure 5. Thyroid cancer worldwide incidence.** Thyroid cancer has a higher incidence in first world countries. Data collected from GLOBOCAN.

### 1.2.2. Survival and prognosis

TC has a 98% 5-years survival-rate, being a neoplasia with good prognosis. This seems to be due to early diagnosis, with most of the cases being localised within the thyroid gland, and the effective treatments options. Treatment for TC includes partial or total thyroidectomy, followed by radioiodine therapy in the advanced cases. In low-risk cases hemi thyroidectomy is performed, followed by prospective follow-up. When facing an aggressive thyroid cancer, which can occur in 10-15% of the cases, surgery may not be curative and radioiodine therapy and/or systemic therapy, such as kinase inhibitors, is used [8, 14, 15].

### 1.2.3. Risk factors

While considering reasons for the augmentation in the diagnosis of TC, risk factors need to be considered. Risk factors can be divided in two categories: modifiable and non-modifiable [8, 13].

### 1.2.3.1. Modifiable risk factors

#### 1.2.3.1.1. Radiation exposure

Radiation exposure by ionisation interplays with desoxyribonucleic acid (DNA), causing damage, such as strand breaks, rearrangements, and, less often, point mutations. This type of damage is a well-known risk factor for development of carcinogenesis, and there are numerous indications that thyroid gland is particularly susceptible to ionizing radiation, as demonstrated in Chernobyl nuclear breakout. Although they have not been hit with direct ionizing radiation, descendants of individuals that have been directly hit can still be affected and damaged by it, as they show genomic instability [8, 16-18].

Many chromosomal rearrangements occur in TC, such as rearranged during transfection/Papillary Thyroid Carcinoma (RET/PTC), and are more commonly found in people exposed to radiation at a young age, opposing to point mutations on V-raf murine sarcoma viral oncogenes homolog B1 (*BRAF*) and Rat sarcoma viral oncogenes homolog (*RAS*) genes, which are frequently found on sporadic thyroid cancer and rarely connected to radiation exposure. Ionising radiation exposure in children is known to be extremely harmful, especially in thyroid, since this organ is very sensitive at young ages. Several evidence show that after the Chernobyl accident, RET/PTC rearrangements were the most prevalent to be found in children [8, 9, 16-18].

Another concerning risk factor in the exposure to radiation is due to the dramatic increase in the use of diagnostic imaging, such as computed tomography (CT). Nearly 30% of CT scans are done on the head and neck, consequently irradiating the thyroid. Recurrent CT scans are a common practise for diagnosis nowadays and with each scan the thyroid becomes at a higher risk of TC [8, 16].

Therefore, a current debate has been established in whether TC is influenced by internal sources of radiation, namely radiopharmaceuticals, in the same way and degree as by external causes, such as Chernobyl incident [8, 17].

#### 1.2.3.1.2. Thyroid imaging and radioiodine

When it comes to diagnosing or treating TC or a thyroid related disease, radioiodine is the most common practice. Radioiodine as a diagnostic setting has not been correlated with higher incidence of TC. However, there are different opinions in whether radioiodine may correlate with a higher risk for TC in patients with hyperthyroidism. For instance, in a meta-analysis performed by Hieu *et al.* (2012) [19], it

was advanced that thyroid imaging with radioiodine in hyperthyroidism may increase the risk for developing TC; but according to a review by Kitahara *et al.* (2022), the correlation between treatment with radioiodine and TC remains vague [8, 16].

#### 1.2.3.1.3. Iodine intake

Iodine intake is regulated by diet, and so, in an unbalanced diet, iodine deficiency may occur and affect normal thyroid function. Therefore, if iodine shortage occurs, a decrease in the level of THs will be present in the bloodstream, consequently, there will be a rise in the amount of TSH secreted and released from the pituitary gland. As mentioned previously, TSH will stimulate the thyroid follicular cells for both function and growth. Endemic goitre is a situation detected in regions with low iodine intake. Ultimately, long-term shortage of iodine, may be considered a risk factor for Follicular Thyroid Carcinoma (FTC), through an unknown mechanism. According to Guan *et al.* (2009) [20], long-term stimulation of thyroid follicular cells by TSH could be intertwined with known *BRAF* mutations in Papillary Thyroid Carcinoma (PTC) [16, 20]. On the other hand, PTC has been associated with high intake of iodine [20, 21].

As recorded in the literature, iodine excess or its deficiency could be matched with a dominant role in distribution of subtypes of TC, such as the ones that are related to follicular cancer in iodine deficiency areas and papillary cancer in areas with iodine excess. Another example from the Chernobyl incident showed that children exposed to radioiodine presented a higher incidence of TC, especially the ones from areas that were already iodine deficient [16].

#### 1.2.3.1.4. Environmental pollutants

Environmental pollutants may come from various sources. Nitrates serve as an incredible case point since they are found in industrialized food additives and water. The first proceed from cured meat and vegetables, which will engage with iodine and alter normal thyroid function, which ultimately may lead to TC. The latter showed that higher nitrate levels in drinking water may also be a factor for increasing thyroid cancer incidence [8, 9, 16, 22].

Another example of environmental pollutants are endocrine disrupting chemicals (EDCs), which are exogenous substances deemed as relevant tumourigenic chemicals. A systematic review reported that the pesticides group comprised the highest number of

retrieved studies, and an increased risk for neoplasia was found in a relatively high percentage of the studies. This risk presented a higher value for thyroid (67%) [23].

#### 1.2.3.1.5. Lifestyle and diet

The relationship between lifestyle and diet with TC incidence is unclear, although many studies try to study and establish it. Per example, one study showed a negative correlation between diet patterns rich in fruits and vegetables with TC, in a population-based case-control [16]; a few studies showed a positive correlation between the intake of cruciferous plants and the risk of TC, while other studies showed a positive correlation in the consumption of oranges and lemons with the risk of development of TC. However, other fruits and vegetables, other than oranges, lemons and cruciferous plants, showed a negative correlation with the risk of TC, in a way that it showed its prevention [8, 16, 24].

Physical activity is also considered to be a distinctive factor regarding prevention of the appearance of cancers in its all. Physical activity has been shown to improve DNA repair ability, reducing body fat content, lowering resistance to insulin, and altering circulating inflammatory factors. There have been some studies addressing the correlation between the practice of physical activity and the risk of TC [16, 25].

The pool of results from five different studies have shown that cigarette smoking diminishes the risk of PTC and even FTC. This is thought to be due to the lowering of TSH levels done by smoking, which in its turn has an anti-oestrogenic effect, leading to a reduced TC risk. Also, a case-control study in Korea showed that female patients with positive family history, higher body mass index (BMI), non-smokers, non-drinkers and with lower monthly income presented a higher risk for TC [8, 9, 16, 24].

#### 1.2.3.1.6. Body weight and BMI

Body weight, especially obesity, is an important aspect associated with the development of cancers. In a study that analysed a multitude of risk factors, the authors verified that in individuals that have been diagnosed with obesity, factors such as younger age, higher TSH serum levels, lower fasting blood glucose levels, smaller diameter nodules and multifocality, are associated with a higher risk for malignancy compared to normal weight patients that present normal values in the factors previously mentioned. Older male patients that present higher BMI usually have a more advanced

Tumour, Node and Metastasis (TNM) stage [24, 26, 27]. Regarding TC, the prevalence of PTC was associated with high BMI in men, but not in women [16, 24, 26].

### 1.2.3.2. Non-modifiable risk factors

#### 1.2.3.2.1. Chromosomal (genetic) alterations

Genetic alterations are the most significant aspect when discussing non-modifiable risk factors for Familial Non-Medullary Thyroid Carcinoma (FNMTTC). These genetic alterations are often related to the understanding of molecular pathways, such as metabolic, or cell cycle, or apoptotic pathways.

In the familiar forms of NMTC, genetic alterations can be divided in two categories: syndromic and non-syndromic, which will be further explored in 2.5. *Familial thyroid cancer*.

#### 1.2.3.2.2. Sex

TC has a higher incidence in women than in men, and that fact along with evidence that oestrogen stimulates the proliferation of TC cells provided from laboratory-based studies, put forward a possible correlation between sex steroid hormones in TC development [8, 28].

In numerous epidemiological studies, it is still on debate if the presence of oestrogen is related to the development of TC. On one hand, some studies show that exogenous oestrogen increases the risk for TC development, while at the same time proving that lower ovarian oestrogen decreases its risk [16]. On the other hand, hormone therapy was associated with none/low risk for the development of TC [16]. Nevertheless, several studies that evaluated factors of hyperoestrogenism in the development of TC, such as lifetime of oestrogen exposure, ages at menarche and menopause, intake of oral contraceptives, or hormone therapy, showed that, if present, the correlation is weak [8, 28, 29].

#### 1.2.3.2.3. Autoimmunity

Some studies show that thyroid autoimmune diseases, such as Graves' disease and Hashimoto's thyroiditis, are correlated with higher incidence of TC, although the evidence is disputable. As such correlation has been made, numerous studies point that

higher TSH levels in patients with thyroid autoimmune diseases stimulate follicular proliferation and, therefore, promoting the development of TC [16, 30].

#### 1.2.4. Classification

The World Health Organization (WHO) [31] has just published a new classification for thyroid neoplasms, which will be the one used throughout this thesis, that will focus in follicular cell-derived neoplasms (**Table 1**).

**Table 1. Thyroid neoplasms classification.** Summary of the thyroid neoplasms classification according to the 5th edition of WHO Classification of Endocrine and Neuroendocrine Tumours [31].

| Developmental abnormalities                                                                                                                                                                                                                                |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Thyroglossal duct cyst                                                                                                                                                                                                                                     | Other congenital thyroid abnormalities                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
| Follicular cell–derived neoplasms                                                                                                                                                                                                                          |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| <b>Benign tumours</b> <ul style="list-style-type: none"> <li>Thyroid follicular nodular disease</li> <li>Follicular adenoma</li> <li>Follicular adenoma with papillary architecture</li> <li>Oncocytic adenoma of the thyroid</li> </ul>                   | <b>Malignant neoplasms</b> <ul style="list-style-type: none"> <li>Follicular thyroid carcinoma</li> <li>Invasive encapsulated follicular variant papillary carcinoma</li> <li>Papillary thyroid carcinoma</li> <li>Oncocytic carcinoma of the thyroid</li> <li>Follicular-derived carcinomas, high-grade <ul style="list-style-type: none"> <li>Differentiated high-grade thyroid carcinoma</li> <li>Poorly differentiated thyroid carcinoma</li> </ul> </li> <li>Anaplastic follicular cell–derived thyroid carcinoma</li> </ul> |
| <b>Low-risk neoplasms</b> <ul style="list-style-type: none"> <li>Non-invasive follicular thyroid neoplasm with papillary-like nuclear features</li> <li>Thyroid tumours of uncertain malignant potential</li> <li>Hyalinizing trabecular tumour</li> </ul> |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| Thyroid C-cell–derived carcinoma                                                                                                                                                                                                                           |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| Medullary thyroid carcinoma                                                                                                                                                                                                                                |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| Mixed medullary and follicular cell–derived carcinomas                                                                                                                                                                                                     |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| Salivary gland–type carcinomas of the thyroid                                                                                                                                                                                                              |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| Mucoepidermoid carcinoma of the thyroid                                                                                                                                                                                                                    | Secretory carcinoma of salivary gland type                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
| Thyroid tumours of uncertain histogenesis                                                                                                                                                                                                                  |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| Sclerosing mucoepidermoid carcinoma with eosinophilia                                                                                                                                                                                                      | Cribriform morular thyroid carcinoma                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
| Thymic tumours within the thyroid                                                                                                                                                                                                                          |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| Thymoma family                                                                                                                                                                                                                                             | Spindle epithelial tumour with thymus-like elements                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |
| Thymic carcinoma family                                                                                                                                                                                                                                    |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| Embryonal thyroid neoplasms                                                                                                                                                                                                                                |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| Thyroblastoma                                                                                                                                                                                                                                              |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |

#### 1.2.4.1. Genetics of sporadic Follicular cell-derived thyroid cancer

Numerous signalling pathways are regulated and implied in the development of TC, although two of them stand-out: mitogen-activated protein kinases (MAPK) and phosphoinositide 3 kinase/V-akt murine thymoma viral oncogene (Pi3k/Akt) [16].

The dysregulation of the first is commonly found in PTC, mainly driven by point mutations in the *BRAF* gene. Chromosomal rearrangements may happen during the *RET/PTC* rearrangement, Neurotrophic tyrosine receptor kinase (*NTRK*) and rarely in *BRAF*. Point mutations in *RAS* genes may also be found in PTC [16, 32].

In FTC, the more commonly found alterations involve *RAS* mutations and Peroxisome Proliferator-Activated Receptors gamma/Paired box 8 (*PPARγ/Pax8*) rearrangements. In poorly differentiated and anaplastic thyroid carcinomas, TP53 mutations are frequent, as well as mutations on the promoter of Telomerase Reverse Transcriptase (*TERTp*). In addition, the dysregulation involves *RAS* mutations, Phosphatase and tensin homolog (*PTEN*) mutations – such as methylation and epigenetic silencing; and point mutations on *PIK3CA*, *PIK3CB*, *PDK1* and *Akt*. Also genomic instability by genomic amplification or copy gain is frequent [16, 33].

### 1.2.5. Familial thyroid cancer

Two main familial thyroid cancer settings can be identified in thyroid. These differ from the cells in which the cancer appears: if thyroid cancer involves follicular cells it is named FNMTc; if thyroid cancer arises from calcitonin-producing C-cells it is named familial medullary thyroid carcinoma (FMTC) [8, 34].

Specific germline mutations on the *RET* protooncogene are key molecular factors related to the development of FMTC and Multiple Endocrine Neoplasia 2 (MEN 2) syndromes phenotype [34-36]. This topic will not be extensively explored, since it is not the focus of this thesis.

FNMTc accounts for between 3% to 9% of all the thyroid cancer that originate from follicular cells [37-40]. FNMTc is harder to differentiate from sporadic NMTC because of three main reasons: FNMTc is known for being an uncommon condition; histologically speaking, FNMTc tumours do not differ much from sporadic cancers; and the genetics behind FNMTc are heterogeneous and poorly understood [39, 41].

In FNMTc, cancer diagnosis occurs at an early age, presenting as a multifocal disease, and some authors advance that the course of the disease can be aggressive, with local invasion, lymph nodes metastasis, increased risk of recurrence and decrease survival rates when compared to sporadic TC [40], however these are still disputable.

Through clinicopathological findings [34, 38, 39], FNMTc is divided into two categories: syndromic and non-syndromic (**Table 2**).

**Table 2. Familial thyroid cancer.** Categorical division of FNMTTC in syndromic and non-syndromic with the respective gene/chromosomal location associated.

| Familial thyroid cancer        |                           |                                                                                |                           |
|--------------------------------|---------------------------|--------------------------------------------------------------------------------|---------------------------|
| Syndromic                      | Gene/Chromosomal location | Non-Syndromic                                                                  | Gene/Chromosomal location |
| Familial Adenomatous Polyposis | <i>APC/5q21</i>           | familial Papillary Thyroid Carcinoma with or without oxyphilia, fPTC           | <i>TCO/19p13.2</i>        |
| PTEN-hamartoma tumour syndrome | <i>PTEN/10q22-23</i>      | familial Papillary Thyroid Carcinoma associated with renal papillary neoplasia | <i>Unknown/1q21</i>       |
| Werner Syndrome                | <i>WRN/8p11-21</i>        | Familial Non-Medullary Thyroid Carcinoma type 1                                | <i>Unknown/2q21</i>       |
| Carney Syndrome                | <i>PRKAR1/17q24</i>       | Familial Multinodular Goitre                                                   | <i>Unknown/14q</i>        |
| DICER1 syndrome                | <i>DICER1</i>             | -                                                                              | -                         |

#### 1.2.5.1. Syndromic familial non-medullary thyroid carcinoma

Syndromic familial non-medullary thyroid carcinoma comprehends several syndromes where thyroid cancer is not the main phenotypic expression, but cases of non-medullary thyroid carcinoma can be one of the manifestations, such as Familial Adenomatous Polyposis (FAP), PTEN-hamartoma tumour syndrome, Werner Syndrome (WS), Carney Syndrome (CS), DICER1 syndrome, and others [37, 38].

FAP is an autosomal dominant syndrome characterised by germline mutations that inactivate the tumour suppressor Adenomatous Polyposis Coli gene (*APC*). This syndrome is known for the development of multiple adenomatous polyps with malignant potential in the gastrointestinal tract mucosa [34, 37, 42]. A particular subtype of TC, the cribriform morular variant, is characteristic of FAP associated TC.

The major form of PTEN-hamartoma tumour syndrome is characterised by germline mutations in the tumour suppressor gene *PTEN* is a complex syndrome that comprehends manifestations that vary in the clinicopathological presentation, such as Cowden syndrome, Bannayan-Riley-Ruvalcaba syndrome, and Proteus-like syndrome [35, 36].

WS is an autosomal recessive disease characterised by mutations in the Werner Syndrome ATP-dependent helicase (*WRN*) gene. It's clinicopathological characteristics comprehend premature aging, muscular atrophy, diabetes, and a high incidence of neoplasms, such as higher incidence of papillary and anaplastic thyroid cancers [34, 35, 37, 42].

CS is an autosomal dominant syndrome caused by mutations in the Protein Kinase cAMP-Dependent Type I Regulatory Subunit Alpha (*PRKAR1A*) gene. In this syndrome, diseases in the thyroid gland are common, such as cysts, or PTC or follicular thyroid carcinoma [34, 37, 42].

DICER1 syndrome is characterised by germline mutations in *DICER1*. It's a syndrome that shows predisposition for multiple childhood tumours such as pleuropulmonary blastoma, as well as multinodular goitre and differentiated thyroid cancer [38, 43].

#### 1.2.5.2. Non-syndromic familial non-medullary thyroid carcinoma

After extensive and complex statistical analysis, Charkes (2006) [44] inferred that about 62% of families that had two individuals diagnosed with the same subtype or variant of TC may be phenocopies (two sporadic cases associated by chance). Even so, thyroid cancer shows a high incidence of familial aggregation and the reasons for that fact remain elusive. According with the WHO classification, non-syndromic FNMTTC is clinically characterised when one of two conditions are found in a family: either the presence of follicular cell derived thyroid carcinoma in at least three first-degree relatives or the presence of papillary thyroid carcinoma in two or more first-degree family members. In either of the cases, there must exist an absence of history of previous

radiation and inherited syndromic cancer [38, 39, 41]. The genetic alterations of non-syndromic FNMTC are heterogeneous, poorly understood and with less well-defined genetic susceptibility [40, 41].

Although the genetic component is poorly understood, there has been a few reports that advance several genes that may play a role in non-syndromic FNMTC, such as germline mutations in Hyaluronan Binding Protein 2 (*HABP2*) gene [45, 46], mutations in SLIT-ROBO Rho GTPase Activating Protein 1 (*SRGAP1*) gene [46, 47], a germline variant of Forkhead Box E1 (*FOXE1*) gene [46, 48], a germline mutation in Thyroid Transcription Factor-1 (*TITF-1*) [46, 49], mutations in Mitogen-Activated Protein Kinase Kinase 5 (*MAP2K5*) gene [46, 50], and germline mutations in USP13 [51].

Non-syndromic familial non-medullary thyroid carcinoma is characterised by thyroid cancer as the main manifestation, and can be sub-categorised in familial Papillary Thyroid Carcinoma (fPTC) with or without oxyphilia, familial non-medullary thyroid carcinoma type 1 (FNMTC1) and familial multinodular goitre (FMNG) [35, 52-54].

fPTC is characterised by multicentric tumours and multiple adenomatous nodules (MANs) with or without oxyphilia [35, 55].

FNMTC1 presents the characteristics of PTC without any different features than the usual [35, 55].

FMNG is a rare variant in which patients with MNG have been associated with the development of classical PTC [34, 35, 55].

#### 1.2.5.3. Research strategies

In many cases, when exploring at the genetic level of a family that fills the criteria of FNMTC, different candidate genes may appear in the panel of each family [41]. It becomes essential to unravel the genetic basis of this disease as well as identifying molecular biomarkers predictive of aggressiveness or useful for screening for other family members [40, 41].

Therefore, diverse search strategies have been sought to search for novel candidate genes related to the appearance of FNMTC. The first strategy was based on the linkage analysis, which refers to the physical association between two loci. Linkage analysis is an indirect method that tests the co-segregation, in a family, of a chromosomal region or uses genetic markers for a disease to find inherited alleles linked to it [38]. Then, Genome-Wide Association (GWA) studies took shape and polymorphisms have

been evaluated. In this strategy, controls are compared to patients to discover predisposing polymorphisms. Per example, in a large GWA study, two single nucleotide polymorphisms (SNP) related to the risk of FNMTC were identified, rs944289 and rs965513 [38, 56]. Next, studies targeting candidate genes and/or specific metabolic pathways in those regions appeared, and as a result, Pereira *et al.* (2015) [48] studied *FOXE1* in 60 families with FNMTC in Portugal, discovering an association between germline variants of this gene and in some of the studied FNMTC families. Recently, Next-Generation Sequencing (NGS) technique is the most used form of genetic study and provided advances in the genetic field since the study of specific genes was changed for the study and exploration of the entirety of the genome [38]. Hence, the implementation of novel sequencing techniques will promote the advance of genetic studies that may identify the genetic causes in the development of FNMTC.

One proven result of the use of NGS as a discovery strategy was the identification of a Ubiquitin-Specific Protease (USP) gene in a familial form of TC, unravelling USP13 in a family with thyroid cancer [51]. As explained by Maria *et al.* (2022) [51], USP13 is a ubiquitin-specific protease, belonging to the biggest class of deubiquitinating enzymes (DUBs), and its role in cancer is contradictory, since it may act as an oncogene on several cancers [57, 58] and as a tumour suppressor on other cancer types [59-61]. In that work, the genetic cause of diverse thyroid dysfunctions in the same family was investigated and related to a variant of *USP13*. In this work, *in vitro* studies were carried out and the authors observed that this variant in USP13 led to the stabilisation of MDA-32 protein, showed a more severe phenotype, and, when USP13 was overexpressed, an increase in the capacity for colonies formation was seen.

### 1.3. Protein ubiquitination

#### 1.3.1. Ubiquitin

Ubiquitin (**Figure 6**) is a protein composed of 76 aminoacids and its function correlates with proteasomal and lysosomal proteolysis. Ubiquitin participates in a complex process named protein ubiquitination, but it also has non-proteolysis functions, such as subnuclear trafficking or DNA damage repair [62-64].

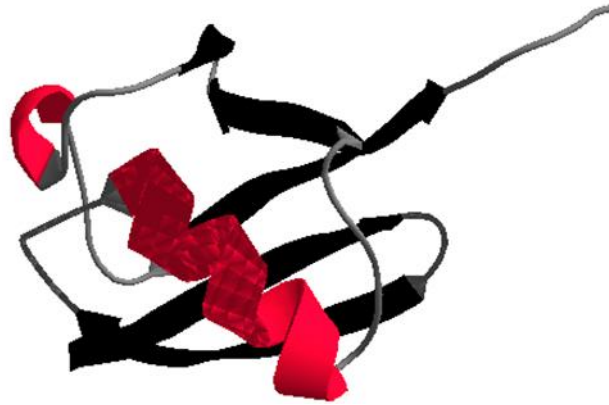


**Figure 6. Ubiquitin.** Ribbon diagram representation, in which the alpha-helix is represented in red, the beta-sheets are represented in black, and the random coils are represented in grey. Created with Swisspdb software.

Ubiquitin is coded by four different genes in the human genome, that can be divided in two types of translational fusions, homomeric and heteromeric fusions. The Ubiquitin B (*UBB*) and Ubiquitin C (*UBC*) genes, that are homomeric fusions, code for polyubiquitin precursor proteins. The Ubiquitin A-52 Residue Ribosomal Protein Fusion Product 1 (*UBA52*) and Ribosomal Protein S27a (*RPS27A*) genes, which code for a single copy of ubiquitin fused to the ribosomal proteins L40 and S27a, respectively, are heteromeric fusions [62, 65]. These four genes encode different sizes' proteins that are non-functional until they are cleaved and the 76 aminoacids protein is released [62].

