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# TELOMERASE RELATED STUDIES IN THYROID CANCER

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# TELOMERASE RELATED STUDIES IN THYROID CANCER
## Thesis for Doctoral Degree (Ph.D.)

By

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To my cousin Maria Carolina, I wouldn't be here without you.

It still saddens me that I have to be here without you.

# Popular science summary of the thesis

The goal of this thesis is to improve the diagnostic workup of thyroid cancer by evaluating a genetic alteration that can be found in thyroid cancer cells.

The thyroid gland is a small, butterfly-shaped organ that is located in the neck, just underneath the "Adam's apple". Its main function is to produce hormones that help control our bodies metabolism.

As a cytopathologist, I often use fine needle aspiration cytology (FNAC) to diagnose thyroid cancer. FNAC involves collecting a small sample of cells from a tumor and examining it under a microscope to determine if the cells are cancerous. If deemed necessary, these tumors are surgically removed. It is then often challenging to accurately diagnose these tumors, based on microscopic slides alone. This is because we can only distinguish benign Follicular Thyroid Adenomas (FTA) from malignant tumors such as Follicular Thyroid Carcinoma (FTC) by evaluating the whole tumor surface for signs of invasive growth. In some cases, we need additional tests to differentiate between benign and malignant.

Recently, researchers have started to focus on the role of the *Telomerase Reverse Transcriptase* (*TERT*) gene in thyroid cancer, specifically in follicular tumors. *TERT* is involved in regulating the length of telomeres, which are the protective caps on the ends of our chromosomes. These caps shorten with every cell division. If they fall below a certain length, the cells will not be allowed to divide anymore. In cancer cells, the *TERT* gene expression is often increased, allowing the cancer cells to divide and grow uncontrollably. This increased expression is usually caused by a mutation in a specific region of the gene, called the promoter region.

To better understand the role of *TERT* in thyroid follicular tumors, we conducted several studies for this thesis:

In **study I** we evaluated the role of the *TERT* promoter mutation in a specific group of borderline follicular tumors, in which the differentiation between benign FTA and malignant FTC is difficult. These tumors are therefore called "Follicular tumors of uncertain malignant potential" (FT-UMP). We were able to show that *TERT* promoter mutations occur in some of these tumors and these especially have a higher likelihood to recur as spread disease in patients after successful surgery.

In **study II**, the goal was to improve the identification of *TERT* promoter mutations in FT-UMPs by using digital droplet polymerase chain reaction (ddPCR) which is a highly sensitive and specific method for analyzing *TERT* promoter mutations. We were able to confirm this technique's superiority over the standard method used, called DNA Sequencing, and to show that *TERT* mutations don't always occur in the whole tumor but can be present in a small group of cells within a tumor.

In **study III** we evaluated the advantage gained by using *TERT* promoter mutational analysis via ddPCR on FNAC to make a more accurate diagnosis and determine the best course of treatment for patients with follicular thyroid tumors. We were able to show that ddPCR can successfully identify *TERT* promoter mutations in this material. Using this technique will allow us to identify a group of malignant follicular thyroid tumors on FNAC material alone before they undergo surgery, which potentially would allow the surgeon to choose a more aggressive approach.

In s**tudy IV** we wanted to identify tumors with *TERT* promoter mutations without having to use advanced molecular analyses, which might not be readily available in every pathology department.

Instead, we used a widely available technique that can identify the loss of the 5hmC protein. Loss of 5hmC has been shown in another type of *TERT* promoter mutated thyroid tumors (papillary thyroid carcinoma). In our cohort of FTCs, we found that 5hmC expression was not a reliable predictor of *TERT* promoter mutations, and further studies are therefore needed.

To summarize, this thesis was able to show that the use of *TERT* promoter mutational screening for follicular thyroid tumors has the potential to improve the diagnostic workup for these patients and provide new insights into the progression of this type of cancer.

# Populärwissenschaftliche Zusammenfassung

Mit dieser Arbeit möchte ich die Diagnostik von Schilddrüsenkrebs durch die Untersuchung eines Gens in den veränderten Schilddrüsenzellen verbessern.

Die Schilddrüse ist ein kleines, schmetterlingsförmiges Organ, das sich am Hals, unterhalb des Adamsapfels befindet. Ihre Hauptaufgabe besteht darin Hormone zu produzieren, die den Stoffwechsel unseres Körpers regulieren.