Ubiquitin has many potential sites for additional post-translational modifications, namely its seven lysine (Lys) residues – K6, K11, K27, K29, K33, K48 and K63, which can all be cleaved to connect with proteins or proteins substrates and/or be ubiquitinated to create isopeptide-linked ubiquitin chains [63, 66-68]. Each ubiquitin linkage type exerts diverse functions: K6 is involved in DNA damage response; K11 is involved in the regulation of cell cycle, proteasomal degradation and membrane trafficking; K27 is involved in the regulation of protein secretion, DNA damage repair, mitochondrial damage response and innate immune system response; K29 is involved in the regulation of 5'-AMP-activated protein kinase pathway (AMPK), proteasomal degradation and innate immune system response; K33 is involved in the regulation of the innate immune system response; K48 is related to proteasomal degradation; and K63 is involved in the regulation of intracellular signalling events in DNA damage repair, cytokine signalling and autophagic degradation signalling [69]. Ubiquitin also has an additional regulation site, one methionine residue – M1, that can generate a linear chain type, and happens specifically when ubiquitin is attached to the N-terminus of a second ubiquitin. As an example of interaction, the M1 site is involved in the regulation of Nuclear Factor kappa B (NF- $\kappa$ B) and Interferons (IFN) signalling [63, 66, 69].

### 1.3.2. Ubiquitination

Protein ubiquitination is a complex, dynamic, and conserved process of post-translational modification [63]. Protein ubiquitination is a process carried out in numerous protein substrates, such as cytosolic, membrane-localised and nuclear proteins, through a cascade of enzymes, which adds ubiquitin to these protein substrates so that they proceed to their functions. These can be linked to proteasomal degradation, cell cycle, apoptosis, localisation, components of signalling pathways, and others [62, 66, 70, 71].

The ubiquitination pathway may lead to monoubiquitination, only one ubiquitin is attached to the protein; multi-monoubiquitination, several ubiquitins can be connected to different lysine site in a single manner; and poly-ubiquitination, a chain of ubiquitins is formed (**Figure 7**) [62, 66, 72].

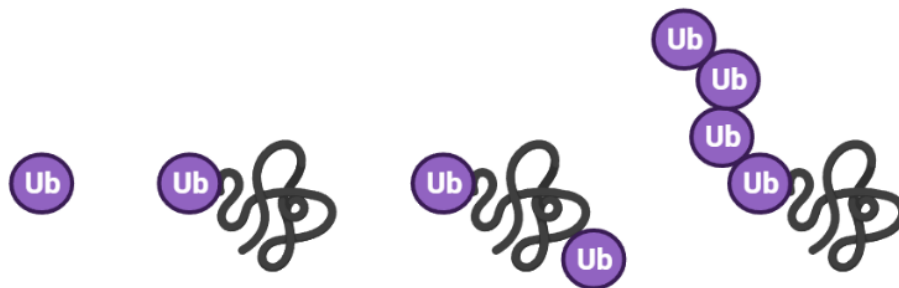


**Figure 7. Ubiquitination products.** On the left a representation of ubiquitin can be found; on the central left a representation of monoubiquitination; on central right a representation of multi-monoubiquitination; and on the right a representation of poli-ubiquitination. Created with Biorender.

The ubiquitination pathway processes in three main biochemical steps (**Figure 8**), which are mediated by three family of enzymes: E1 (ubiquitin-activating enzymes), E2 (ubiquitin-conjugating enzymes) and E3 (ubiquitin ligases) [62, 66, 70].

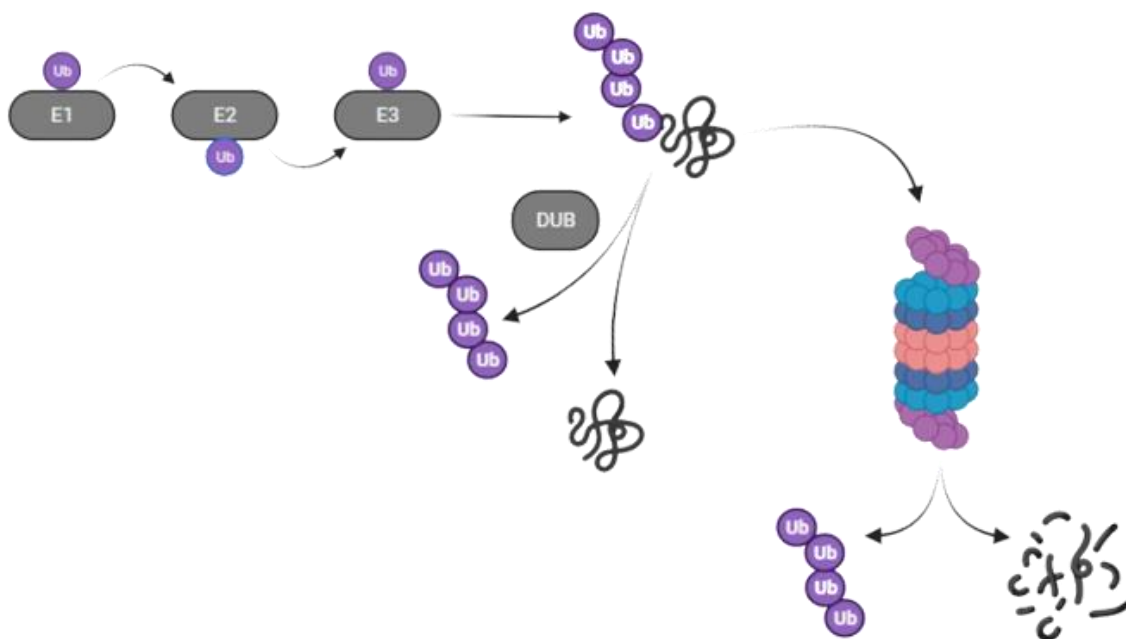


**Figure 8. Ubiquitination pathway.** Created with Biorender.

The first step of the pathway is performed by E1, which catalyses ubiquitin activation by performing adenylation in the C-terminal carboxyl group and forming an intermediate. This intermediate forms a thioester bond, and then is transferred to an active site on a cysteine residue and forms a thioester bond between ubiquitin and E1 (ubiquitin-activating enzyme). This first step is energy-dependent, which means it requires Adenosine Triphosphate (ATP) and magnesium ( $Mg^{2+}$ ) [62, 66, 67, 70]. In the second step of the pathway, the activated ubiquitin is transferred from the E1 to a cysteine residue in a E2 enzyme (ubiquitin-carrier proteins or ubiquitin-conjugating enzymes) and then forms a new thioester bond [62, 66, 70]. The third and last step of the pathway, the activated ubiquitin is transferred to the protein. This is accomplished through an E3 enzyme, which have been advanced that may play a role in cancer progression [69]. This enzyme will also dictate if the transfer occurs in a direct or indirect manner and determine the specificity of the ubiquitin system [62, 66, 70, 73, 74]. The E3 enzymes can be classified in four types, depending on their structure and function: HECT type, RING type, U-box type and RBR type [69, 70].

The first type, HECT (homologous to the E6AP carboxyl terminus) type, contains a common HECT domain, where the E2 enzyme can transfer ubiquitin to the active cysteine site before the binding to the target protein (**Figure 9A**) [69].

The second type, RING type, are the biggest class of E3 enzymes, which can be proven by the more than 600 different RING type found expressed in human cells [75]. The RING type are characterised by their RING domain, which binds to the E2, and the ubiquitin is directly transferred from the E2 to the protein. The RING type can be divided into two separated classes: monomeric RING and multi-subunit RING (**Figure 9B**) [69].

The third type, U-box type, are, to this day, known as the smallest class of E3, but much necessary to control of quality of Post-Translational Modifications [76]. The U-box type has a U-box domain that interacts with the E2, which will directly transfer the ubiquitin from itself to the lysine site of the protein (**Figure 9C**) [69, 77].

The last type, RING-IBR-RING (RING-in between RINGs-RING) type, are a distinctive class, in which the RING1 interacts with the E2 first, the ubiquitin is transferred to the IBR and then to the RING2, and finally to the protein. Moreover, it has been reported [78] that different RING-IBR-RING may be involved in intermolecular autoinhibited protein interactions, such as phosphorylation (**Figure 9D**) [69].

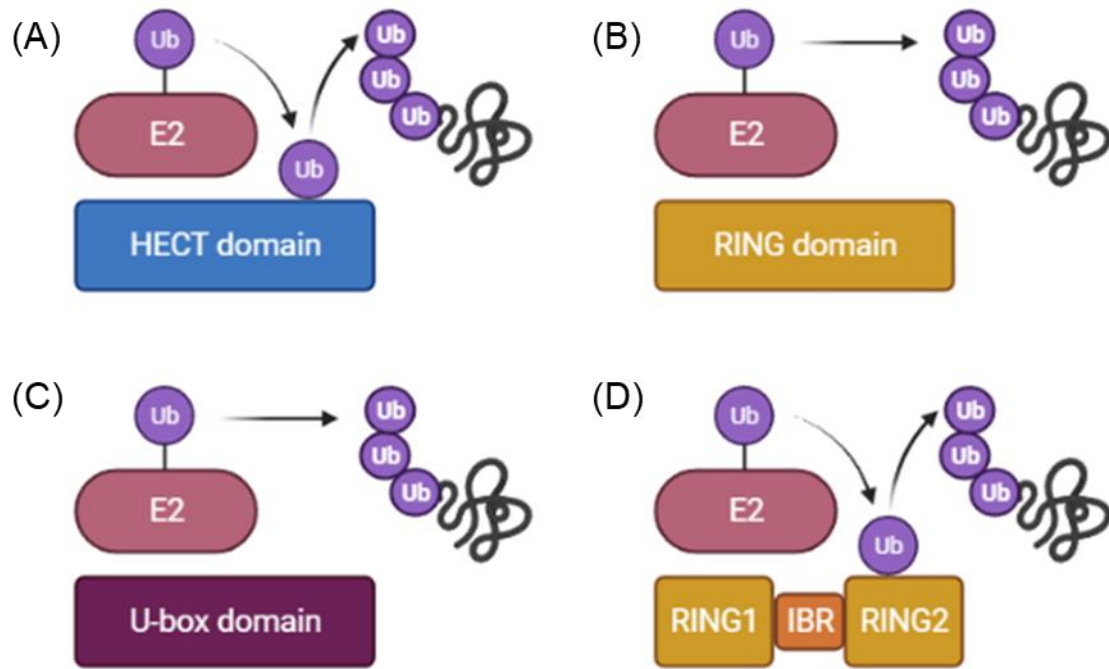


**Figure 9. Types of E3 ligases.** (A) E3 ligase HECT type, (B) E3 ligase RING type, (C) E3 ligase U-box type, (D) E3 ligase RING-IBR-RING type Adapted from ref 57. Created with Biorender.

### 1.3.3. Deubiquitinating enzymes (DUBs)

The ubiquitination pathway, as previously stated, is a dynamic and complex process. It is regulated by E3 enzymes and reversible through DUBs [66, 79, 80]. DUBs are enzymes capable of removing ubiquitin from ubiquitinated proteins, which may regulate their stability and activity [81-83]. The degradation of the bond between ubiquitin and the protein is essential in three processes: protein degradation, ubiquitin synthesis and control of protein traffic [70, 84]. It has been reported that more than one hundred DUBs regulate protein deubiquitination in humans [80, 85].

DUBs are classified in two major classes: Metalloproteinases and Cysteine proteinases.

#### 1.3.3.1. Metalloproteinases

The metalloproteinase has only one known family: JAMM/MPN domain-associated metalloproteinases (JAMMs) [66, 71, 80, 84-88].

This class of DUBs catalyses the hydrolysis of the bond between ubiquitin and proteins through a catalytic serine and a zinc ion factor [89, 90], which means this class incorporates zinc-dependent enzymes. The human genetic code has 14 genes for

metalloproteinases DUBs, only seven of them are capable of acquire the zinc ion, of which six have been proven to possess the ability to perform hydrolysis [71, 90, 91].

### 1.3.3.2. Cysteine Proteinases

The cysteine proteinases class of DUBs has seven families that are differentiated by their catalytic domain and are named: Ubiquitin-Specific Proteases (USPs), Ovarian Tumours Proteases (OTU), Ubiquitin C-terminal Hydrolases (UCHs), Machado-Joseph disease protein domain proteases (MJD), Monocyte Chemotactic Protein-Induced Proteins (MCPIP), Motif Interacting with ubiquitin-containing novel DUB (MINDY), and Zn finger and UFSP domain protein (ZUFSP). [66, 71, 80, 84-88].

On **Table 3**, an example of each family of DUBs can be found.

**Table 3. DUBs families.** Example for each family of DUBs, with their respective class and ubiquitin linkage site.

| DUB           | Class               | Family | Ubiquitin linkage site | Reference |
|---------------|---------------------|--------|------------------------|-----------|
| <b>USP25</b>  | Cysteine proteinase | USP    | K48, K63               | [92]      |
| <b>Otulin</b> | Cysteine proteinase | OTU    | M1                     | [93]      |
| <b>UCHL5</b>  | Cysteine proteinase | UCH    | K48                    | [94]      |
| <b>JOSD1</b>  | Cysteine proteinase | MJD    | K48, K63               | [95]      |
| <b>MINDY1</b> | Cysteine proteinase | MINDY  | K48                    | [96]      |
| <b>ZUFSP</b>  | Cysteine proteinase | ZUFSP  | K11, K48, K63          | [87]      |
| <b>CSN5</b>   | Metalloproteinase   | JAMM   | K63                    | [97]      |

This class of DUBs has a catalytic triad as catalytic domain, which is composed of an active cysteine site, a histidine, and an asparagine (in most of the cases) or an aspartate. Cysteine proteinases' catalytic domain catalyses the hydrolysis of the bond between ubiquitin and proteins [71, 98]. Due to the catalytic site having a cysteine, cysteine proteinases may also be regulated by reactive oxygen species (ROS), therefore adding complexity to the DUBs system [71, 99, 100].

The OTU family is characterised by its seventeen members. These members have specificity for ubiquitin substrates through the usage of other ubiquitin interaction sites, leading them to be known for the regulation of signalling pathways [71, 101].

The MJD family has four members with a highly conserved catalytic domain. This catalytic domain contains two ubiquitin binding sites, two histidines and the catalytic cysteine [71, 102].

The MINDY family has five members with a unique catalytic triad composed of the catalytic cysteine, the histidine and one glutamine, which makes it particularly specific for the K48 ubiquitin cleavage site [71, 86, 102, 103].

Known to date, the ZUFSP family only has one member, which contains two ubiquitin-binding domains. Therefore, it has a particular specificity to the K63 ubiquitin cleavage site [71, 87].

The USP family is the largest family of cysteine proteinases with 58 genes present in the human genome. The USP family has the most conserved catalytic domain amongst its peers and have several accessory domains attached. This large family regulates many cellular functions, such as cell cycle progression, apoptosis, stem cell differentiation, protein turnover, DNA repair, chromosome segregation, cell signalling, and others [66, 71, 84].

A variable number of published studies indicate that the dysregulation of DUBs may be correlated with the development of cancer by either increasing the effects of oncogenes or diminishing the activity of tumour suppressor genes, such as p53 [66, 85].

#### 1.3.4. Association of USPs with cancer

As previously mentioned, the dysregulation of DUBs may be involved in carcinogenesis. Specifically, USPs have been indicated as regulators of tumour formation and proliferation, mainly because they are involved in cell cycle progression, regulation of apoptosis related factors, DNA damage repair activity, and others important cellular functions [66, 85].

Regarding thyroid cancer, there has been some studies that report the involvement of USPs with tumourigenesis. Liang *et al.* (2021) reported that patients with low expression of USP18 had worse survival rate than patients with high expression of USP18, meaning that the downregulation of this USP may be meaningful for PTC proliferation [104]. Jianing *et al.* (2022) reported that USP26 interacts with TAZ protein, stabilising it, and the stabilisation of this protein leads to the proliferation of Anaplastic Thyroid Carcinoma (ATC) [105]. An *et al.* (2015) showed that the knockdown of USP39 decreased cell proliferation and caused cell cycle arrest at G2/M phases, naming that USP39 could be a possible therapeutic target against the proliferation of MTC [106]. Lastly, in the investigation of a family with FNMTTC, through Whole-Exome Sequencing (WES), Maria *et al.* (2022) identified a heterozygous missense variant, in the USP13 gene, p.V495M, and resorting to *in silico* and *in vitro* analysis, concluded that the variant

found would cause the overexpression of USP13, which would consequently play a role in the tumourigenesis of PTC [51].

On **Table 4** a summary of some known associations of USPs with cancer are summarised.

**Table 4. USPs association with cancer.** USPs interaction with different proteins in several cancer types.

| USPs  | Cancer type     | Consequence                                                                               | Reference |
|-------|-----------------|-------------------------------------------------------------------------------------------|-----------|
| USP7  | Cervical cancer | Upregulation of USP7 mediate MDC1 stabilization and promote cervical cancer cell survival | [107]     |
|       | Prostate cancer | Downregulation of USP7 inhibit cell migration and invasion                                | [108]     |
|       |                 | USP7 regulates EZH2 via its deubiquitination and stabilization                            |           |
| USP13 | Thyroid cancer  | USP13 overexpression increases the capability of a single cell to form colonies           | [51]      |
| USP18 | Thyroid cancer  | Downregulation of USP18 in PTCs has an anti-proliferative effect                          | [104]     |
| USP26 | Thyroid cancer  | USP26 promotes ATC progression by deubiquitinating and stabilizing TAZ                    | [105]     |
| USP39 | Thyroid cancer  | Knockdown of USP39 inhibited cell growth due to inactivation of the CDK1/Cyclin B complex | [106]     |
|       |                 | May play a role in MTC proliferation                                                      |           |
| USP42 | Breast cancer   | Upregulation of USP42 promote progression by inhibition of JNK/p38-mediated apoptosis     | [109]     |
|       | Gastric cancer  | USP42 expression was significantly increased in GC tissues                                | [110]     |
|       |                 | Upregulation of USP42 might be important for tumour progression and metastasis of GC      |           |

### 1.3.5. Deubiquitinating enzyme 42 (USP42)

USP42 (**Figure 10**) is a cysteine proteinase DUB that belongs to the family of USPs. USP42 is in the human genome in the chromosome 7 p22.1 [111]. It is broadly expressed in mRNA in human tissues, particularly in the brain, testis, bone marrow and lymphoid tissues [112]. According to the information found on Alliance of Genome Resources, USP42 is predicted to be in the nucleoplasm and be actively functional in the cytosol and nucleus.

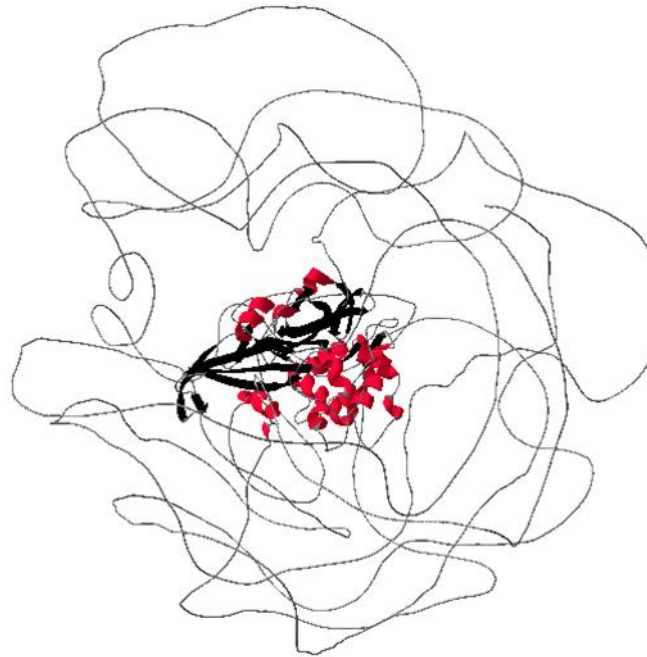


**Figure 10. USP42.** Ribbon diagram representation, in which the alpha-helix is represented in red, the beta-sheets are represented in black, and the random coils are represented in grey. Created with Swisspdb software.

There are several evidences that connect USP42 to the development of cancer, as previously mentioned on **Table 4**. On breast cancer, it was showed that USP42 was upregulated in a series of patients, and *in vitro* results revealed promotion of cell growth, migration, and invasion when USP42 was upregulated, while when USP42 was downregulated, this promotion was not visible and there was a significant increase in the apoptotic rate in cancer cells [109]. On gastric cancer, it was reported that USP42 was more expressed in tumour tissues than non-tumour tissues, and there was a correlation with more aggressive clinicopathological findings. *In vitro* studies of USP42 silencing showed induction of cell cycle arrest in G1 phase, while also promoting the downregulation of proteins that promote the progression of the cell cycle, such as Cyclin D1 and Cyclin E1 [110]. Hock *et al.* (2011) have reported that USP42 controls the levels of p53 ubiquitination, and that is necessary to allow a rapid activation of a p53-dependent cell cycle arrest [113].

As far as we are aware, no research has been done on USP42's biological roles and expression pattern in thyroid cancer.

## Aims

FNMTTC accounts for 3 to 9% of all the thyroid cancer and its genetic causes are heterogeneous and poorly understood. This work is based on the study of a small family (a mother and her twin daughters) that was diagnosed with thyroid cancer, more specifically, they were diagnosed with Oncocytic Variant of Papillary Thyroid Carcinoma (OVPTC). By NGS analysis of the family members, a mutation in the *USP42* gene was found (pG486R). Recent studies have been showing the correlation between USPs mutations and several cancer types.

With this work, the main aim was to validate USP42 alteration as an underlying cause for FNMTTC. Hence, the specific objectives were drawn:

1. **To study in *in silico* databases, public and in-house, the relevance of USPs in thyroid cancer.** The databases were obtained through NGS, and the data provided insights on alterations in USPs, as well as other mutations already described as involved in thyroid cancer pathology. Mutational data will be cross-referenced with available clinicopathological data from the individuals under study.
2. **To carry out *in vitro* studies to evaluate the impact of USP modulation in thyroid cell lines.** In these studies, transient (siRNAs) and stable (CRISPR-RNP) USP42 genetic modification were done in Nthy-Ori 3-1 cell line. More specifically, it was aimed to obtain a knockout (KO) line for this USP gene. To assess its impact in the oncological field and in the clones, USP42 expression levels, proliferation and apoptosis assays will be carried out.

## Chapter 2. Materials and methods

### 2.1. Genetic characterisation of a family

#### 2.1.1. Biological samples and bioinformatics analysis

Peripheral blood samples and Formalin Fixed Paraffin Embedded (FFPE) tumour samples were obtained from three family members diagnosed with OVPTC. A preliminary evaluation by NGS was performed and two germinative variants (*EVC2* – p.R335W, and *USP42* – p.G486R), that were present in the family, were found through the analysis of the *in silico* outputted data. All analysed mutations were confirmed by conducting a new and independent analysis.

Additional bioinformatics analysis was performed using two different databases, one online and one in-house. The online databases were accessed through *cbioportal for Cancer Genomics*. Thyroid cancer databases – namely The Cancer Genome Atlas (TCGA) Cell 2014, TCGA Firehose Legacy and TCGA PanCancer Atlas, were used in the analysis. In particular, 59 genes were evaluated, namely 4 USPs genes (*USP8*, *USP13*, *USP39*, *USP42*), 5 sporadic thyroid cancer associated genes (*BRAF*, *HRAS*, *NRAS*, *KRAS* and *TERTp*), 40 voltage-gated potassium channels genes (*vgKCN*) and 10 voltage-gated potassium channels modulators genes. The two last sets of genes mentioned were included in the analysis but were related to other projects. For this reason, they will not be mentioned throughout this thesis.

The in-house database was from a series of 18 cases of thyroid tumours retrieved from Hospital de São João, that were evaluated by NGS for the same 59 genes aforementioned.

Afterwards, both the databases were evaluated for copy number variation.

An additional independent analysis was performed by a member of the group, for *in silico* results validation.

##### 2.1.1.1. Whole-exome sequencing

DNA extracted from the three affected individuals was subjected to WES using the Illumina HiSeq2000 sequencing platform (San Diego, CA, USA). Sanger sequencing was used to validate the candidate variants identified by WES in these affected family members.

### 2.1.1.2. Variant calling and annotation

For each of the studied samples, raw sequence files were prepared using the Genome Analysis Tool Kit (GATK) (PMID:20644199). Each fastq file was aligned against the human hg19/GRCh37 reference genome using the Burrows-Wheeler Aligner (BWA, v0.7.10) (PMID:19451168) and a variant call format (VCF) file was generated for each sample. PCR duplicates were removed using Picard (<http://broadinstitute.github.io/picard/>); reads around known and detected indels were realigned, and base quality was recalibrated using GATK (PMID:20644199).

All generated VCF files were analysed using The Cancer-Related Analysis of Variants Toolkit (CRAVAT) (PMID:29092935), which is a web-based suite of informatics tools for genomic variant interpretation that includes variant mapping, extensive integrated annotation of variants, genes and proteins, and variant impact scoring, including joint prioritization of all nonsynonymous variants. In addition, the annotations of the built-in variant effect predictors such as ClinVar (PMID:31777943), SIFT (PMID:19561590) and Polyphen-2 (PMID:20354512) were also taken into consideration, as were the allele frequencies of each germline variant present in the overall population, given by gnomAD (PMID:32461652).

### 2.1.1.3. Variant prioritisation

A step-by-step approach was used to filter germline variants that did not meet the following criteria: (i) having an AF threshold > 0.01, (ii) not being present in all three affected individuals, (iii) not being present on Integrative Genomics Viewer (IGV) (PMID:21221095) when visualising the sequence reads, and (iv) not being classified as damaging/probably damaging by Polyphen-2 (PMID:20354512) or deleterious by SIFT (PMID:19561590).

### 2.1.2. DNA extraction

DNA from FFPE material of the family members was obtained in a previous work of the group.

DNA from blood of the family members were obtained through the GRS Total DNA Kit – Blood & Cultured Cells (GrisP Research Solutions, Porto, Portugal) – that allows an efficient and fast method for extraction and purification of high-quality DNA, based on chaotropic salts to lyse cells and to denature proteins, and was also used for

cells lines' DNA extraction. The DNA extraction was performed according to the protocol provided by the manufacturer.

The cultured cells were trypsinised and transferred to a 1,5mL microcentrifuge RNase-free tube. The tubes were centrifuged (5 minutes at 300g), the supernatant was discarded, and the formed pellet was stored at -20°C overnight. The stored pellet was resuspended with 150µL of Red Blood Cell Lysis Buffer and mixed by inversion. Then, 200µL of Buffer BC1 was added to the tube, vigorously shaken for five seconds to mix the components, and incubated for 10 minutes at 70°C in the thermoblock (Thermal Shake *lite*, VWR, USA). After incubation, 200µL of 100% ethanol was added to the tube and shaken vigorously for ten seconds to mix the added component.

Afterwards, 500µL of the total mix was transferred to the genomic DNA mini spin column (provided in the Kit) with a 2mL collection tube. The column was centrifuged (2 minutes at 16 000g), the flow-through in the collector tube was discarded and the genomic DNA mini spin column was put back in the collection tube. The remaining sample was added to the column and centrifuge in the same conditions. Then, the used collection tube was discarded, and the genomic DNA mini spin column was placed in a new collection tube.

For the several washing steps, 400µL of Wash Buffer 1 was added to the genomic DNA mini spin column and centrifuged (30 seconds at 16 000g). The flow-through was discarded and the genomic DNA mini spin column placed back in the collection tube. Then, 600µL of Wash Buffer 2 was added and centrifuged (30 seconds at 16 000g). The flow-through was discarded, the genomic DNA mini spin column placed in the collection tube, and it was centrifuged for 3 more minutes, to dry the column matrix.

The genomic DNA mini spin column was then transferred to a new 1,5mL DNase/RNase-free tube. Then, 20µL of pre-heated Elution Buffer was added in the centre of the column and incubated at room temperature for 5 minutes. After the incubation period, the genomic DNA mini spin columns were centrifuged (1 minute at 16 000g) to elute the extracted and purified total DNA. The column was discarded, and the DNA was stored at -20°C.

### 2.1.3. DNA quantification and purity evaluation

Total DNA concentration was measured using Nanodrop 1000 (Thermo Fisher Scientific, USA). The absorbance ratios were 260/280, which is used to investigate the integrity of DNA and/or RNA, and 230/260, which is used to investigate the purity of the

sample in regard to possible contamination from alcohol or phenols. The first ratio must be between 1.8-2.0 and the second must be between 2.0-2.2 for the sample to be accepted as a pure quality sample.

#### 2.1.4. Polymerase chain reaction (PCR)

The PCR technique was used to check, in DNA samples extracted from blood and tumours of the patients, if the mutations found were present in all the family members. To perform PCR, we used Master Mix, Q solution, Primer Forward (F), Primer Reverse (R), distilled water (dH<sub>2</sub>O) and DNA (**Table 5**). The primes used in the analysis are presented on **Table 6** and were acquired from Integrated DNA Technologies – IDT.

**Table 5. Components of mix for PCR reaction.**

| Components        | For 1 reaction |
|-------------------|----------------|
| Master Mix        | 5µL            |
| Q solution        | 2µL            |
| Primer Forward    | 1µL            |
| Primer Reverse    | 1µL            |
| dH <sub>2</sub> O | 5µL            |
| DNA               | 0,5 - 2µL      |

**Table 6. Primers forward and reverse used in PCR reactions.**

| Primer    | Sequence             | Melting Temperature (T <sub>m</sub> ) |
|-----------|----------------------|---------------------------------------|
| USP42 (F) | CAGCCAAAATGGGTTCTGTT | 53.8°C                                |
| USP42 (R) | TTTGGACAGAAGCTGAAGCA | 54.6°C                                |
| EVC2 (F)  | CACAGAACTGGGGGATGAAC | 55.6°C                                |
| EVC2 (R)  | CTCCTCTCCCTTGTGCTGAC | 57.3°C                                |

Through  $T_m$ , the annealing temperatures ( $T_a$ ) chosen to perform the PCR reaction test were 52°C and 54°C, to verify which one was the most adequate for each set of primers. The followed protocol is described in **Figure 11**.

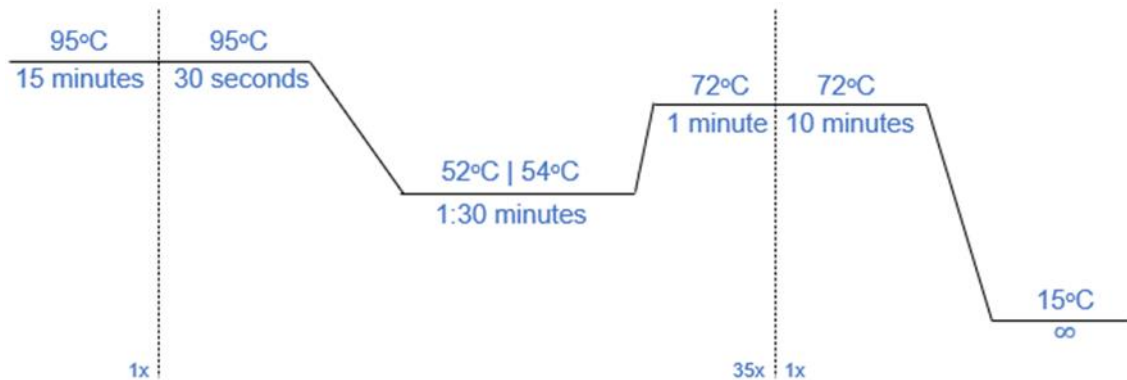


**Figure 11.** Thermocycler conditions applied in the PCR.

The PCR reactions were conducted in the SimplyAmp Thermal Cycler (Applied Biosystems, Life Technologies, USA).

### 2.1.5. Electrophoresis

Electrophoresis was performed to evaluate DNA amplification. The electrophoresis gel was made using 1% agarose (GRS Agarose LE, GrisP Research Solutions, Porto, Portugal) in 0.5x concentrated SGTB buffer (20 X SGTB agarose electrophoresis buffer, GrisP Research Solutions, Porto, Portugal). 2,5µL of the PCR amplified products were mixed with 1µL of Loading Buffer dye containing Gel Red, which intercalates with DNA and gains fluorescence under UV light (VWR International – Material de Laboratório, Lda.). To assess the size of the PCR products in the electrophoresis gel, a DNA Ladder (Invitrogen, CA, USA) was used for each run. Gel revelation was performed by UV light transillumination using the ChemiDoc™ XRS+ System, BIORAD (Universal Hood II, Hercules, CA, USA).

### 2.1.6. PCR product purification

After assessment through electrophoresis, PCR product purification was performed. For that, 2µL ExoSAP was added to the samples. ExoSAP is a mix of two hydrolytic enzymes: Exonuclease I (2 000U, 20U/µL; Thermo Fisher Scientific, USA), which degrades residual single-stranded primers and/or single-stranded DNA from the PCR reaction; and Fast AP Thermosensitive Alkaline Phosphatase (1 000 U, 1 U/ µL;

Thermo Fisher Scientific, Lithuania), which hydrolyses any dNTPs that were not used in the PCR reaction.

The entire protocol was performed on ice, since ExoSAP is active at room temperature. After adding ExoSAP to all the samples, SimplyAmp Thermal Cycler (Applied Biosystems, Life Technologies, USA) was used for the purification process to occur correctly, according with the established protocol (**Figure 12**).

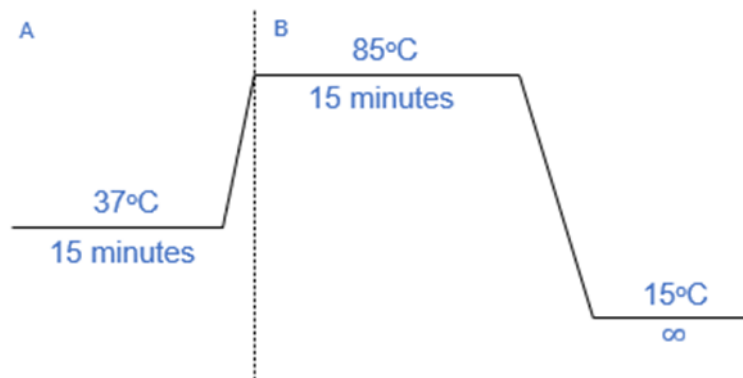


**Figure 12. Thermocycler condition applied for ExoSap reaction.** The first part of the process, designated by A, is the performance of the enzymes. The second part of the process, designated by B, is the inactivation of the enzymes.

### 2.1.7. Sanger sequencing PCR

After PCR product purification, the samples were submitted to Sanger sequencing PCR. A mix containing BigDye, Sequencing Buffer (Applied Biosystems, Life Technologies, USA), the primer of interest, in this case, the primer used was the forward primer, DNase/RNase-free water and the purified PCR product (**Table 7**).

**Table 7. Mix components for Sanger sequencing reaction.**

| Components             | For 1 reaction |
|------------------------|----------------|
| BigDye                 | 0,25μL         |
| Sequencing Buffer      | 3,40μL         |
| Primer of interest     | 0,30μL         |
| DNase/RNase-free water | 3,65μL         |
| Purified PCR product   | 0,5 – 1,5μL    |

The samples were amplified using a specific protocol (**Figure 13**) in SimplyAmp Thermal Cycler (Applied Biosystems, Life Technologies, USA).

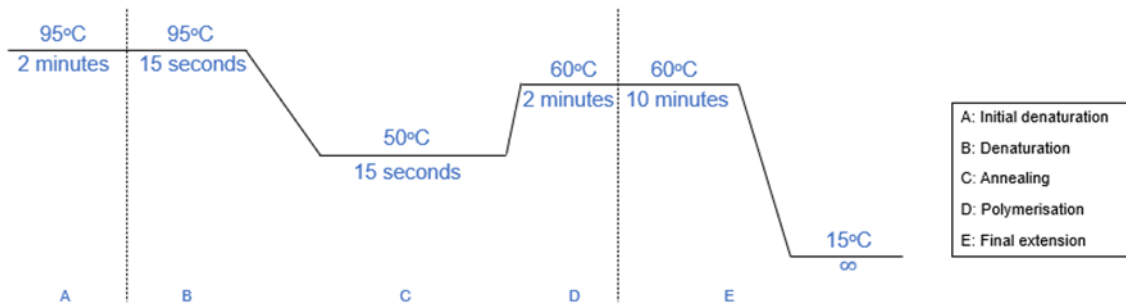


**Figure 13. Thermocycler conditions for the Sanger sequencing reaction.**

### 2.1.8. Sanger sequencing PCR purification and precipitation

The products of Sanger sequencing PCR were then purified using Sephadex™ G-50 resin fine columns (GE Healthcare Life Sciences, Little Chalfont, UK) to eliminate all nucleotide triphosphates (dNTPs and ddNTPs – DNA nucleotides made up of: a nitrogenous base, a sugar and three phosphate group) that did not incorporate the DNA chain while amplification occurred and that could affect the reading signal. All the product of Sanger sequencing PCR was loaded in the column of Sephadex and centrifuged (4 minutes at 3200rpm). After the centrifugation, 15µL of Hi-Di™ Formamide (Applied Biosystems, Life Technologies, USA) was added to each sample to keep the DNA denaturated.

The denaturated samples were analysed at the Genomic Service at i3s by capillary electrophoresis using the Applied Biosystems 3130/3130xl Genetic Analysers (Applied Biosystems, Life Technologies, USA).

## 2.2. Cell culture

The Nthy-Ori 3-1 cell line, used along this study, was gently provided by Dr. Ana Luisa from IPO, Lisbon. The cells were cultured in T75 flasks (TPP Techno Plastic Products AG, Switzerland) in RPMI-1640 medium (Capricorn scientific, Germany), supplemented with 10% foetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, USA) and 1% penicillin/streptomycin (Gibco, Thermo Fisher Scientific, USA). The cells were maintained at 37°C with 5% CO<sub>2</sub> humidified environment in an incubator (HERACell 150i, Thermo Fisher Scientific, USA).

### 2.2.1. Cell cryopreservation

For cryopreservation, the cells were trypsinised with TrypLE Express reagent (Gibco™, Thermo Fisher, USA), incubated for 5 minutes to allow the cells to detach, and then centrifuged at 1500 rpm for 5 min (CompactStar CS4, VWR, USA). Cells were then resuspended in freezing medium (consisting in 10% DMSO [PanReac, AppliChem, Germany] and 90% FBS) and aliquoted in cryogenic storage vials, which were kept in a cryofreezing container containing isopropanol (Nalgene™, Thermo Fisher, USA) and stored at -80°C, allowing a stable decrease in temperature (-1°C per minute).

### 2.2.2. Cell thawing

Cell culture was started from cryopreserved cells, which were in a cryogenic vial containing, and placed in a 37°C water bath. Immediately after defrosting, the cells were resuspended in complete medium. The cell suspension was then centrifuged at 1500 rpm for 5 minutes (CompactStar CS4, VWR, USA). After resuspending the pellet in 1mL of complete medium and then transferred to a T75 culture flask, and then incubated at 37°C.

## 2.3. Silencing USP42 gene using small interference RNA (siRNA)

Predesigned siRNAs directed against Human USP42 were obtained from Qiagen (Quiagen, The Netherlands): Hs\_USP42\_2 – SI00758618 (target sequence: TTGGAGGATACCTAAGATCTA) and Hs\_USP42\_7 – SI03068583 (target sequence: CAGCTTCAAAGAGGTTCACTA), designated throughout this thesis as S2 and S7, respectively. The siRNAs were prepared to a concentration of 20µM. A negative control was also used, Allstars Negative Control (Qiagen, The Netherlands).

In 6-well plates (TPP Techno Plastic Products AG, Switzerland), Nthy-Ori 3-1 cells were plated at a concentration of  $1 \times 10^5$  cells/well. The plating concentration was obtained by previous concentration tests in Nthy-Ori 3-1, in order to establish a 70% confluence after 24h, the time of siRNA transfection. The medium containing the cells was slowly dropped in the wells for a uniform distribution. At the microscope, it was verified that in each well the cells were evenly distributed.

On the next day, the siRNAs were transfected to Nthy-Ori 3-1 cells using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, USA). The transfection process starts by removing the medium from the cells, carefully washing the wells with 1mL of RPMI plain medium to remove remaining FBS and adding 1mL of Opti-MEM to the cells. Afterwards, the siRNAs complexes, whose final concentration in each well is

25nM, are made. To make the complexes two tubes are prepared: one with Lipofectamine 2000 diluted in Opti-MEM (tube 1) and one with the siRNA diluted in Opti-MEM (tube 2). Then, tube 2 is added to tube 1, in that specific order, and incubated for 20 minutes in the dark to form the complexes properly. Nearly at the end of the incubation period, the Opti-MEM was removed from the cells. Then, 250µL of complexes was added alongside 750µL of plain medium. The cells were incubated for 4 hours in standard culture conditions. Afterwards, the medium with the complexes was removed and 2mL of fresh medium (RPMI 1640 + 10% FBS) was added to each well, which counted as the equivalent of the 0 hours timepoint. The untreated cells were kept in complete medium (RPMI 1640 + 10% FBS + 1% Penicillin/Streptomycin)

The cells were collected by trypsinization at the 24, 48 and 72 hours' timepoints. For the trypsinization, the medium in the wells was removed and discarded, 1mL of PBS was added to clean the well from the remnants of FBS and then discarded, and 500µL of trypsin was added and incubated. After 5 minutes, the trypsin was inactivated by adding 800µL of medium. Then, the medium containing the cells was transferred to a DNA/RNA-free microcentrifuge tube and centrifuged (5 minutes at 300g) to form pellets. These were either stored at -20°C to subsequent analysis of RNA and protein, or fixed with 70% ethanol for cell cycle analysis, or immediately used for apoptosis analysis.

## 2.4. RNA extraction

### 2.4.1. Total RNA extraction with Trizol

For total RNA extraction, the formed pellet was resuspended with 500µL of Trizol, in the hood, and stored at -20°C.

For RNA purification, the samples were defrosted on ice and the entirety of the protocol was done on ice, in the hood. After defrosting, 100µL of chloroform was added, the tubes were inverted 15 times to homogenise, and then incubated for 5 minutes at 4°C. After the incubation period, the samples were centrifuged (15 minutes at 12 000g at 4°C), in order to separate the phases. Then, while been extremely careful, the aqueous phase was removed (the first phase) and transferred to a new 1,5mL RNase/DNase-free microcentrifuge tube. Equal volume of RNase-free 100% isopropanol were added to the tube with the aqueous phase (typically 250µL). The mix was incubated for 10 minutes at room temperature, and then centrifuged for 15 minutes at 12 000g at 4°C. The supernatant was removed while being cautious of the pellet. Then, the pellet was washed, without being resuspended, with 900µL of RNase-free 75% ethanol and

centrifuged (15 minutes at 12 000g, 4°C). The supernatant was removed, while being extremely cautious of the pellet, which at this point is almost not visible. The entirety of the ethanol must be removed to have RNA with quality. The formed pellet was air dried for 5 minutes, and then suspended with 20µL of pre-heated RNase-free water.

#### 2.4.2. RNA extraction by the GRS Total RNA Kit – Blood & Cultured Cells

Using total RNA extraction with Trizol did not provide good quality and quantity of RNA. An alternative method was tested, and RNA extraction started to be performed with the GRS Total RNA Kit – Blood & Cultured Cells (GrisP Research Solutions, Porto, Portugal).

The GRS Total RNA Kit – Blood & Cultured Cells (GrisP Research Solutions, Porto, Portugal) – stipulate an efficient and fast method for extraction and purification of high-quality RNA. The RNA extraction was performed according to the protocol provided by the manufacturer. All the reagents utilized were included on the Kit, except ethanol 100%, ethanol pro-analysis 70% and β-mercaptoethanol.

The stored pellet was resuspended with 100µL of Red Blood Cell Lysis Buffer, to lyse the cells. Then, 400µL of Buffer R1 and 4µL of β-mercaptoethanol was added to the microcentrifuge tube. The tube was shaken vigorously to mix the added components and then, incubated for 5 minutes at room temperature. Then, 500µL of 70% ethanol was added to the tube and shaken vigorously to mix the added component.

Afterwards, 500µL of the total mix was transferred to the RNA mini spin column (provided in the Kit) in a 2mL collection tube. The RNA mini spin column was centrifuged (1 minute at 16 000g), the flow-through in the collection tube was discarded and the RNA mini spin column was put back in the collection tube. The remaining sample was added to the RNA mini spin column and centrifuge in the same conditions. Then, the used collection tube was discarded, and the RNA mini spin column was placed in a new collection tube.

For the washing steps, 400µL of Wash Buffer 2 was added to the RNA mini spin column and centrifuged (30 seconds at 16 000g). The flow-through was discarded and the RNA mini spin column placed back in the collection tube. A mix containing 45µL of DNase I reaction buffer and 5µL of DNase I solution was prepared for each sample, a volume of 50µL was pipetted in the centre of each column and incubated at room temperature for 10 minutes. After, 400µL of Wash Buffer 1 was added and centrifuged (30 seconds at 16 000g), the flow-through was discarded, the RNA mini spin column

placed back in the collection tube, and 600µL of Wash Buffer 2 was added and centrifuged (30 seconds at 16 000g). The flow-through was discarded, the RNA mini spin column placed in the collection tube, and additional 600µL of Wash Buffer 2 were added and centrifuged (30 seconds at 16 000g). The flow-through was discarded, the RNA mini spin column placed in the collection tube, and it was centrifuged for 3 more minutes to dry the column matrix.

The RNA mini spin column was then transferred to a new 1,5mL RNase free centrifuge tube, 20µL of RNase-free water was added in the centre of the column and incubated at room temperature for 2 minutes. After the incubation period, the RNA mini spin columns were centrifuged (1 minute at 16 000g) to elute the extracted and purified total RNA. The column was discarded, and the RNA was stored at -80°C for subsequent use.

## 2.5. RNA quantification and purity evaluation

Total RNA concentration was measured using Nanodrop 1000 (Thermo Scientific, USA). The absorbance ratios were 260/280, which is used to investigate the integrity of DNA and/or RNA, and 230/260, which is used to investigate the purity of the sample in regard of possible contamination from alcohol or phenols. The first ratio must be between 1.8-2.0 and the second must be between 2.0-2.2 for the sample to be accepted as a pure quality sample.

## 2.6. DNase I treatment and conversion of RNA to cDNA

DNase I treatment and conversion of RNA to cDNA was performed in a reaction volume of 10µL, composed of 1µL of 10x RB (ThermoScientific, USA), 1µL of DNase I (ThermoScientific, USA), 1000ng of total RNA extracted, and the remaining volume was completed with RNase-free water. After, an incubation for 30 minutes at 37°C was performed in the SimplyAmp Thermal Cycler (Applied Biosystems, Life Technologies, USA).

Then, an inhibitor, in this case 1µL of EDTA (ThermoScientific, USA), was added to the sample and a 10 minute incubation at 65°C was done in the SimplyAmp Thermal Cycler (Applied Biosystems, Life Technologies, USA).

A random primer, in this case 1  $\mu$ L of Random Hexamer Primer (ThermoScientific, USA), was added to the sample. The SimplyAmp Thermal Cycler (Applied Biosystems, Life Technologies, USA) was used for a further incubation at 65°C, for 5 minutes.

Afterwards, the samples were immediately put on ice, and two different reverse transcriptase mixes were made: one for all the samples and one for the negative control, which is used to control the purity of the reaction. The first mix is composed of H<sub>2</sub>O, 5x RB, Ribolock – inhibits the activity of RNases A, B and C by binding them in a non-competitive mode – dNTPs, and RT enzyme – enzyme used to catalyse cDNA synthesis (Table 8).

Table 8. Mix components for RNA to cDNA conversion.

| Components for mix 1 | For 1 sample | Components for mix 2 | For 1 sample |
|----------------------|--------------|----------------------|--------------|
| dH <sub>2</sub> O    | 0,5 $\mu$ L  | dH <sub>2</sub> O    | 0,5 $\mu$ L  |
| 5x RB                | 4,0 $\mu$ L  | 5x RB                | 4,0 $\mu$ L  |
| Ribolock             | 0,5 $\mu$ L  | Ribolock             | 0,5 $\mu$ L  |
| dNTPs                | 2,0 $\mu$ L  | dNTPs                | 2,0 $\mu$ L  |
| RT enzyme            | 1,0 $\mu$ L  | dH <sub>2</sub> O    | 1,0 $\mu$ L  |

Both mixes were added to the iced samples and then put in the SimplyAmp Thermal Cycler (Applied Biosystems, Life Technologies, USA) (Figure 14). All the components were from ThermoScientific (USA).

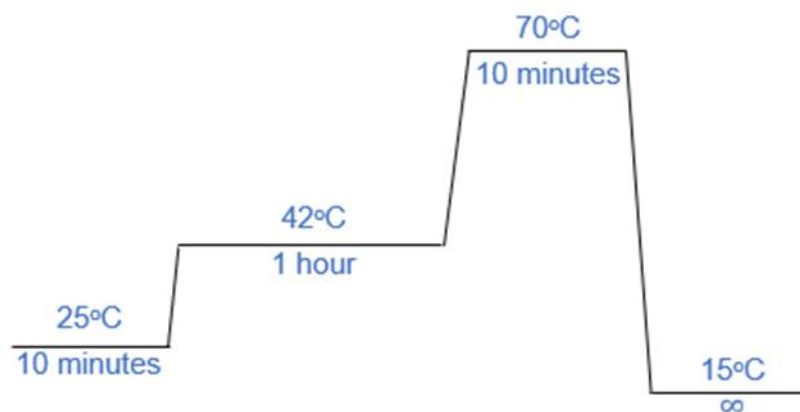


Figure 14. Thermocycler condition applied for RNA to cDNA conversion.

## 2.7. Reverse Transcriptase-quantitative Polymerase Chain Reaction

After RNA conversion to cDNA, Reverse Transcriptase-quantitative Polymerase Chain Reaction (RT-qPCR) analysis was performed on QuantStudio5 (Applied Biosystems, Life Technologies, USA). To perform this analysis, an endogenous gene,

TATA Binding Protein (TBP), was used as a control gene. Two bulk mixes were prepared for all the samples, one for TBP expression (**Table 9**) and one for USP42 expression (**Table 9**). The first was composed of an enzyme – TaqMan® Universal PCR Master Mix - No AmpErase® UNG (Ref: 4324018, ThermoFisher Scientific, USA); a TBP probe – Hs.PT.39a.22214825 (Ref: 379288377, Integrated DNA Technologies – IDT, USA); and H<sub>2</sub>O. The latter was composed of an enzyme – TaqMan® Universal PCR Master Mix - No AmpErase® UNG (Ref: 4324018, ThermoFisher Scientific, USA); a USP42 probe – Hs.PT.58.670189 (Ref: 378619960, Integrated DNA Technologies – IDT, USA); and H<sub>2</sub>O.

**Table 9.** Mix components for RT-qPCR for TBP and USP42 genes.

| Components       | For 1 sample | Components       | For 1 sample |
|------------------|--------------|------------------|--------------|
| TaqMan           | 5µL          | TaqMan           | 5µL          |
| TBP probe        | 0,5µL        | USP42 probe      | 0,5µL        |
| H <sub>2</sub> O | 3,5µL        | H <sub>2</sub> O | 3,5µL        |
| cDNA             | 1µL          | cDNA             | 1µL          |

The samples – Negative control (NC), S2, S7 and Untreated (U) – were distributed in triplicate in MicroAmp™ Optical 96-Well Reaction Plate (Ref: N8010560, Thermo Fisher Scientific, USA).

The protocol used on QuantStudio™ 5 Real-Time PCR System for Human Identification (Applied Biosystems, Life Technologies, USA) is described on **Figure 15**.

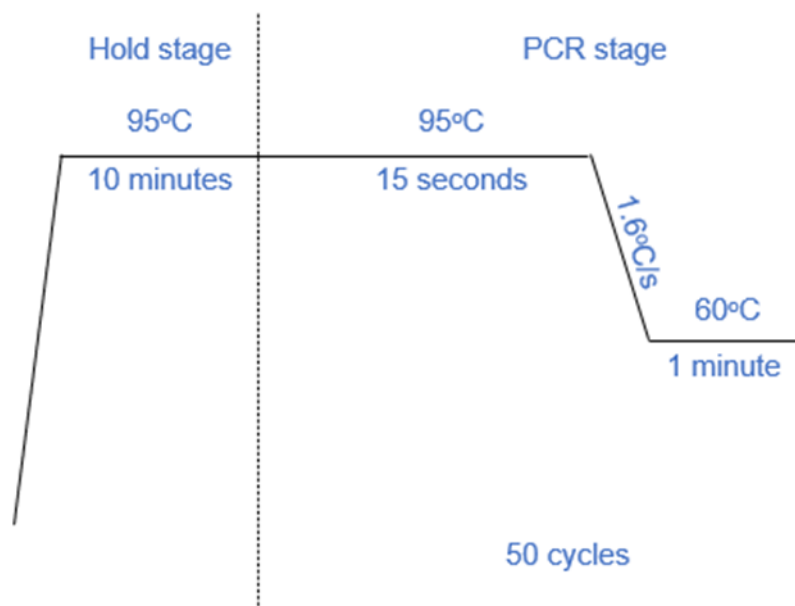


**Figure 15.** Thermocycler condition applied for RT-qPCR reaction.

## 2.8. Protein extraction and quantification for Western Blot

The cultured cells were trypsinised and transferred to a 1,5mL RNase-free tube. The microcentrifuge tubes were centrifuged (5 minutes at 300g), the supernatant was discarded, and the formed pellet was stored at -20°C.

For protein extraction, the stored cell pellet was defrosted on ice, and a specific buffer was used to extract the total protein – radioimmunoprecipitation assay buffer (RIPA buffer) mix. The final mix, for one sample, is composed of 95µL of RIPA buffer, 1µL phosphatases inhibitor and 4µL proteases inhibitor. So, a total of 100µL RIPA mix was added to each sample, incubated on ice for 15 minutes, and centrifuged for 10 minutes at 14 000rpm at 4°C. After the centrifugation, the lysate was transferred to a new 1,5mL microcentrifuge tube and the pellet was discarded. From the lysate, 3µL were removed to a new 1,5mL microcentrifuge tube and store at -20°C, the rest of the lysate was preserved at -80°C.

For protein quantification modified Lowry assay was performed on ice. To prepare the samples, the 3µL of lysate were diluted with dH<sub>2</sub>O in a proportion of 1:5. So, to the 3µL of lysate 12µL of dH<sub>2</sub>O was added. On a 96-well plate (TPP Techno Plastic Products AG, Switzerland), in the first two columns, a blank sample and a series of standard solutions with known protein concentrations were added in duplicate. These standard solutions were 7 different solutions with increasing concentrations of BSA: 0,25µg/mL, 0,50µg/mL, 0,75µg/mL, 1,0µg/mL, 1,25µg/mL, 1,5µg/mL, 3µg/mL. To the remaining wells, 5µL of each sample were added in duplicate (**Figure 16**).

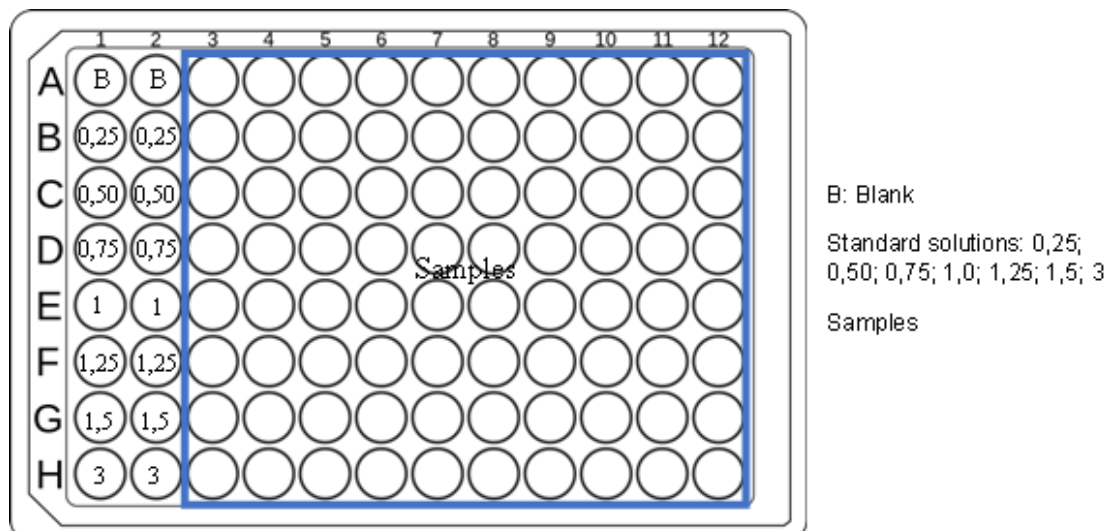


**Figure 16. Protein quantification plate.** Example of protein quantification plate with wells, in duplicate, for blank sample, BSA standard solutions with known concentration and samples.

For this specific assay, a master mix solution was prepared at room temperature, containing Reagent A and Reagent S. The total volume of Reagent A used was 25µL of

Reagent A times the number of samples to be quantified; for each 1000µL of Reagent A, 20µL of Reagent S was added. From this mix, 25µL was added to each well containing sample. Then, 200µL of Reagent B was added to each well and incubated for 10 minutes in the dark. After the incubation, the absorbance of the samples was read at 650nm on SpectraMax® iD3 (Molecular Devices, USA). The output data from SpectraMax® iD3 was collected and a calibration curve was created. Through the calibration curve, the quantity of protein in each well was extrapolated.

## 2.9. Western Blot

For Western Blot analysis, the samples were prepared at the concentration of 15µg of protein per well for a final volume of 20µL in the previous day and stored at -20°C. This volume was composed of 5µL of LB marker, the equivalent volume for 10µg of protein from each sample and the remaining was dH<sub>2</sub>O.

Acrylamide gels were prepared with 7,5% of resolving gel (**Table 10**) and 4% of stacking gel (**Table 10**) in previous day and put on the fridge overnight, soaked in Running Buffer (for 1 litre: 100mL of TGS 10x [GrisP Research Solutions, Porto, Portugal] + 900mL of dH<sub>2</sub>O).

**Table 10. Components of Acrylamide gels for Western Blot technique.**

| Resolving 7,5%        |         |         | Stacking 4%           |         |         |
|-----------------------|---------|---------|-----------------------|---------|---------|
| Number of gels        | 1x      | 2x      | Number of gels        | 1x      | 2x      |
| Acrylamide 30%        | 2,5 mL  | 5 mL    | dH <sub>2</sub> O     | 2,39 mL | 4,78 mL |
| Tris HCl 1,5M, pH=8.8 | 2,5 mL  | 5 mL    | Tris HCl 0,5M, pH=6.8 | 1 mL    | 2 mL    |
| dH <sub>2</sub> O     | 4,84 mL | 9,68 mL | Acrylamide 30%        | 530 µL  | 1060 µL |
| SDS-PAGE              | 100 µL  | 200 µL  | SDS-PAGE              | 40 µL   | 80 µL   |
| APS                   | 100 µL  | 200 µL  | APS                   | 40 µL   | 80 µL   |
| Temed                 | 10 µL   | 20 µL   | Temed                 | 4 µL    | 8 µL    |

On the next day, the prepared samples were defrosted and warmed at 95°C with shaking, in order to denature the proteins. Then, a spin-down is made and the samples are loaded in the gel. The gel ran for about 20 minutes at 80V for the samples to enter

the resolving gel, and then the voltage was augmented to 100/120V, and the gel ran for about 1:30h.

After the gel run, the material on the gel was transferred to a nitrocellulose membrane. To perform the transfer, for each individual gel, the following material, in this exact order, is needed: a transference cassette with the black side facing down, 1 foam, 2 filter papers of 3mm, the gel, 1 nitrocellulose membrane, 2 filter papers of 3mm, 1 foam and the other side of the transference cassette. While assembling, every component was wet in Transfer Buffer (for 1 litre: 100mL of Transfer Buffer 10x + 700mL of dH<sub>2</sub>O + 200mL of methanol) and rolled out to avoid any bubbles in between components. Then, the cassettes were put inside the transfer system, which was filled with Transfer Buffer. A magnet was added inside the system, which was put on a tray filled with ice on top of a magnetising machine. Then, the transfer system ran at 500mA for 2h.

After the transfer, membranes were removed from the transfer system and washed with dH<sub>2</sub>O for 5 seconds. Then, Ponceau S (Sigma-Aldrich, USA) was poured over the membranes to reveal the total protein loaded for 30 seconds with slow shaking. After scanning the stained membrane, the Ponceau S was removed, and the excess pigment was washed away with several dH<sub>2</sub>O washes. When no staining was visible, the membranes were put to dry on top of a filter paper for approximately an hour.

Afterwards, the membranes were cut and rehydrated, and the blockage of the membrane was performed with either 5% milk (5g of non-fat powder milk with 100mL of TBS 1x) or 5% BSA (5g of BSA with 100mL of TBS 1x), for an hour. Then, the membranes were incubated with the primary antibody (**Table 11**) overnight at 4°C with rotation.

**Table 11. Antibodies used for Western Blot technique.**

| Primary antibody                   | Dilution | Animal of origin | Product reference | Brand                    |
|------------------------------------|----------|------------------|-------------------|--------------------------|
| <b>USP42</b>                       | 1:1000   | Mouse            | sc-390604         | Santa Cruz Biotechnology |
| <b><math>\alpha</math>-Tubulin</b> | 1:5000   | Mouse            | T5168             | Sigma                    |
| <b><math>\beta</math>-Actin</b>    | 1:1000   | Mouse            | sc-47778          | Santa Cruz Biotechnology |
| <b>Vinculin</b>                    | 1:3000   | Mouse            | V9131             | Sigma                    |
| <b>HSP70</b>                       | 1:8000   | Mouse            | sc-32239          | Santa Cruz Biotechnology |
| <b>GAPDH</b>                       | 1:1000   | Mouse            | sc-32233          | Santa Cruz Biotechnology |
| <b>p53</b>                         | 1:3000   | Mouse            | NCL-L-p53-DO7     | Leica                    |
| <b>Cyclin D1</b>                   | 1:10000  | Rabbit           | ab134175          | Abcam                    |

On the next day, the membranes were washed 4 times, 5 minutes for each wash, with TBS-Tween 0,1% (TBS-T) on a rolling shaker. After the washes, the membranes were incubated with the secondary fluorescent antibody (800nm) at room temperature with rotation for 1 hour.

Afterwards, the bands on the membranes were visualised using Odyssey<sup>®</sup> CLx Infrared Imaging System (LI-COR Biosciences, USA) and read at 800nm absorbance.

## 2.10. Cell cycle analysis

For cell cycle analysis, the cells were cultured in 6-well plates and followed the same transfection protocol described in the section 2. *Silencing USP42 gene using small interference RNA (siRNA)*. For the collection process, the medium in which the cells were cultured was collected to a 15mL falcon. The cells were washed with PBS, which was collected to the previous falcon. Then, the cells were trypsinised, collected to 15mL falcon and centrifuged (5 minutes at 300g). The supernatant was discarded, and the pellet resuspended in 2mL of freezing 70% ethanol, the ethanol was added drop by drop, using the vortex at the same time, and left overnight at 4°C. Then, the falcons were centrifuged (5 minutes at 300g), the supernatant was discarded, and the droplets of

ethanol remaining in the falcons were airdried. Afterwards, the cells were resuspended in 200 $\mu$ L of DNA staining solution, which is a mix of PBS 1x, propidium iodide 5 $\mu$ g/mL (Sigma-Aldrich), and RNase 100 $\mu$ g/mL. The falcons were incubated for 15 minutes. After the incubation period, 300 $\mu$ L of the samples were filtered to eppendorfs, and read in the flow cytometer, BD Accuri C6 (BD Biosciences, USA). The analysis of the output data was performed on FlowJo (FlowJo™ Software version 10.9, BD Biosciences, USA).

### 2.11. Annexin-V assay

For apoptosis analysis, Annexin-V assay was performed. The cells were cultured in 6-well plates and followed the same transfection protocol described in the section 2. *Silencing USP42 gene using small interference RNA (siRNA)*. For the collection process, the medium in which the cells were cultured was collected to a 15mL falcon. The cells were washed with PBS and collected to the previous falcon. Then, the cells were trypsinised, collected to 15mL falcon and centrifuged (5 minutes at 300g). The supernatant was discarded, and the pellet resuspended in 400 $\mu$ L of Binding Buffer 1x, which was composed of a dilution of Binding Buffer 5x (Thermo Fisher Scientific, USA) in DNase/RNase-free water. Then, 195 $\mu$ L of the samples were put on a 1,5mL microcentrifuge tubes. The tubes with the samples were put on ice, 5 $\mu$ L of Annexin-V FITC (ImmunoTools, Germany) was added to each tube, and incubated for 30 minutes at room temperature in the dark. Then, 10 $\mu$ L of Propidium Iodide 50 $\mu$ g/mL, obtained by mixing 5 $\mu$ L of Propidium Iodide 1mg/mL and 95 $\mu$ L of Binding Buffer 1x, was added to the samples and incubated for 5 minutes in the dark. After the incubation period, 300 $\mu$ L of the samples were filtered to new tubes, and read in the flow cytometer, BD Accuri C6 (BD Biosciences, USA). The analysis of the output data was performed on FlowJo (FlowJo™ Software version 10.9, BD Biosciences, USA).

### 2.12. Knockout of *USP42* gene using CRISPR-RNP

For CRISPR-RNP technique to be performed, purified Cas9 Protein can be combined with guide RNA to form a ribonucleoprotein (RNP) complex to be delivered to cells for rapid and highly efficient genome editing. For this, the Invitrogen™ TrueGuide™ Synthetic gRNA was used to perform CRISPR-RNP, and all the products used were bought from Thermo Fisher Scientific (USA). To obtain all the cellular clones necessary for the experimental conditions, a positive control – CDK4, a negative control – NC, USP42 knockout, a single-guide RNA (sgRNA) for each condition and a donor DNA

(dDNA) for USP42 were created using TrueGuide Synthetic gRNA tool from Thermo Fisher Scientific, specified on **Table 12**. The protocol provided by the Thermo Fisher Scientific was optimised regarding the plating concentrations used.

**Table 12. Sequences of USP42 donor DNA and sgRNAs for USP42 knockout using CRISPR-RNP technique.**

| Material                      | Sequence                                                                                           |
|-------------------------------|----------------------------------------------------------------------------------------------------|
| <b>USP42 Donor DNA</b>        | ATGTGTCAAATCACACACTTTCTTTAGGACCAGTA<br><b>TGATGATGATGACCTGGTGCTGTA</b><br>GTTTATTTCGAGTTCATCTGTACC |
| <b>sgRNA USP42</b>            | 5'-TAAACTACAGCACCAGGTAC-3'                                                                         |
| <b>sgRNA CDK4</b>             | 5'-CACUCUUGAGGGCCACAAAG-3'                                                                         |
| <b>sgRNA Negative Control</b> | 5'-AAAUGUGAGAUCAGAGUAAU-3'                                                                         |

The cells were cultured in a 96-well plate at the concentration of  $2,5 \times 10^3$  cells/well with RPMI 1640 + 10% FBS and incubated for 24 hours. For the transfection steps, since the protocol provided was being used, some of the volumes were not pipettable and the necessary dilutions were made. Therefore, four mixes were prepared, according to the protocol (**Table 13**).

**Table 13. Components of each mix for USP42 knockout using CRISPR-RNP technique.**

| Components                     | First mix – CDK4 | Second mix – NC | Third mix – USP42 | Fourth mix |
|--------------------------------|------------------|-----------------|-------------------|------------|
| <b>Opti-MEM</b>                | 10,5µL           | 10,5µL          | 10,5µL            | 31µL       |
| <b>Cas9 protein</b>            | 2,1µL            | 2,1µL           | 2,1µL             | -          |
| <b>sgRNA</b>                   | 3,15µL           | 3,56 µL         | 3,15µL            | -          |
| <b>dDNA</b>                    | -                | -               | 3,57µL            | -          |
| <b>Lipofectamine CRISPRMAX</b> | -                | -               | -                 | 1,86µL     |

The fourth mix was the last to be made, since after adding Lipofectamine CRISPRMAX to Opti-MEM, an incubation for 1 minute at room temperature was necessary. Then, the content of the last mix was equally divided in the three eppendorfs and incubated for 10 minutes to form the complexes. While the complexes were forming, the medium in the plate was removed and 100µL of fresh medium was added. After the incubation period, 10µL of the complexes were added to the wells. The cells rested for 2 days, and on the third day the medium was exchanged.

## 2.13. Clonal selection

The CRISPR-RNP transfected cells that were cultured in a 96-well plate were allowed to grow and sequentially moved from a 96-well plate to 12-well plate, T25 flask and T75 flask. When the cells of each condition – CDK4, NC and USP42 knockout – achieved a T75 flask, the clonal selection started.

The cells were trypsinised, collected and counted in a Neubauer chamber. A dilution was performed in order to have 100 cells/mL. The diluted cells were distributed and cultured in a 96-well plate, having 100µL of medium. Then, later in the same day, after giving some time for the cells to adhere to the bottom of the well, the plate was observed to try to identify which wells had a single cell in them and the corresponding well was marked. This protocol was performed for each CRISPR-RNP condition.

## 2.14. Molecular characterisation of USP42 knockout clones

### 2.14.1. Polymerase chain reaction

The Polymerase chain reaction (PCR) technique was used to check the purity of the clones, as to verify that the cells that grown on a specific well all came from one cell only, and that the clone had the knockout that was intended. To perform PCR, Master Mix, Q solution, Primer Forward, Primer Reverse and dH<sub>2</sub>O was used. The primers used in the analysis are represented on **Table 14**. The annealing temperature was 62°C and was determined after protocol optimisation (**Figure 17**).

**Table 14.** Primers forward and reverse used for PCR reaction.

| Primer         | Sequence             | Melting Temperature (T <sub>m</sub> ) |
|----------------|----------------------|---------------------------------------|
| <b>USP42 F</b> | ATCTTCAGACCCATCAGCCT | 58.1°C                                |
| <b>USP42 R</b> | ATACAACTCTGCTGGCCACT | 59.0°C                                |

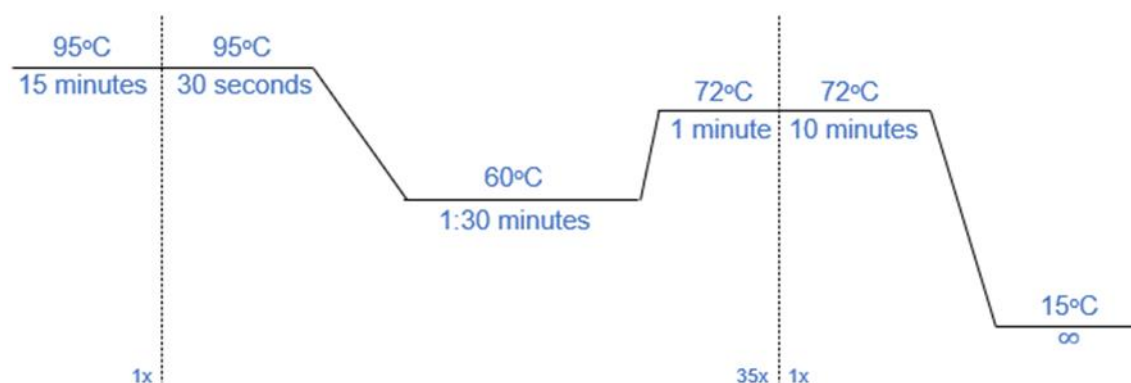


**Figure 17.** Thermocycler conditions applied for PCR reaction.

All PCR reactions were conducted in the SimplyAmp Thermal Cycler (Applied Biosystems, Life Technologies, USA).

The entire process after the PCR reaction is the same as aforementioned on the topics 2.1.5. to 2.1.8..

## 2.15. Statistical analysis

Data analyses were performed using GraphPad Prism 8.0.2. The results are presented with its respective means and standard errors. Tukey's multiple comparisons tests (non-parametric two-way ANOVA) was performed to compare the samples in mRNA expression analysis and apoptosis analysis. Dunnett's multiple comparisons tests (non-parametric two-way ANOVA) was performed to compare the samples in protein expression analysis. The symbol (\*) was used to signify the statistical significance between samples and the colour blue was used for the comparison untreated versus treated cells, while the colour black was used for the comparison between treated cells. Samples were considered statistically significant when  $p\text{-value} < 0.05$  (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\*  $p \leq 0.001$ ; \*\*\*\*  $p \leq 0.0001$ ) and confidence interval  $\neq 1$ .

## Chapter 3. Results

### 3.1. Molecular characterisation of a family to confirm *in silico* data

#### 3.1.1. Biological samples

Biological samples were collected from a family with FNMTTC, a mother (R1) and her two twin-daughters (R2 and R3), that were diagnosed with OVPTC. The mother was the proband, being diagnosed at the early age of 36 years old, and, later, the twin-daughters were diagnosed when they were both 19 years old.

Through a NGS analysis conducted in the blood and tumour tissues of the patients, a new validation of all the data was performed and two possible gene mutations were deemed as likely to be causative of tumourigenesis in this family: *EVC2* and *USP42* (Table 15).

**Table 15. Filtered NGS results.** Filtering the NGS results from the tumour and blood samples from the family, two genes, *EVC2* and *USP42*, remained as possible to potentiate tumourigenesis, since they were found in the three affected individuals.

| Gene  | Chromosome | Loss of homozygosity | Reference base(s) | Alternate base(s) | Protein sequence change | ClinVar                | FATHMM    | SIFT        | POLYPHEN          |
|-------|------------|----------------------|-------------------|-------------------|-------------------------|------------------------|-----------|-------------|-------------------|
| EVC2  | chr4       | Yes                  | G                 | A                 | R335W                   | Uncertain significance | Cancer    | Deleterious | benign            |
| EVC2  | chr4       | Yes                  | G                 | A                 | R335W                   | Uncertain significance | Cancer    | Deleterious | benign            |
| EVC2  | chr4       | Yes                  | G                 | A                 | R335W                   | Uncertain significance | Cancer    | Deleterious | benign            |
| USP42 | chr7       | Yes                  | G                 | A                 | G486R                   | -                      | Passenger | Deleterious | Probably damaging |
| USP42 | chr7       | Yes                  | G                 | A                 | G486R                   | -                      | Passenger | Deleterious | Probably damaging |
| USP42 | chr7       | Yes                  | G                 | A                 | G486R                   | -                      | Passenger | Deleterious | Probably damaging |

To validate the presence of the mutations in the biological samples available, PCR and Sanger sequencing was performed using DNA extracted from the blood and the tumour tissues from the three elements of the family.

#### 3.1.2. PCR optimisation

DNA from blood and different tumour samples from each family member was evaluated by PCR in order to validate whether or not the mutated genes were present in the three family members. The DNA had already been extracted and was preserved at -20°C until further processing, since this project started in 2011. Since DNA quantification could not be found, a new quantification was performed (Table 16).

**Table 16. DNA quantification of the selected samples from the three affected individuals.** DNA quantification of tumour and non-tumour samples from the mother (R1) and one of the twins (R3), and DNA quantification of tumour samples from the other twin (R2).

| Family member | Histological number | DNA quantification (ng/ $\mu$ L) |
|---------------|---------------------|----------------------------------|
| R1            | 1115490 - 6         | 20.38                            |
| R1            | 1115490 - 7         | 118.60                           |
| R2            | 1115491 - 6         | 80.40                            |
| R2            | 1115491 - 7         | 14.96                            |
| R2            | 4325/11 --1         | 26.80                            |
| R2            | 4325/11 --2         | 75.36                            |
| R2            | 4328/11             | 25.08                            |
| R3            | 4464                | 184.33                           |
| R3            | 4465                | 162.99                           |

With the purpose of confirming which mutations were present in the family, the PCR protocol was optimised to have better amplification for each gene. So, the annealing temperatures calculated and tested were 52°C and 54°C. As observed in **Figure 18**, for the *EVC2* gene the best annealing temperature was 54°C and for the *USP42* gene was 52°C.

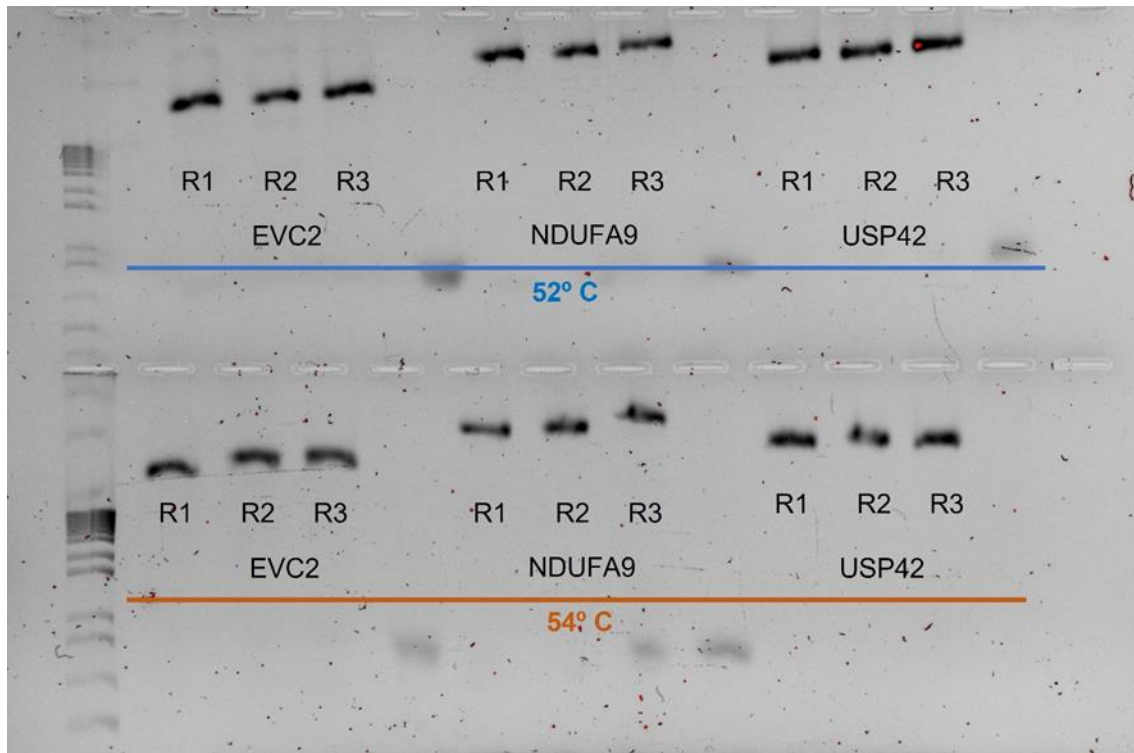


**Figure 18. Electrophoresis gel from the touchdown PCR of the histological and blood samples.** Based on the  $T_m$  of the primers used for the PCR reaction, two temperatures (52°C and 54°C) were tested to assess which temperature amplified better for each gene.

A PCR was performed to see if both blood, tumour, and non-tumour samples expressed *EVC2* and *USP42* (Figure 19).

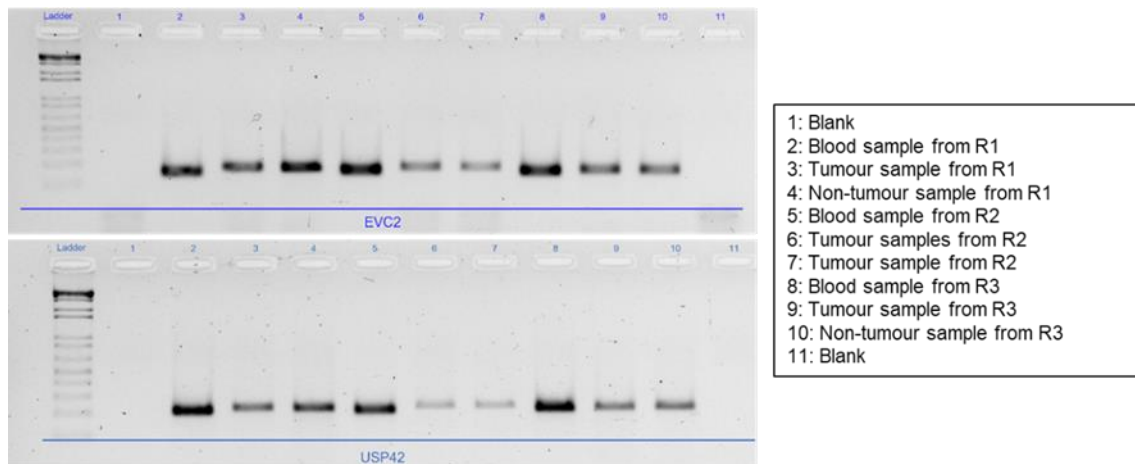


**Figure 19. Electrophoresis gel from the histological and blood samples.** Blood and tissue samples were amplified with the selected temperature for each gene.

Through Sanger sequencing, it was validated that the *EVC2* mutation was present in homozygosity in the mother and in heterozygosity in the twin-daughters, while *USP42* mutation was present in all family members as a heterozygous mutation, in the blood and in the tumour samples (Figure 20).

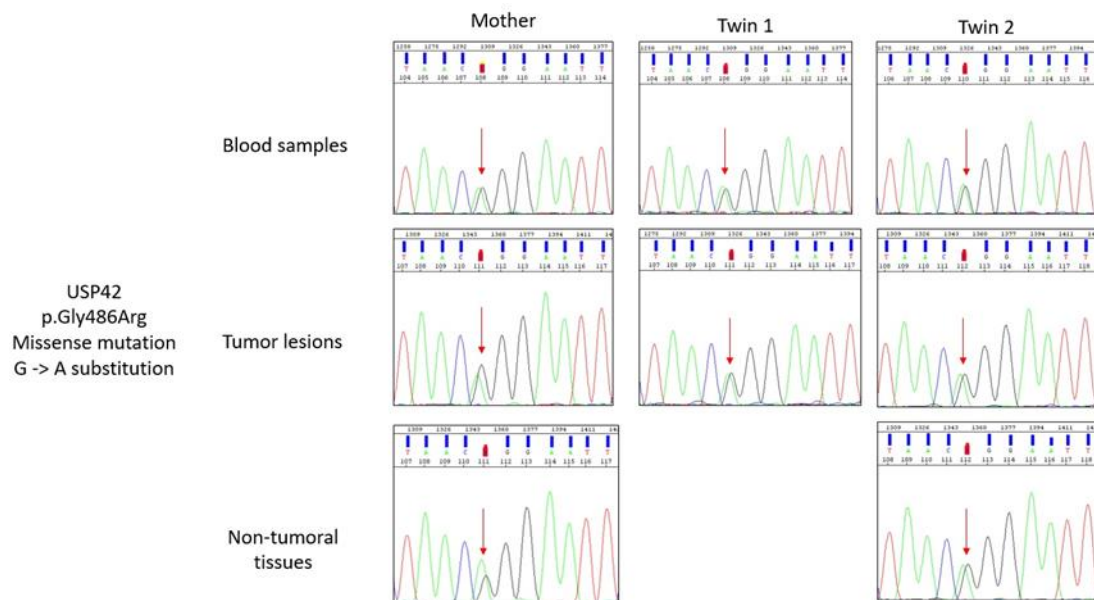


**Figure 20. Sequences from the histological and blood samples.** All the tested samples had the *USP42* mutation (p.G486R).

### 3.2. Bioinformatic analysis

In order to know if any other USP alterations could be present in thyroid cancer, two *in silico* analysis were performed.

The first bioinformatic analysis was performed on a series of 18 patients, 15 females and 3 males, that had been diagnosed with thyroid cancer. For this analysis, 59 genes were evaluated through NGS: 4 USPs genes; 5 thyroid cancer associated genes – *BRAF*, *NRAS*, *HRAS*, *KRAS*, and *TERTp*; 40 *vgKCNs* coding genes; and 10 *vgKCNs* modulator/regulator genes. The two last set of genes were analysed for another ongoing project within the group. With the obtained results from the NGS, copy number variation (CNV) was analysed, and a threshold was determined: normal CNV is between -0.2 and +0.2, genic amplification is above +0.2, and genic deletion is below -0.2. The results (**Figure 21**) showed an increased CNV of USP42 and a reduced CNV of USP39.

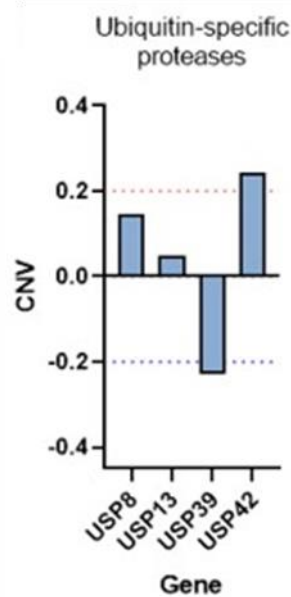


**Figure 21. Copy number variation analysis.** Copy number variation analysis from in-house database, in which genic deletion was detected for USP39 and genic amplification was detected for USP42.

In the second *in silico* analysis, a merged database was created with the three online databases available in *cbioportal*, related to well-differentiated thyroid cancer. From this merge of databases resulted the removal of duplicates and triplicates, the removal of cases without clinical information, the collection of the most informative data relating to each case, and, in the end, the information of 513 patients was analysed for the same 59 genes evaluated in the first *in silico* analysis. From this analysis, in the 513 patients, only 2 other *USP42* mutations were found and both cases had *BRAF* mutation alongside it: *USP42 p.Pro669Glu* with *BRAF p.Val600Glu*, and *USP42 p.Leu687Met* with *BRAF p.Val600Glu*. Since no other alterations were found, statistical analysis regarding patient clinicopathological characteristics and prognosis could not be performed.

### 3.3. Silencing of USP42 gene in cell culture through siRNAs

#### 3.3.1. Protocol optimisation

The protocol of siRNAs transfection was optimised 4 times, each time for a different aspect. The optimisations were regarding the number of siRNAs treatments used, the conditions of the wells, the concentration of siRNA used, and the incubation period.

Originally there were 4 siRNAs treatments: siRNA 2 (S2), siRNA 5 (S5), siRNA 6 (S6) and siRNA 7 (S7). An experiment was conducted to observe if the silencing that these treatments produced were similar at 24 hours (**Figure 22A**) and 48 hours (**Figure 22B**). The results showed that S2 and S7 silenced the expression of USP42 about 60% and S5 and S6 silenced the expression of USP42 about 30%. Therefore, the following experiences were conducted using only S2 and S7 treatments.

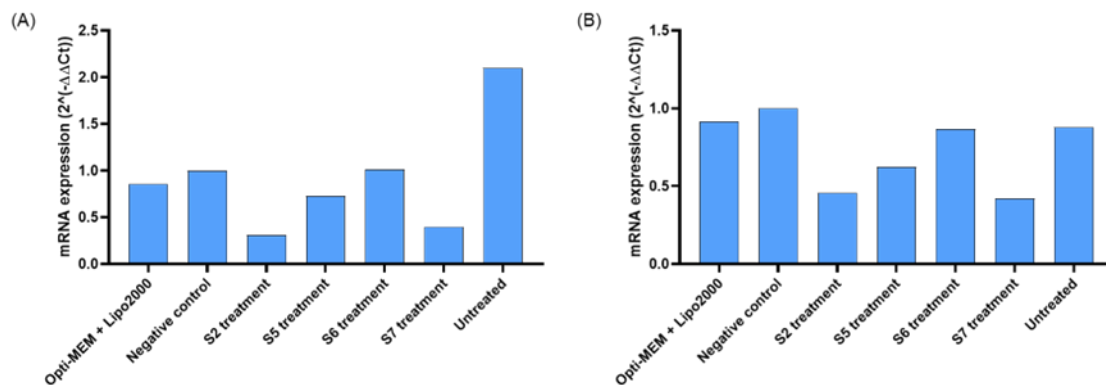


**Figure 22. mRNA expression after siRNA treatments.** (A) USP42 mRNA expression at the 24h timepoint. (B) USP42 mRNA expression at the 48h timepoint.

The second optimisation was the conditions of the wells. Initially there were 5 conditions presented in the experiment – Opti-MEM Gibco™ Opti-MEM™ I Reduced Serum Medium and Lipofectamine2000 (Opti+Lipo), S2 treatment, S7 treatment, and Untreated cells (U). The Opti-Lipo condition was used as a control for toxicity of Lipofectamine2000 in the cells. The results showed that Lipofectamine2000 presented some toxicity to the cells, since there was visible cell death in the well, but not enough to change this particular transfection reagent to another.

The third optimisation was the concentration of siRNA treatment used. Two concentrations were tested 25nM and 50nM. The results from this experiment showed that at both concentrations, the same silencing of USP42 gene in the cells was obtained

(Figure 23). For this reason, the 25nM concentration was applied for the rest of the experiments, since it resulted in a better cost/benefit ratio.

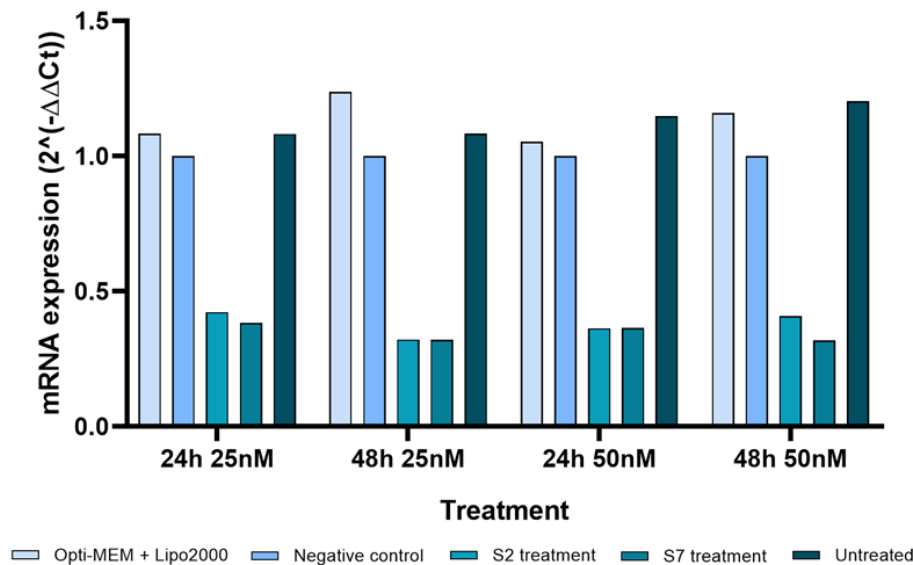


**Figure 23. USP42 mRNA expression after siRNA treatments.** USP42 mRNA expression after testing two concentrations of siRNA treatment, 25nM and 50nM, at the 24h and 48h timepoints.

The fourth and last optimisation was the incubation period. The incubation periods tested were 4 hours and 6 hours with the transfection complexes in the wells. The obtained results indicated that 4 hours with the transfection complexes was not severely toxic for the cells, while the 6 hours incubation period seemed to present more cytotoxicity in the cells (Figure 24).

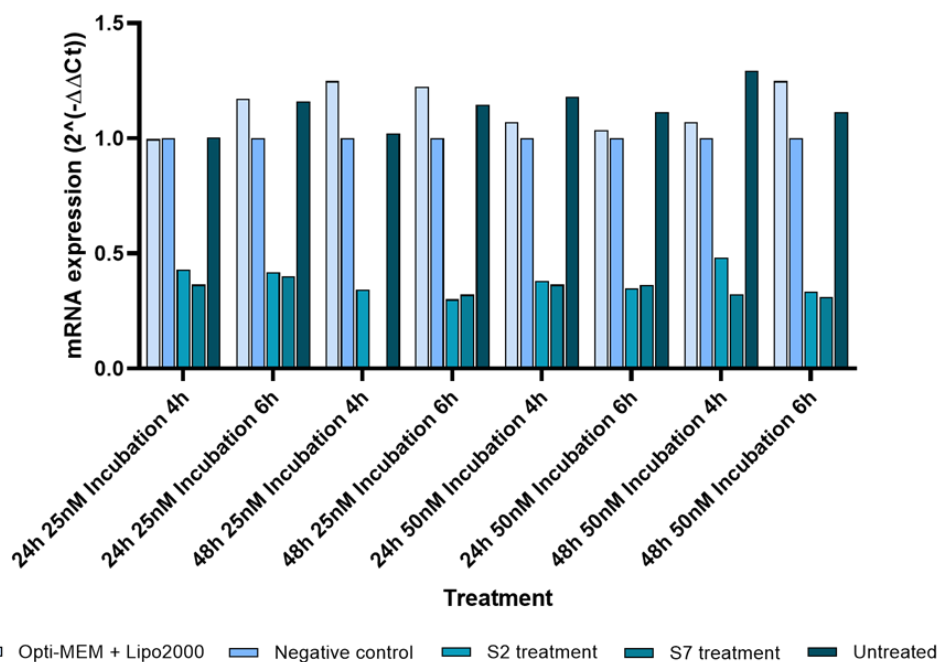


**Figure 24. USP42 mRNA expression after siRNA treatments.** At the 24h and 48h timepoints, USP42 mRNA expression after testing two concentrations of siRNA treatment, 25nM and 50nM, and two different incubation periods, 4h and 6h.

### 3.3.2. RNA extraction protocol optimisation

Initially, RNA extraction was being performed using Trizol. As shown on **Table 17**, the quantity and quality of RNA were not satisfactory.

**Table 17. RNA quantification from total RNA extraction using Trizol method.** RNA quantification from Trizol method of treated cells with siRNAs and its purity and quality ratios.

| Sample | Quantity (ng/ $\mu$ L) | 280/260 ratio | 260/230 ratio |
|--------|------------------------|---------------|---------------|
| NC1    | 34,3                   | 0.858         | 0.403         |
| S2 (1) | 61,25                  | 1.531         | 0.816         |
| S7 (1) | 140,37                 | 3.509         | 1.831         |
| U1     | 125,12                 | 3.128         | 1.652         |
| NC2    | 64,6                   | 1.615         | 0.814         |
| S2 (2) | 90,51                  | 2.263         | 1.240         |
| S7 (2) | 90,28                  | 2.257         | 1.184         |
| U2     | 95,85                  | 2.396         | 1.233         |

For this specific reason, RNA extraction by Trizol was switched to GRS Total RNA Kit - Blood & Cultured Cells kit, from GrisP Research Solutions. As shown on **Table 18**, by using the kit, high quantity and quality of RNA were obtained with 280/260 ratios comprised between 2,03-2,11 and 260/230 ratios comprised between 2,02-2,42.

**Table 18. RNA quantification from GRS Total RNA Kit – Blood & Cultured Cells.** RNA quantification of treated cells with siRNAs and its purity and quality ratios, using the extraction kit.

| Sample | Quantity (ng/ $\mu$ L) | 280/260 ratio | 260/230 ratio |
|--------|------------------------|---------------|---------------|
| NC1    | 230,15                 | 2,11          | 2,07          |
| S2 (1) | 252,78                 | 2,10          | 2,34          |
| S7 (1) | 192,21                 | 2,09          | 2,38          |
| U1     | 213,66                 | 2,08          | 2,42          |
| NC2    | 259,35                 | 2,10          | 2,38          |
| S2 (2) | 196,62                 | 2,11          | 2,02          |
| S7 (2) | 403,86                 | 2,03          | 2,19          |
| U2     | 410,98                 | 2,04          | 2,41          |

### 3.3.3. RT-qPCR evaluation

After RNA extraction, RNA was converted to cDNA. This cDNA was used to perform RT-qPCR. RT-qPCR was used to evaluate mRNA expression through the fold-change ( $2^{-\Delta\Delta C_t}$ ) comparing to negative control samples. At the 24h timepoint (**Figure 25**), S2 and S7 treatments had a statistically significant 60% silencing effect on the gene when compared to negative control ( $p < 0.0001$ ), and when compared to untreated cells, they also showed a statistical difference ( $p = 0.0047$  and  $p = 0.0051$  for S2 and S7 treatments, respectively). At the 48h timepoint (**Figure 25**), S2 treated cells still presented a statistically significant decrease ( $p = 0.0002$ ) to negative control cells, and S7 treated cells also presented a statistically significant decrease ( $p = 0.0005$ ) to negative control cells; when compared to untreated cells, S2 and S7 treated cells presented a statistically significant decrease in the expression of USP42 mRNA ( $p < 0.0001$ ). At the 72h timepoint, the mRNA expression of USP42 is higher, showing a recuperation in the cells against the transfection procedure (**Figure 25**). Still, S2 and S7 treated cells showed a statistically significant silencing effect of 50% ( $p = 0.0004$ ) and 40% ( $p = 0.0027$ ), respectively, when compared to negative control cells; when compared to untreated cells a statistically significant difference is observed for S2 ( $p = 0.0002$ ) and S7 ( $p = 0.0067$ ) treated cells.

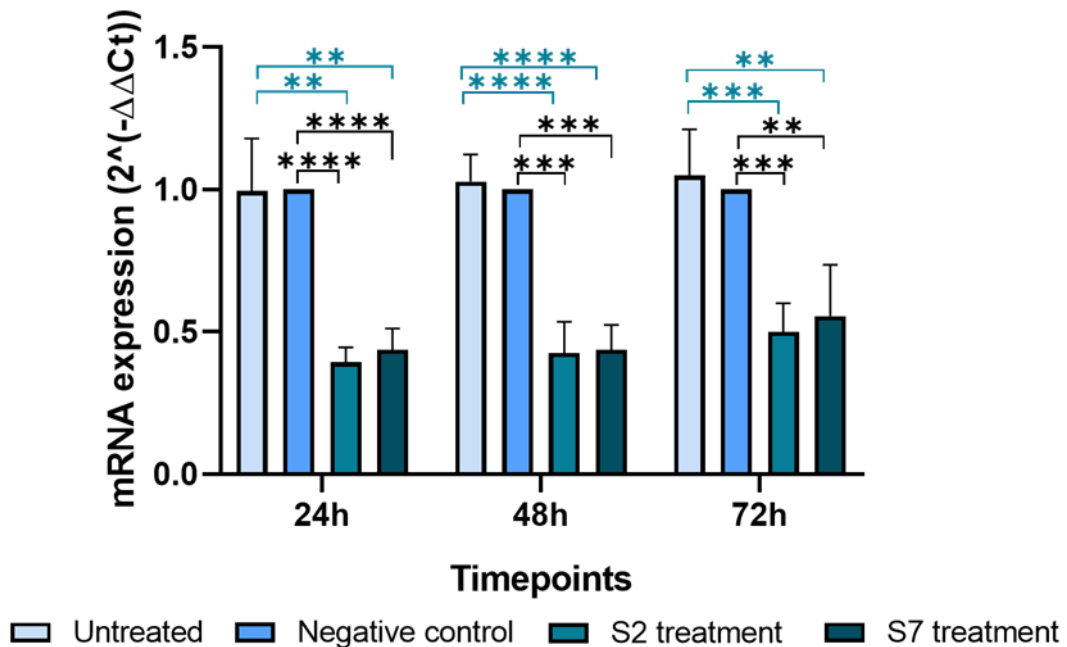


**Figure 25. USP42 mRNA expression.** USP42 mRNA expression in Nthy-Ori 3-1 cell line after treatment with siRNAs.

### 3.3.4. Western Blot analysis

Following the protein extraction and protein quantification protocols, the readings of absorbance from each protein was compiled in a file. With this file, a calibration curve was calculated, and an equation was obtained. Through the equation, the amount of total protein to have 15µg in the Western Blot gel was calculated for each sample.

For the initial attempts of quantification of the Western Blot gels, the endogenous control genes used were  $\alpha$ -Tubulin (52 kDa) and  $\beta$ -Actin (42 kDa). It was observed that both these proteins were also silenced upon siRNA transfection protocol with USP42 S2 and S7 (**Figure 26**). The endogenous gene was switched to Vinculin (117 kDa), and it was seen that Vinculin was also silenced (**Figure 26**).

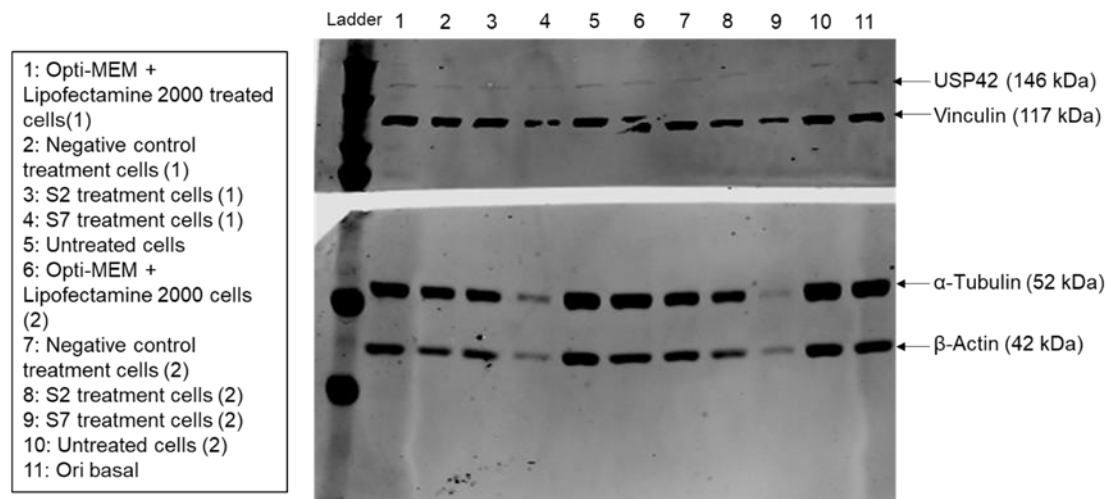


**Figure 26. Protein expression.** After treatment with siRNAs, USP42 protein expression in Nthy-Ori 3-1 cell line. Assessing which endogenous control to use,  $\alpha$ -Tubulin,  $\beta$ -Actin, and Vinculin.

Since all the attempts to select an endogenous gene for control were related to the cytoskeleton, GAPDH and HSP70 were tested to see if they could be used for endogenous control. Both these genes were also silenced (**Figure 27**).

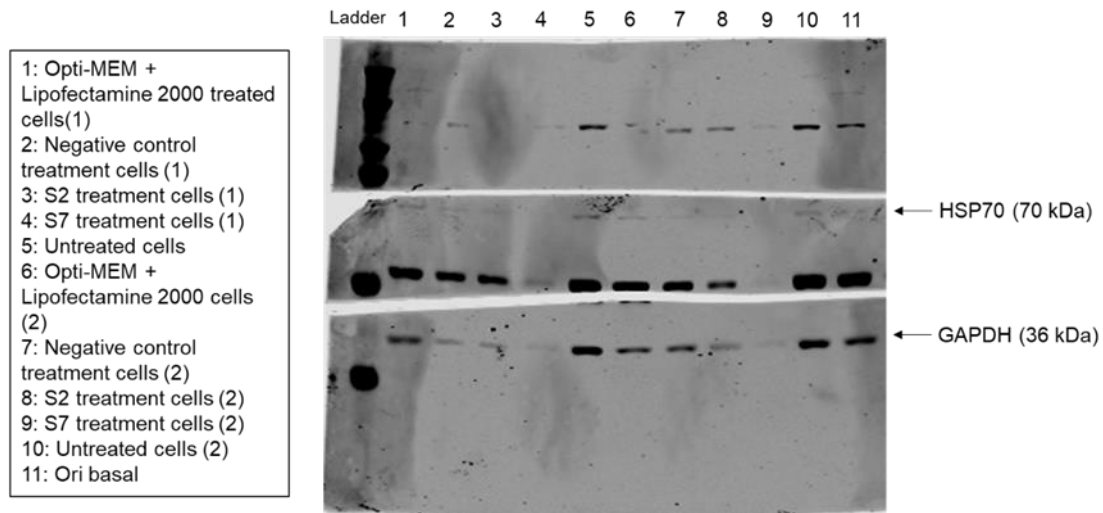


**Figure 27. Protein expression.** After treatment with siRNAs, USP42 protein expression in Nthy-Ori 3-1 cell line. Assessing which endogenous control to use, GAPDH and HSP70.

After these attempts, the quantification of the Western Blot gels was achieved using the total protein staining provided by the Ponceau S staining. Even though there was no statistically significant difference, these quantifications showed that at the timepoints of 24h (N=2) (**Figure 28A**) and 48h (N=3) (**Figure 28B**), there was no decrease in the expression of USP42 for both treatments, when compared to the negative control treatment. Analysing the 72h timepoint (N=3) (**Figure 28C**), a decrease in the expression of USP42 protein is seen by 20% and 25% for S2 treatment and S7 treatment when compared to the negative control treatment, respectively, although not statistically significant.

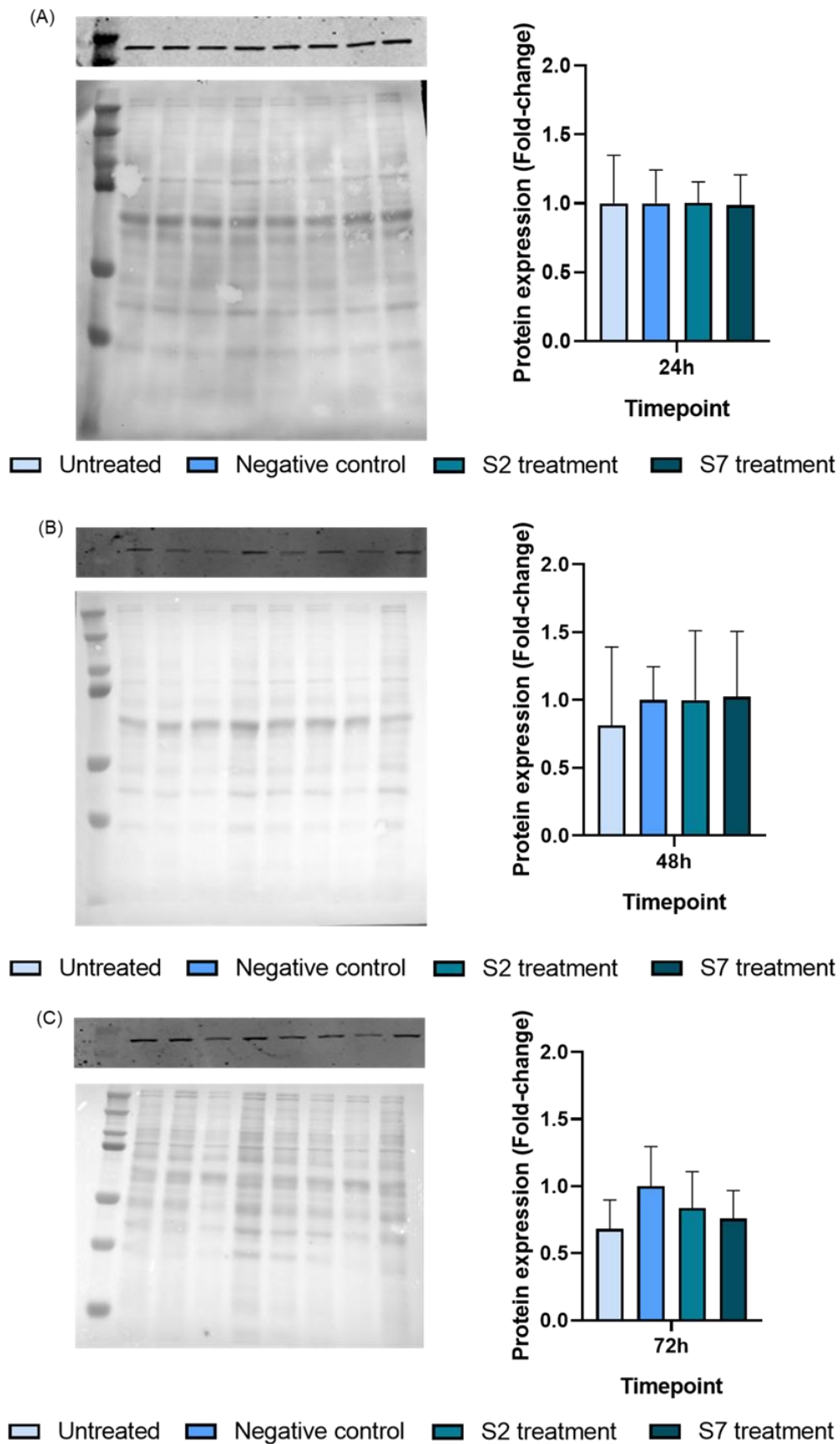


**Figure 28. USP42 protein expression.** (A) USP42 protein expression against the total protein, marked with Ponceau S, at the 24h timepoint. (B) USP42 protein expression against the total protein, marked with Ponceau S, at the 48h timepoint. (C) USP42 protein expression against the total protein, marked with Ponceau S, at the 72h timepoint.

Since USP42 can interfere with TP53 and Cyclin D1 expression, the expression of those two proteins was evaluated after siRNA treatment. For p53, at the 24h timepoint (**Figure 29A**), it is possible to observe that there was a difference, even if not statistically significant, between p53 expression in Untreated cells and treated cells; between treated cells, the difference between negative control and S2 treated cells was almost non-existent, and between negative control and S7 treated cells, the latter showed a little more expression. At the timepoint of 48h (**Figure 29B**), the untreated cells showed a slightly statistically significant difference with S7 treated cells ( $p=0.0433$ ), having the latter less expression than the first; between treated cells it is possible to observe a decrease in p53 protein expression in S2 and S7 treated cells when compared to negative control cells. For the 72h timepoint (**Figure 29C**), between untreated cells and S7 treated cells, there was no difference in the expression of p53, but negative control and S2 treated cells showed a higher protein expression of p53 than untreated; the same decreased pattern showed at the 48h timepoint could be observe between treated cells.

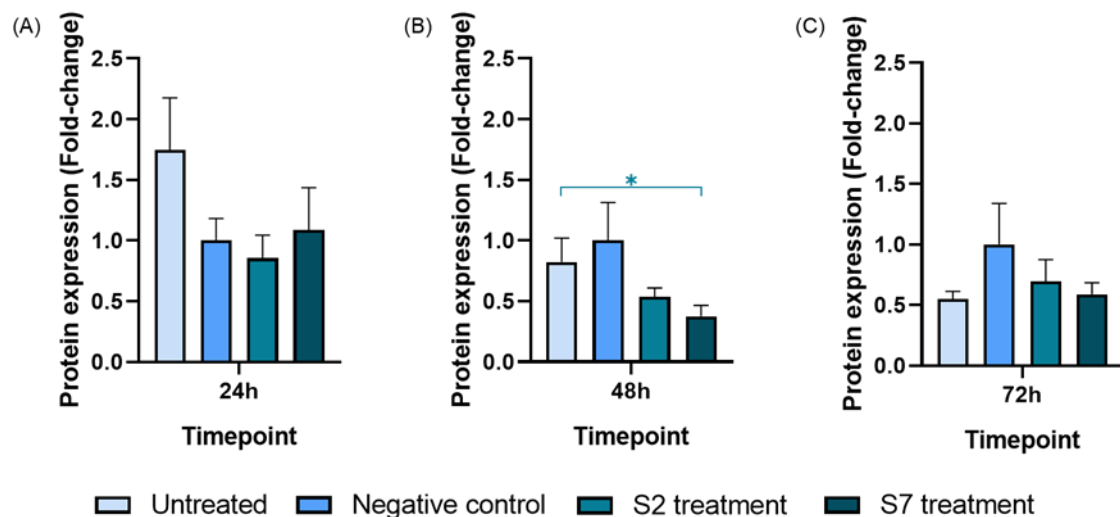


**Figure 29. p53 protein expression.** After treatment with siRNAs, p53 protein expression in Nthy-Ori 3-1 cell line, against the total protein, marked with Ponceau S. (A) 24h timepoint. (B) 48h timepoint. (C) 72h timepoint.

Regarding the protein expression of Cyclin D1, at the 24h timepoint (**Figure 30A**), there was a statistically significant difference between untreated cells and S7 treated cells ( $p=0.0071$ ), between the untreated cells and negative control cells there was no difference in protein expression, and S2 treated cells showed less protein expression of Cyclin D1 than untreated cells, even though there is not a statistically significant difference; for the treated cells, there was a decrease in the expression of Cyclin D1 protein in S2 and S7 treated cells when compared to negative control cells, and the latter was statistically significant ( $p=0.0028$ ). At the 48h timepoint (**Figure 30B**), negative

control and S2 treated cells showed a decrease in protein expression compared to untreated cells, which was not statistically significant, and S7 treated cells showed a statistically significant decrease in the expression of Cyclin D1 protein when compared to untreated cells ( $p=0,0020$ ); as for the difference between negative control and S7 treated cells, there was a statistical significant decrease in protein expression of the latter compared to the first ( $p=0,0407$ ), and for negative control and S2 treated cells, there was no difference in protein expression, concerning these groups.

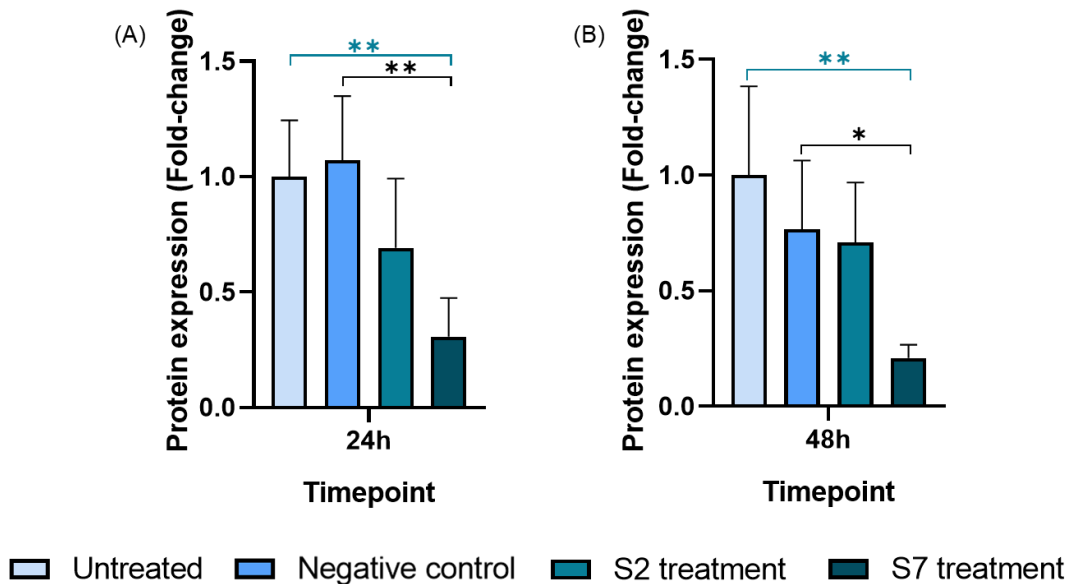


**Figure 30. Cyclin D1 protein expression.** After treatment with siRNAs, Cyclin D1 protein expression in Nthy-Ori 3-1 cell line, against the total protein, marked with Ponceau S. (A) 24h timepoint. (B) 48h timepoint.

### 3.3.5. Apoptosis analysis

Following the protocol for siRNA transfection, an analysis for apoptosis was performed by flow cytometry and with posterior analysis in the FlowJo software. This analysis aimed to verify if there was any difference between untreated cells and treated cells (negative control, S2 and S7 treatments) in the timepoints previously established.

Regarding the viable cellular population (**Figure 31**), at the timepoint of 24h, it was possible to observe that there was a significant difference between the amount of viable cells in untreated cells and all the treated cells (Negative control:  $p<0.0001$ ; S2:  $p<0.0001$ ; S7:  $p=0.0007$ ); there was a slight difference of viability between negative control cells and S2 treated cells ( $p=0.0166$ ). At the timepoint of 48h, there was no significant difference between untreated cells and treated cells, which was also seen in-between treated cells. At the timepoint of 72h, it was seen a significant difference between untreated cells and negative control ( $p<0.0001$ ) and S7 ( $p<0.0001$ ) treated cells, having the two last less viable cells; between negative control treated cells and S2

treated cells, there was a statistically significant difference ( $p < 0.0001$ ), in which the S2 treated cells had more viability than negative control cells.

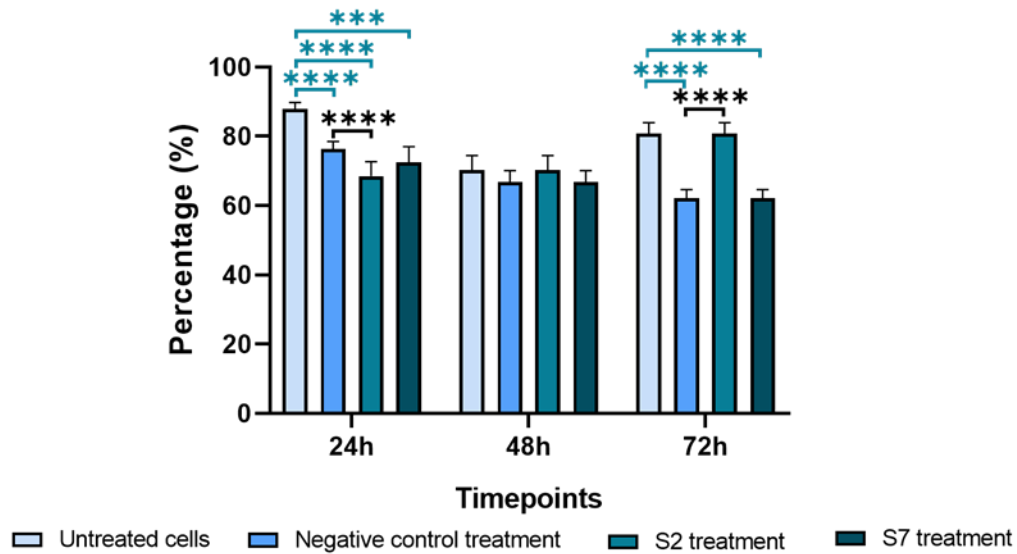


**Figure 31. Apoptosis analysis.** Percentage of viable cellular population, at the studied timepoints.

For the apoptotic cellular population (**Figure 32**), at the 24h timepoint, the untreated cells showed a statistically significant less apoptotic cellular population when compared to negative control ( $p < 0.0001$ ), S2 ( $p = 0.0005$ ) and S7 ( $p = 0.0028$ ) treated cells; and among treated cells, S2 and S7 treated cells had a higher apoptotic population than the negative control cells, with the S2 treatment showing a statistically significant slightly higher number of apoptotic cells ( $p = 0.0414$ ). At the timepoint of 48h, the untreated cells showed a statistically significant decrease in apoptotic population when compared to S2 ( $p = 0.0074$ ) and S7 ( $p = 0.0095$ ) treated cells, and the negative control treated cells showed a slightly higher apoptotic population than untreated cells; between treated cells, negative control cells showed a lower apoptotic population when compared to S2 and S7 treated cells. At the 72h timepoint, the apoptotic population of negative control ( $p = 0.0009$ ), S2 ( $p = 0.0013$ ) and S7 ( $p < 0.0001$ ) treated cells was statistically higher than the untreated cells population; regarding the treated cells, S7 treated cells presented a statistically higher apoptotic population than the negative control ( $p = 0.0001$ ).

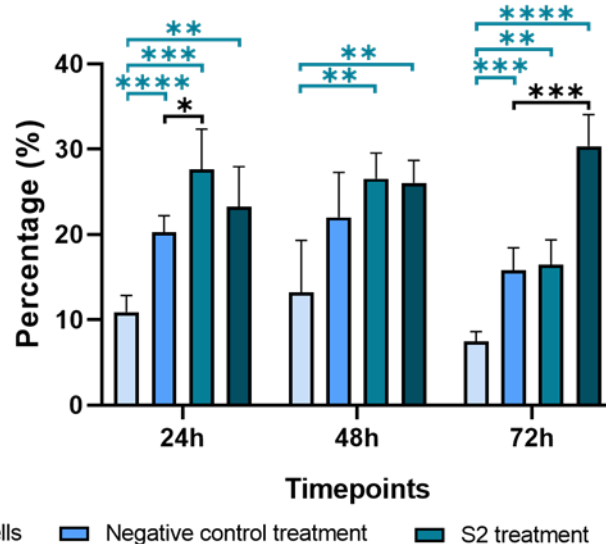


**Figure 32. Apoptosis analysis.** Percentage of apoptotic cellular population, at the studied timepoints.

As for the necrotic cellular population (**Figure 33**), at the timepoint of 24h, S2 ( $p=0.0209$ ) and S7 ( $p=0.0094$ ) treated cells presented a statistically higher necrotic population compared to the untreated cells; when looking into the difference between negative control, S2 and S7 treated cells, no significant difference could be observed. At the timepoint of 48h, there was no noticeable difference between the necrotic population of untreated cells and negative control and S2 treated cells, whilst comparing untreated cells and S7 treated cells, a statistically higher population of necrotic cells could be observed in the S7 treated cells population ( $p<0.0001$ ); comparing the treated cells, no difference could be seen amongst negative control and S2 treated cells, while S7 treated cells showed a statistically higher number of necrotic cells compared to the negative control cells ( $p=0.0088$ ). At the 72h timepoint, there wasn't a noticeable difference amongst negative control and S2 treated cells when compared to untreated cells, and, at the same time, comparing S7 treated cells and untreated cells, there was a slightly statistical significant difference between these populations ( $p=0.0144$ ); comparing the treated cells, there was a statistical significant difference between the S7 and the negative control cells ( $p=0.0095$ ), while S2 treated cells showed no noticeable difference when sided with the negative control necrotic population.

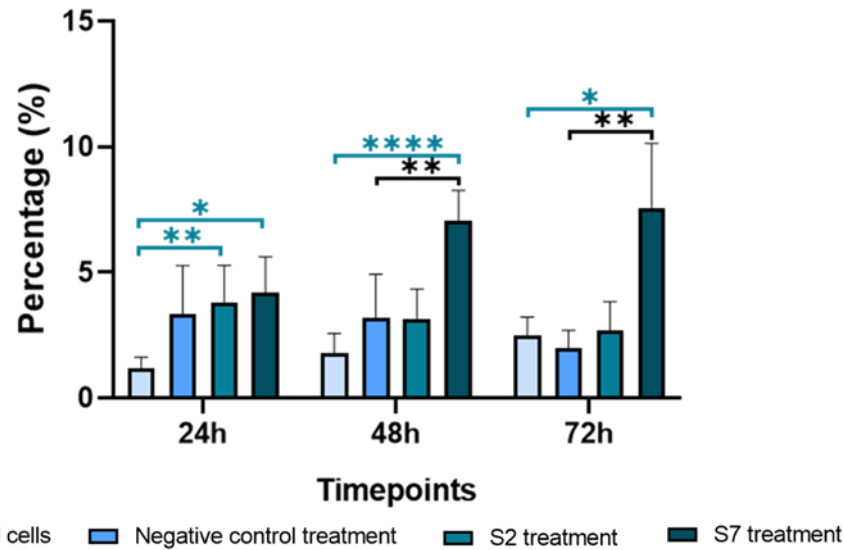


**Figure 33. Apoptosis analysis.** Percentage of necrotic cellular population, at the studied timepoints.

### 3.4. CRISPR-RNP mediated knockout targeting USP42

#### 3.4.1. CRISPR-RNP protocol

The previously designed sgRNAs and donor DNA for CRISPR-RNP were used to transfect the Nthy-Ori 3-1 cell line, following the protocol previously described.

After transfection in a 96-well plate, the cells grew and were transferred to bigger flasks (96-well plate to 12-well plate to T25 flask) until they reached the T75 flask. Upon reaching the T75 flask, the cells were photographed and differences in morphology were observed.

While some cells presented similar morphology to wild-type cells, others acquired an elongated (fibrotic) morphology (**Figure 34A**). Another remark is that the cells started to gather in small colonies (**Figure 34B**), something that was not previously observed.

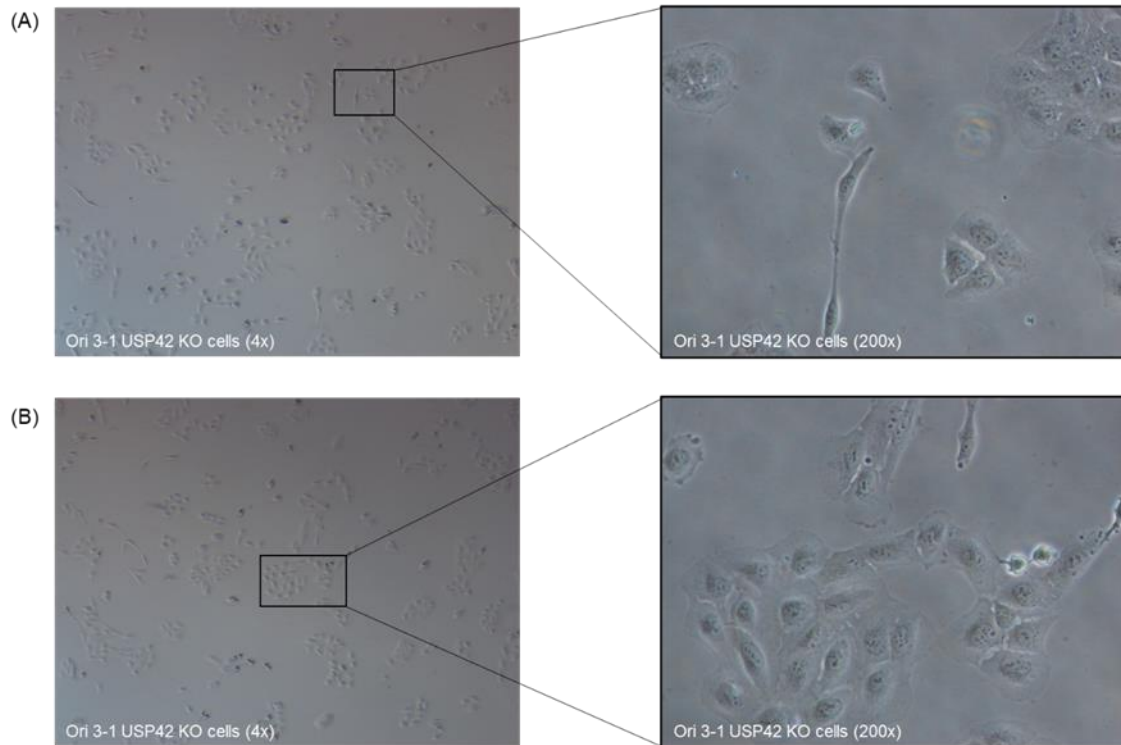


**Figure 34. CRISPR clones.** Different morphologies observed in Nthy-Ori 3-1 cell line after USP42 KO with CRISPR-RNP. (A) Elongated morphology. (B) Colony morphology.

### 3.4.2. Single cell clonal selection

After observing the different cell morphology and behaviour, single cell clonal selection was the chosen method to isolate the cells, in which a dilution of 100 cells/mL was performed and distributed in five 96-well plates.

Afterwards, through the microscope, each well was observed to note which appeared to have only one clone. Several wells seemed to have only one clone, but only a few survived. A few days after the first observation, the plates were observed again and a few of the previous wells seemed to have more than one pool of cells. Since it was not known if the pools were due to cell migration or if there were more than one clone, these wells were appointed as dubious, but still proceeded to let them grow.

The clones grew and were transferred to bigger flasks until they reached the T75 flask. When they reached the T75 flask, morphological changes could be observed in some of the clones, such as elongation and growth in colonies (**Figure 35**), and each clone was cryopreserved in 2 vials and a pellet for molecular characterisation was stored at -20°C until further processing.

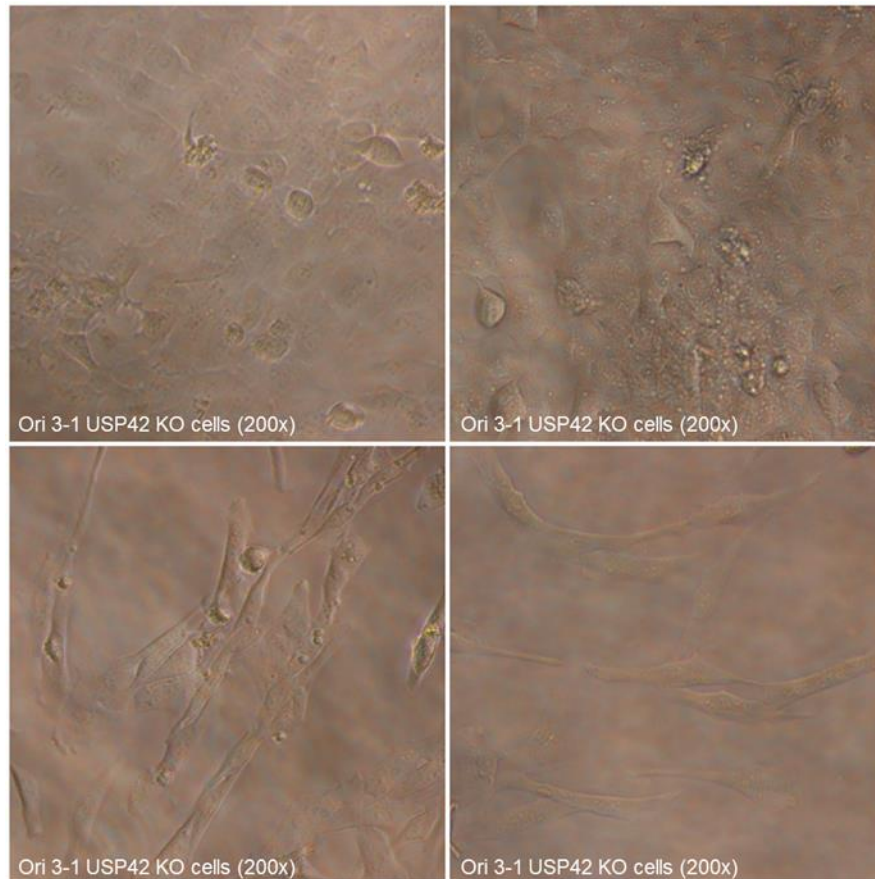


**Figure 35. CRISPR clones after clonal selection.** Different morphologies observed in Nthy-Ori 3-1 cell line after single cell clonal selection, such as colonies formation and elongated cells.

### 3.4.3. Molecular characterisation of CRISPR-RNP clones

From the stored pellet of each clone, DNA was extracted, and PCR technique was performed to validate which clones had the desired KO, meaning what clones had the insertion of the 12 base pairs. The files that came from the Genomic Service were uploaded to an online SeqScreener analysis software provided from Thermo Fisher.

On this software, the files from the clones were compared to a file of basal Nthy-Ori 3-1 cells, and an analysis file (**Table 19**) was created in which the percentage of HDR for each clone was presented.

**Table 19. Clonal selection.** With the information provided with Thermo Fisher's SeqScreener software, the clones more likely to be USP42 KO were selected.

| Clones | Editing (%) | HDR (%) | Frameshift (%) |
|--------|-------------|---------|----------------|
| P1C12  | 100,00      | 70,91   | 13,08          |
| P2D12  | 20,17       | 0,00    | 20,04          |
| P2F5   | 24,64       | 22,30   | 0,35           |
| P2F8   | 77,37       | 11,38   | 57,94          |
| P2F10  | 100,00      | 0,00    | 7,04           |
| P2F12  | 100,00      | 42,94   | 56,69          |
| P3F9   | 30,88       | 0,00    | 30,88          |
| P3G6   | 25,27       | 22,96   | 0,00           |
| P4B5   | 100,00      | 14,16   | 80,54          |
| P4C5   | 24,94       | 22,62   | 0,32           |
| P5D2   | 99,72       | 85,29   | 5,00           |

Further analysis was performed, resorting to a second software (Mutation Surveyor) to validate this analysis. In this software, it was attempted to separate both DNA chains to better evaluate genomic insertions and deletions that might be present in the clones' genome. It was confirmed whether or not the sequence of 4 STOP codons (TGATGATGATGA) has been inserted after Valine 57 (GTA) along with the designed donor DNA (**Figure 36**).

From the two software analysis and aligned with our annotations for clones that had one clone or had two pools uncertain if they came from migration, we concluded that the clones of interest were P1C12, P2F5, P2F12, P4C5 and P5D2 with a HDR percentage of 71%, 22%, 43%, 23% and 85% respectively.

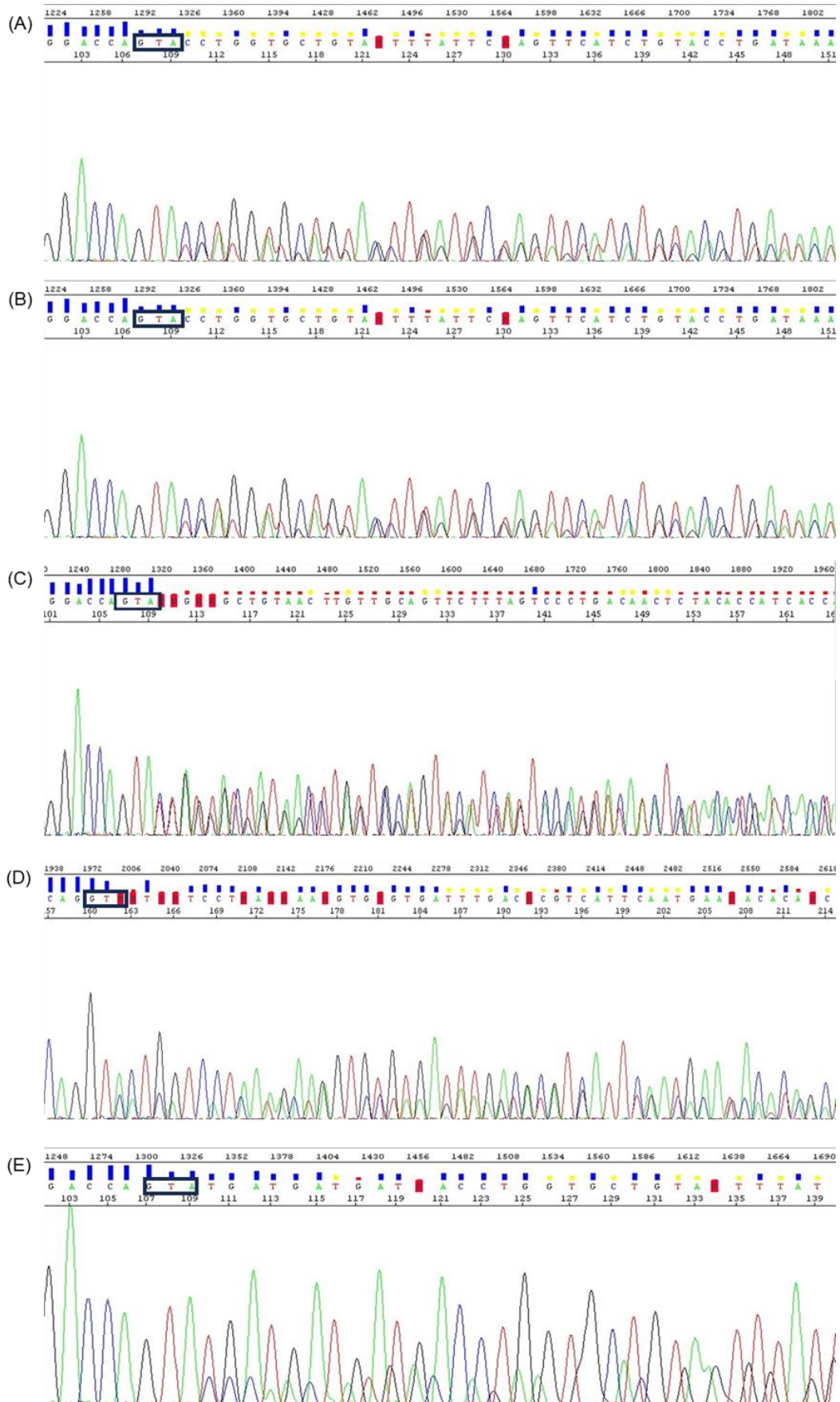


Figure 36. CRISPR clones sequences. (A) P1C12. (B) P2F5. (C) P2F12. (D) P4C5. (E) P5D2.

#### 3.4.4. Preliminary analysis

In order to ascertain whether these clones are of interest, a preliminary analysis was carried out for mRNA expression, protein expression, cell cycle analysis and apoptosis analysis.

To perform the preliminary analysis, a vial from each clone was thawed and cultured in a T75 flask each. After 2 passages, the cells seemed stable and a T25 flask, for each analysis, was cultured for each of the clones.

Regarding the morphology presented by each clone, P1C12 was the most similar to basal cells, P2F12 presented a mix of morphologies between rounded clustered cells and elongated cells, and P5D2 presented elongated cells (**Figure 37**).

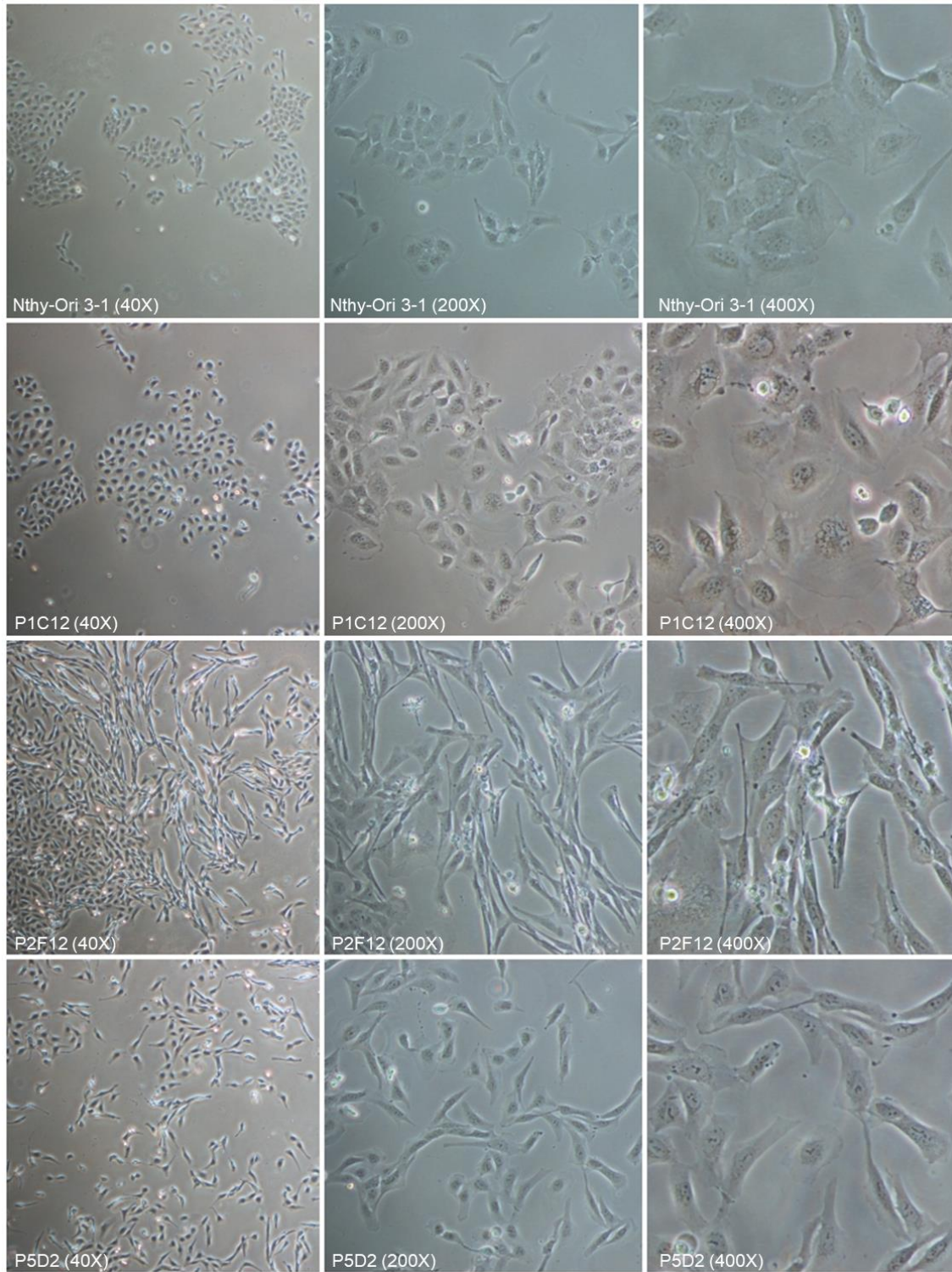


**Figure 37. CRISPR clones selected for studies.**

In terms of USP42 mRNA expression, there was no significant difference between Nthy-Ori 3-1 basal cells and P1C12 clone, a slight difference between P2F12 and P5D2 clones, 10% and 8%, respectively when compared to Nthy-Ori 3-1 basal cells (**Figure 38**).

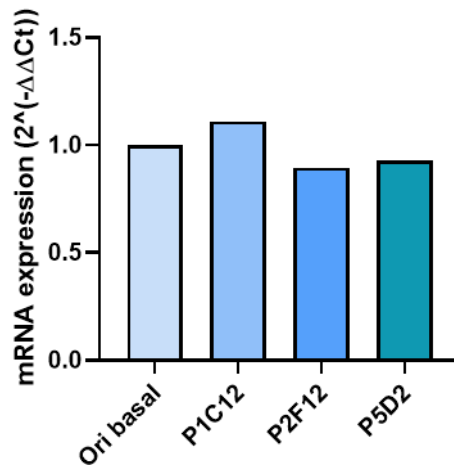


**Figure 38.** USP42 mRNA expression in the first set of selected clones.

Regarding protein expression, when compared to basal cells, all clones showed a knockdown in the production of USP42 protein (**Figure 39**).

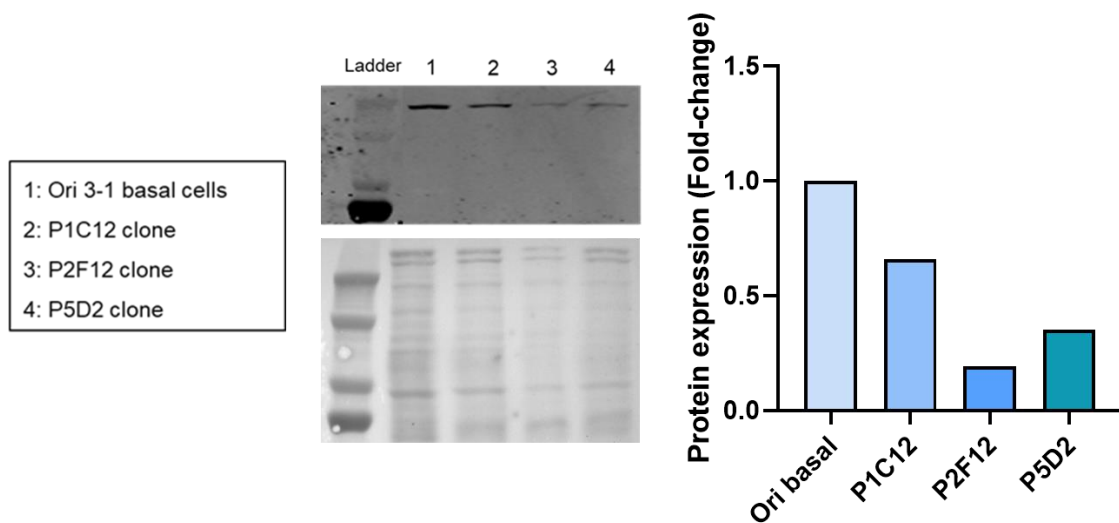


**Figure 39.** USP42 protein expression in the clones.

For the cell cycle analysis (**Figure 40**), the cell cycle presented by the clones showed an almost equivalent percentage of G1 and S phase, while the G2 phase percentage was much lower than the expected. The findings suggest that there was an arrest at G1/S phases.

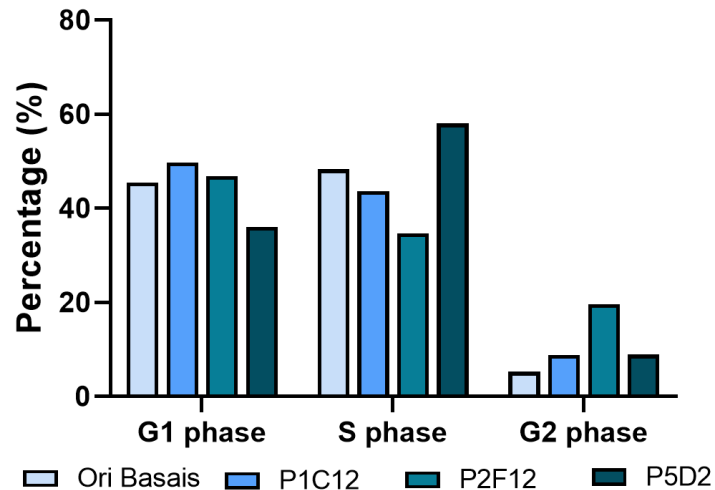


**Figure 40. Cell cycle analysis in the clones.** Percentage of G1 phase, S phase, and G2 phase cellular populations.

For the apoptosis analysis, P1C12 clone presented a similar pattern to Nthy-Ori 3-1 basal cells with a more pronounced viable population, and less apoptotic and necrotic populations when compared to P2F12 and P5D2 clones (**Figure 41**); P2F12 presented the population with the least viable cells and the apoptotic and necrotic populations were higher when compared to the other clones and the Nthy-Ori 3-1 basal cells (**Figure 41**); and P5D2 clone showed a population with less viable cells and more apoptotic and necrotic cells when compared to basal cells, but not as significant as P2F12 clone (**Figure 41**).

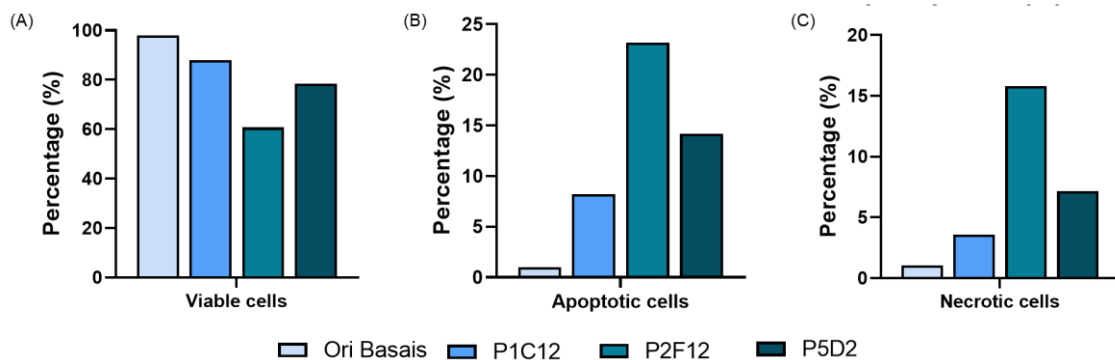


**Figure 41. Apoptosis analysis in the clones.** (A) Percentage of viable cellular populations. (B) Percentage of apoptotic cellular populations. (C) Percentage of necrotic cellular populations.

A second set of analysis was performed, in which two more clones were added, P2F5 and P4C5.

The morphology presented by P2F5 was similar to Nthy-Ori 3-1 basal cells and P1C12 clone with a follicular pattern more present, while P4C5 presented a more elongated morphology (**Figure 42**).

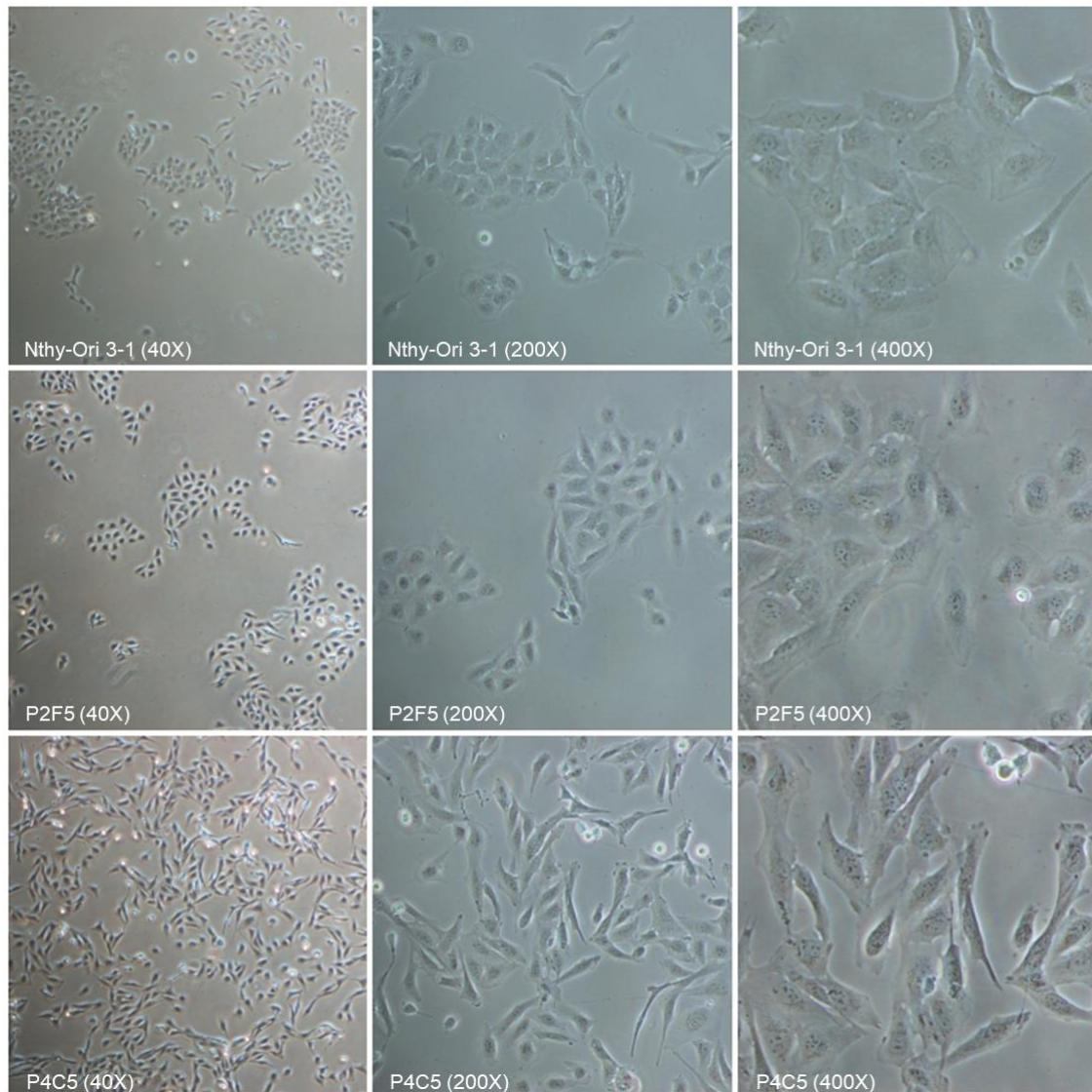


**Figure 42. CRISPR clones selected for studies.**

In this analysis (**Figure 43**), all the selected clones showed to have a similar USP42 mRNA expression when compared to basal cells.

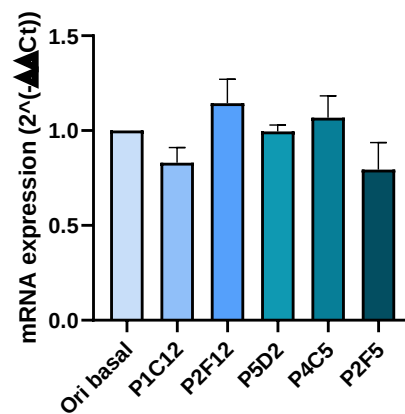


**Figure 43. USP42 mRNA expression in the second set of selected clones.**

## Chapter 4. Discussion

FNMTTC accounts for 3 to 9% of all the thyroid cancer and is known for being an uncommon condition. In terms of histology, FNMTTC tumours do not differ much from sporadic cancers and the genetics behind FNMTTC are heterogeneous and poorly understood [39-41].

Non-syndromic FNMTTC is clinically characterised when one of two aspects are found: either in the presence of follicular cell derived thyroid carcinoma in at least three first-degree relatives or in the presence of Papillary Thyroid Carcinoma in two or more first-degree family members. In both categories, an absence of history of previous radiation and inherited syndromic cancer should exist [39, 41]. Contrary to the syndromic forms, non-syndromic FNMTTC is characterised by having thyroid cancer as the main manifestation [35, 52-54].

The family of this study was diagnosed with thyroid cancer that fell into the category of non-syndromic FNMTTC, since it presented in three first-degree family members, who were diagnosed with Oncocytic Variant of Papillary Thyroid Carcinoma (OVPTC).

With this work, it was aimed to validate USP42 alteration as an underlying cause for non-syndromic FNMTTC. And while USP42 alterations have been described in breast [109] and gastric [110] cancers, to our knowledge, its biological function and tumourigenic potential has not been investigated in thyroid cancer.

### 4.1. Molecular characterisation of a family to confirm *in silico* data

As previously mentioned, the mother was diagnosed at 36 years of age and her two twin daughters were diagnosed at the age of 19 years old, and were submitted to total thyroidectomy.

Since known syndromic genes or the usual thyroid related genes (*BRAF*, *RAS* and *TERTp*) were found to not be mutated in this family, with the exception of *HRAS* sporadic *p.Q61R* mutation. NGS was performed from the preserved samples. The results from NGS showed two possible underlying mutated genetics, *EVC2* and *USP42* genes. *In silico*, these genes were found mutated in all the affected family members.

Then, PCR validation was performed to assess if these genes were actually mutated in the blood, tumour and non-tumour tissue samples. The results showed that

*EVC2* gene was mutated in homozygosity in the mother and in heterozygosity in the twins, and *USP42* gene was mutated in heterozygosity on all the affected family members.

The *EVC2* gene is known for being related to Ellis-van Creveld syndrome (EvC), an autosomal recessive chondrodysplasia. This syndrome is characterised by a broad spectrum of manifestations, such as shorter limbs and ribs, polydactyly, and cardiovascular malformations [114, 115]. Although there is one evidence relating *EVC2* mutations and metastatic breast cancer [116], this gene is widely associated with EvC, while the mutation in this family is classified as likely benign by polyphen, therefore, a less likely impacting factor in the development of OVPTC in this family. Moreover, despite being present in homozygosity in the mother, the same disease phenotype was observed in the twins, who just possessed a mutated allele.

The *USP42* gene, as previously mentioned, belongs to the cysteine proteinase DUBs family, more specifically to the USPs category, which are known for regulating tumour formation by modulating proliferation and death of tumoural cells [85, 109, 110, 113].

The decision of following with *USP42* for *in vitro* studies was done after analysing several factors. These factors were that the affected individuals presented the same diagnosis, OVPTC; the *USP42* germinative missense mutation was present in the three elements of the family and predicted to lead to loss of heterozygosity. This mutation was further validated by Sanger sequencing analysis. Moreover, it also included in this decision the reports connecting the dysregulation of USPs and the development and/or proliferation of cancer. In fact, it was reported that the knockdown of *USP39* decreases cell proliferation and lowers protein levels of cell cycle proliferation related proteins in Medullary Thyroid Carcinoma [106]; *USP42*, our target gene, was reported to promote the proliferation of tumoural cells in breast cancer [109] and to be overexpressed in gastric cancers tissues [110]. Notably, *USP13* was found to be mutated in a family diagnosed with FNMTTC [51] and demonstrated to be the possible gene underlying familial TC in the family. Finally, the mutation found in *USP42* is predicted to be damaging, impacting the activity of *USP42*.

## 4.2. Bioinformatic analysis

For the first *in silico* analysis, a review in the literature was necessary in order to assess which USPs were deemed the most interesting to explore in our in-house

database. This research concluded that USP8, USP13, USP39 and USP42 would be the most interesting to study, due to the fact that these proteins were described to be altered in thyroid cancer.

Regarding USP8, it was shown, in several cancer cell lines, that its upregulation promoted endothelial-mesenchymal transition (EMT) through TGF- $\beta$ /SMAD pathway [117]. USP8 upregulation in breast cancer was correlated with the upregulation of TGF- $\beta$  receptor II (T $\beta$ RII), which upregulated the TGF- $\beta$  pathway, and, consequently, promoted cancer cells' steaminess, migration, invasion and metastasis [117]. Corno *et al.* (2022) revealed that in ovarian cancer, the upregulation of USP8 led to a decrease in apoptotic rate, which resulted in tumour cell proliferation. The authors also showed that tumour tissues had higher expression of USP8, which led them to hypothesised that it could be a possible explanation for resistance in neoadjuvant therapies [118]. Another study regarding tumour cell migration reported that USP8 promoted the activation of c-Jun N-terminal kinase (JNK) pathway, so its upregulation consequently led to the upregulation of the pathway. More specifically, the authors showed that USP8 promoted metastasis in human breast cancer cells [119]. Additionally, somatic mutations in USP8 were found in 35% to 63% of pituitary Cushing adenomas [120].

For USP13, in gastric cancer, it was reported that its upregulation leads to the stabilisation of Cyclin D1. The stabilisation of Cyclin D1 promoted the cell cycle progression and cell proliferation [121]. The authors also performed an *in silico* analysis, in which they revealed that tumour samples had higher expression of USP13 when compared to normal samples, and higher expression of USP13 was correlated with worst prognosis [121]. In a family diagnosed with thyroid cancer, it was reported that the upregulation of USP13 may play a role in the tumourigenic process of PTC [51], more specifically, the authors reported that WES analysis identified a heterozygous missense variant, and *in vitro* studies showed that the variant found led to overexpression of USP13 and preserved protein stability for longer than the non-mutated USP13 protein. Evidence in non-small cell lung carcinoma (NSCLC) showed that tissue samples had higher expression of USP13 when compared to normal adjacent tissues. The authors also reported that the downregulation of USP13 in cell lines led to the inhibition of cell proliferation by AKT/MAPK pathway [122].

As for USP39, Zhang *et al.* (2022) reported that USP39 stabilises Forkhead box protein M1 (FOXM1), which promoted breast cancer cells' proliferation [123]. In colorectal cancer, it was reported higher expression of USP39 in tumour tissues from patients; when studied in cancer cell lines, USP39 has higher expression in all lines; and

*in vitro* studies showed that the downregulation of USP39 promoted the inhibition of cell growth, cell migration and invasion [124]. *In vitro* studies, related to Medullary Thyroid Carcinoma, reported that the downregulation of USP39 led to less cellular proliferation induced by cell cycle arrest at G2/M phase. It was also shown that alongside cell cycle arrest at G2/M phase, Cyclin B1 and CDK1 were less expressed when USP39 was downregulated [106].

Concerning USP42, it was shown in gastric cancer that tumour tissues had a higher expression of USP42 than normal adjacent tissues, and in *in vitro* studies, it was reported that the downregulation of USP42 resulted in cell cycle arrest at G1 phase in gastric cancer cell lines [110]. In breast cancer, Wang *et al.* (2022) reported that in tumour tissues samples, USP42 was more expressed than in normal tissues samples; and *in vitro* studies, showed that the regulation of USP42 promoted cell growth, migration and invasion, while its downregulation increased the apoptosis of tumoural cells by activating JNK-p38 apoptotic pathway [109]. Hock *et al.* (2011) described that the upregulation of USP42 led to the stabilisation of the levels of p53, and, consequently, the apoptotic rate diminished [113].

When comparing the results of the first *in silico* analysis with the literature, it is possible to observe that CNV of USP8 and USP13 are within normal range, which is not what is reported for other cancers. As for USP39, CNV was below the normal range threshold, indicating a genomic deletion. This genomic deletion is according to what was found in the literature for thyroid cancer [106], while disagreeing the articles found for other cancer histological types [123, 124]. Regarding USP42, the articles found reported only its overexpression, which is according to the results, since CNV is above the normal range threshold in this analysis.

Assessing the second bioinformatic analysis performed, the analysis was more extensive, since it included clinicopathological data acquired and alongside with studied USPs in the first *in silico* analysis, more USPs were added to this research, namely, USP18, USP20, USP21 and USP26.

The downregulation of USP18 was reported to potentially be involved in PTC proliferation [104]. USP20 was demonstrated to have functions related to migration, invasion and metastasis in breast cancer [125]; and it is also known that USP20 deubiquitinates the hormone-activating type II deiodinase (D2), which is a precursor of T3, meaning that its dysregulation would dysregulate THs synthesis[126]. USP21 was reported to be recruited by Small Nucleolar RNA Host Gene 5 (SNHG5) to stabilise Forkhead Box O3 (FOXO3), which would lead to activation of autophagy pathways and

inhibition of cell proliferation in PTC [127]. USP26 depletion was demonstrated to diminish cell proliferation, migration and invasion in ATC [105].

In this analysis only information concerning mutation status was obtained, and only two *USP42* mutations were found, *USP42 p.Pro669Glu* and *USP42 p.Leu687Met*, in the 513 patients from the TCGA database, hence statistical analysis relating the mutational status of USPs and clinicopathological data could not be performed.

### 4.3. Silencing of USP42 in cell line through siRNAs

#### 4.3.1. Protocol optimisation

The protocol for siRNAs transfection proved to be more challenging than what it was initially expected. Four optimisations were required for the best outcome in terms of results.

The first optimisation, as previously mentioned, was concerned with the number of transfection treatments used in the experiment. The initial transfection kit that was acquired contained 4 different siRNAs and, therefore, all the treatments were initially tested at the same time (as a scramble). Since this methodology resulted in high cell cytotoxicity, siRNAs were attempted to test one by one to assess silencing efficacy and cell viability. As it can be seen in **Figure 22**, at the 24h and 48h timepoints, two of the siRNAs, S5 and S6, did not perform a knockdown as efficient as S2 and S7, which was the reason why they were discarded from the following experiments. This decision was done according to what was also found in the literature, for example, Zhang *et al.* (2015) used two siRNAs to perform the knockdown of USP28 [128], while Hu *et al.* (2017) only used one siRNA scramble to perform the knockdown of USP32 [129].

The second optimisation performed was regarding the number of conditions in the experiment. Initially, there were five conditions – Opti-MEM and Lipofectamine 2000, negative control treatment, S2 treatment, S7 treatment and untreated cells. The condition Opti-MEM and Lipofectamine 2000 was used to assess the cytotoxicity that Lipofectamine 2000 would have in the cell line that was used, Nthy-Ori 3-1. This condition was reproduced four times in independent experiments and the visual results were consistent, Lipofectamine 2000 had some cytotoxic effect in the cells. This cytotoxic effect was visible in the wells, but this effect was not significant enough to make the experiment unfeasible.

The third optimisation was related to the concentration of siRNA used during the procedure. Two concentrations were tested, 25nM and 50nM (**Figure 23**). Comparing to Nolte *et al.* (2009) [130], more concentrations should have been tested, but since these reagents were limiting, only these two concentrations were tested. Both concentrations exhibited transfection efficiency with 60% silencing of USP42 mRNA expression at the 24h and 48h timepoints. Hence, the 25nM siRNA concentration was used throughout the remaining experiments.

Finally, the fourth optimisation was targeted to the incubation period with the transfection complexes. Since Lipofectamine 2000 presented cytotoxicity in Nthy-Ori 3-1, it was observed that an incubation period of 6h with the complexes gave rise to high cytotoxicity. Therefore, and following Nolte *et al.*'s protocol (2009) [130], the incubation period of 4h was tested. This period provided with the best outcomes without jeopardising the transfection efficiency, as seen in **Figure 24**.

#### 4.3.2. RNA extraction protocol optimisation

In the first few sets of transfection experiments, total RNA extraction was being performed by following a protocol that used Trizol. Several problems arose, namely poor separation of the aqueous and intermediate phases, which consequently led to deficient quantity and poor quality of RNA, as described in **Table 17**. For this particular reason, an alternative was sought and the GRS Total RNA Kit - Blood & Cultured Cells kit was used to perform total RNA extraction. **Table 18** presents a good example of one of the experiments in which RNA was extracted with the kit, as it shows good quantity and quality of the extracted RNA.

#### 4.3.3. RT-qPCR evaluation

Regarding mRNA expression, Hu *et al.* (2017) [129] showed that in small cell lung cancer the effect of transfection with siRNAs was 60%. The same result was reported by Zhang *et al.* (2015) [128]. The results of the transfection procedure with both siRNAs provided an efficient knockdown of 60% at the 24h and 48h timepoints (**Figure 25**), which is aligned with the literature. At the timepoint of 72h, the transfection effect is beginning to be reversed by the cellular machinery as demonstrated in **Figure 25**.

#### 4.3.4. Western Blot analysis

To analyse the proteins levels of USP42 in the transfected cells with siRNAs, Western Blot technique was used. Initially, the endogenous controls tested were  $\alpha$ -Tubulin and  $\beta$ -Actin, which are the usual endogenous controls used. Researching in the literature, Pan *et al.* (2021) [131] used  $\alpha$ -Tubulin as the endogenous control, while Ma *et al.* (2023) [121], Zhang *et al.* (2022) [123] and Wang *et al.* (2022) [109] used  $\beta$ -Actin. As shown in **Figure 26**, the tested endogenous controls were as silenced as USP42. Therefore, an alternative was sought, and Vinculin was tested as an endogenous control, since Corno *et al.* (2022) used it as an endogenous in their work [118]. Vinculin was also silenced as demonstrated in **Figure 26**. These results led to the hypothesis that the siRNAs used may also be silencing cytoskeleton proteins. On other hypothesis is that the downregulation of USP42 protein could interfere with the stability of cytoskeleton proteins.

In order to test the formed hypothesis and to be able to quantify the USP42 protein level in the transfected samples, other endogenous controls non-related to the cytoskeleton were used, namely GAPDH and HSP70. GAPDH is an enzyme that belongs to the glycolysis process [132] and has been widely used as an endogenous control for the knockdown of USPs [106, 110, 122, 124], and HSP70 helps with protein folding and functions as an anti-inflammatory molecule [133]. These metabolism related proteins were also silenced as shown in **Figure 27**.

Given the fact that the usually used endogenous controls tested presented as also being silenced, total protein quantification was performed using Ponceau S staining. This was the best alternative, which was utilised by Xie *et al.* (2022) [134]. Therefore, protein quantification was performed opposing total protein quantification.

The results showed that at the timepoints of 24h (**Figure 28A**) and 48h (**Figure 28B**), the protein levels of USP42 were equal in negative control, S2 and S7 cells. At the 72h timepoint (**Figure 28C**), a quantitative decrease is observed in S2 and S7 treated cells, with the latter presenting a slightly higher silencing effect at protein level, although this decrease is not statistically significant. These findings are seen in the literature, where Hou *et al.* (2016) [110] and Hu *et al.* (2017) [129] also reported a silencing effect in cell lines when transfecting them with siRNAs for USP42 and USP32, respectively.

Regarding p53, several USPs [113, 135, 136] have been reported to stabilise it, meaning that the overexpression of USPs led to the stabilisation of p53 and consequently to cell cycle arrest or apoptosis; and the opposite strategy, the knockdown, presented

cell growth and proliferation. As seen in **Figure 29**, at the 48h and 72h timepoints, p53 protein expression were decreased in S2 and S7 treated cells, which is aligned with the literature findings.

Ma *et al.* (2023) [121] reported that USP13 expression stabilised Cyclin D1, therefore promoting cell cycle progression; and when they performed the knockdown of USP13, Cyclin D1 protein levels were decreased. **Figure 30** shows that the downregulation of USP42 led to a decrease of Cyclin D1 protein levels, which is according to the study described above.

Hence, the results point that the knockdown of USP42 would lead to cell cycle arrest. Cell cycle analysis in siRNA treated cells are being conducted at the present time to test this hypothesis.

#### 4.3.5. Apoptosis analysis

Apoptosis analysis was measured by flow cytometry using Annexin-V and PI double staining.

In the literature, Wang *et al.* (2022) [109] showed that the knockdown of USP42 led to an increase in the apoptotic rate of MDA-MB-231 cell line. Pan *et al.* (2021) [131] also reported the increase of apoptotic cellular population in SHG-140 and T98G cell lines. Hu *et al.* (2017) [129] described that the knockdown of USP32 with siRNAs led to an increase in the apoptotic cellular population.

The achieved results showed siRNA treated cells presented a decrease in viable cells population (**Figure 31**) and an increase in apoptotic and necrotic cells populations (**Figure 32 and 33**), throughout the studied timepoints. Therefore, the results obtained are in alignment the literature, and with the observations of the cells made during the course of the silencing experiments.

### 4.4. CRISPR-RNP mediated knockout targeting USP42

#### 4.4.1. CRISPR-RNP protocol

In order to achieve a stable downregulation of USP42, preliminary studies were done with an alternative methodology. CRISPR is a technique widely used for genome editing. Traditionally, CRISPR-Cas9 system is composed by a sgRNA, which is a hybridisation of tracrRNA and crRNA, and is conducted by an endonuclease, Cas9

enzyme [137, 138]. The original system presents the disadvantage of presenting many off-targets, and CRISPR-Cas9 using RNP complexes (CRISPR-RNP) avoid this disadvantage, and has the advantage of having high cleavage efficiency [139]. CRISPR-RNP is based on the same principal, however, in this technique, active Cas9 protein is precomplexed with the sgRNA and Cas9-RNP complex is delivered in its entirety inside the cells [137, 140]. This method is more efficient and provides with less off-targets, which is the reason why it was chosen.

#### 4.4.2. Preliminary analysis

The CRISPR technique was performed in order to assess if the transient effect seen with siRNAs was permanently seen in the cells and if the results obtained would show the same patterns.

In a primary analysis, the first set of chosen clones was analysed for morphological changes, mRNA expression, protein expression, cell cycle and apoptosis analysis.

When the selected clones were thawed, morphological changes could be observed (**Figure 37**). These morphological changes could indicate that these clones could have significant difference in USP42 expression. As observed in **Figure 38**, these clones seemed to not be affected at the mRNA level, since mRNA was being detected in RT-qPCR analysis, and no significant difference was seen when compared to basal cells. Nevertheless, protein levels were assessed and, as seen in **Figure 39**, despite the fact that these clones were not USP42 knockouts, a clear knockdown of the gene in the CRISPR transformed cells could be observed. Similar findings had already been reported in the literature by Yuan *et al.* (2022) [141], who demonstrated in osteosarcoma that even with the depletion of USP1, TAZ mRNA expression remained unchanged, while its protein levels appeared to be decreased. In one hand, in the obtained clones of this study, the CRISPR induced alterations seemed to be present in heterozygosity and it was hypothesised that WT mRNA is still being produced. On the other hand, although the alterations introduced affect the exon 3, and could potentially induce the RNA premature decay, that could not be the case. In this situation, mRNA is still being detected, but due to the presence of STOP codons, it will interfere with protein synthesis. On other hypothesis is that the USP42 antibody used in the Western Blot may be recognising USPs other than USP42, since the backbone of these proteins might be similar. Further studies will be necessary to understand these results.

Regarding cell cycle (**Figure 40**), in published works was reported that USP13 depletion led to cell cycle arrest at G1 phase in gastric cancer [121], and USP32 knockdown presented cell cycle arrest at G0/G1 phase in small cell lung carcinoma [129]. As for the results obtained in cell cycle analysis, G1 and S phases seem to have a normal percentage, while G2 phase had a lower percentage when compared with control cells. These results suggest that the knockdown of USP42 can lead to a cell cycle arrest in G1/S phases.

As for apoptosis analysis (**Figure 41**), the obtained results showed a lower viable cellular population, while apoptotic and necrotic cellular populations were higher. These results are in alignment with the siRNA treatment results, as well as with the literature found and above described.

In a second set of analysis, two more clones were added. When thawed, these clones also presented different morphology when compared to Nthy-Ori 3-1 basal cells (**Figure 42**), which was a good preview since they showed a lower HDR percentage than the first set of clones. For the time being, only mRNA expression has been analysed for the five clones of interest (**Figure 43**).

## Conclusion

According to the literature, USPs have been described to play a role in tumourigenesis. A family was diagnosed with FNMTC and a mutation in USP42 gene was found through NGS.

Through *in silico* analysis, this work reports that USPs can be found, even if rarely, mutated in thyroid cancer. *In vitro* studies with siRNAs showed that the silencing of USP42 led to less expression of mRNA and protein, and an increase in the apoptotic rate. Alterations in the expression of p53 and Cyclin D1 proteins were also noted.

In the preliminary results using CRISPR-RNP, an increased apoptotic rate was also observed. Further studies are ongoing to establish the role of USP42 in tumourigenesis in FNMTC.

As for future perspectives, it would be necessary to perform additional assays with siRNAs and with CRISPR clones, in order to better understand USP42 role and how it impacts the cancer cells. The same siRNAs, CRISPR-RNP and cellular function assays should be performed in other cell lines, preferably cancer cell lines. Lastly, it would be interesting to perform the knock in of the USP42 mutation found in cell lines.

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