Als Zytopathologe nutze ich häufig die Feinnadelaspirationszytologie (FNAC), um Erkrankungen der Schilddrüse, allen voran Schilddrüsenkrebs, zu diagnostizieren. FNAC beinhaltet das Sammeln einer kleinen Zellprobe aus einem Tumor und deren Untersuchung unter einem Mikroskop, um festzustellen, ob die Zellen bösartig sind. Nachdem diese Knoten chirurgisch entfernt wurden, ist es immer noch schwierig, diese aufgrund von mikroskopischen Schnitten alleine zuverlässig zu diagnostizieren. Dies liegt daran, dass man zwischen gutartigen, sogenannten follikulären Schilddrüsenadenomen (FTA) und bösartigen Tumoren wie dem follikulären Schilddrüsenkarzinom (FTC) nur unterscheiden kann, wenn man den gesamten Tumor im Mikroskop auf zerstörerisches Wachstum untersucht hat. Dieser Prozess ist sehr arbeitsaufwendig und stark vom Untersucher abhängig. In manchen Fällen benötigen wir zusätzliche Tests, um zwischen den beiden zu unterscheiden.

In jüngster Zeit haben Forscher begonnen, sich auf die Rolle des *TERT*-Gens („Telomerase Reverse Transkriptase") im Schilddrüsenkrebs, insbesondere in follikulären Tumoren, zu konzentrieren. *TERT* ist an der Regulierung der Länge der Telomere beteiligt. Telomere sind die Schutzkappen am Ende unserer Chromosomen, die bei jeder Zellteilung kürzer werden. Wenn eine gewisse Länge unterschritten wird, sorgen sie dafür, dass die Zelle sich nicht mehr weiter teilen kann. Bei Krebszellen ist das *TERT*-Gen häufig hochreguliert, was die Krebszellen zur unkontrollierten Teilung und Wachstum befähigt. Diese Hochregulierung wird von einer Mutation in einem speziellen Teil des Gens, dem Promotor, verursacht.

Um die Rolle von *TERT* in follikulären Schilddrüsentumoren besser zu verstehen, haben wir im Rahmen dieser Dissertation mehrere Studien durchgeführt:

In **Studie I** haben wir die Rolle dieser *TERT*-Promotormutation in einer spezifischen Gruppe von follikulären Tumoren untersucht, bei denen die Unterscheidung zwischen gutartigen FTA und bösartigen FTC besonders schwierig ist und deshalb als follikulärer Tumor unklaren malignen (bösartigen) Potenzials bezeichnet wird. Wir konnten zeigen, dass *TERT*-Promotormutationen bei einigen dieser Tumore vorkommen und diese eine höhere Wahrscheinlichkeit haben, bei einem Patienten trotz einer erfolgreichen Operation als bösartige Karzinome wieder aufzutreten.

**Studie II** zielte darauf ab, die Identifizierung von *TERT*-Promotormutationen durch die Verwendung der digitalen Tröpfchen-Polymerasekettenreaktion (ddPCR) zu verbessern, die eine sehr hohe Sensitivität und Spezifität gegenüber anderen Methoden zur Analyse von *TERT*-Mutationen aufweist. Wir konnten bestätigen, dass diese Technik im Vergleich zur Standardmethode DNA-Sequenzierung überlegen ist, und dass *TERT*-Mutationen nicht immer im gesamten Tumor vorliegen, sondern nur in einer kleinen Gruppe von Zellen innerhalb eines Tumors vorkommen können. Mit dieser Methode konnten auch diese Untergruppen identifiziert werden.

In **Studie III** haben wir den Vorteil untersucht, den wir durch die Verwendung der *TERT*-Analyse via ddPCR in Feinnadelbiopsien erzielen können, um eine genauere Diagnose zu stellen und den Behandlungsverlauf für Patienten mit follikulären Schilddrüsentumoren zu verbessern. Wir konnten zeigen, dass die ddPCR Technik auch bei diesem Material erfolgreich *TERT* Promoter Mutationen identifizieren kann. Dadurch könnte man in diesen Fällen auch schon vor einer Operation bösartige Karzinome erfolgreich identifizieren.

In **Studie IV** wollten wir Tumore mit *TERT*-Promoter-Mutationen identifizieren, ohne auf eine fortgeschrittene molekulare Analyse zurückgreifen zu müssen, die nicht in jedem Pathologielabor verfügbar sein könnte. Stattdessen wollten wir eine weit verbreitete Technik verwenden und den Verlust des Proteins 5hmC identifizieren. Dieser Verlust wurde zuvor bei einem anderen Typen von *TERT*-Promoter-mutierten Schilddrüsenkarzinomen (papillären Schilddrüsenkarzinomen) beobachtet. In unserer Kohorte von FTCs konnten wir diesen Effekt jedoch nicht nachweisen oder nachhaltige Schlüsse daraus ziehen.

Zusammenfassend konnten wir mit diesen Studien zeigen, dass die Verwendung des *TERT*-Promoter-Mutationsscreenings für follikuläre Schilddrüsenknoten das Potenzial hat, die Diagnostik für diese Patienten zu verbessern und neue Einblicke in den Verlauf dieser Art von Krebs zu geben.

# Abstract

Follicular thyroid neoplasms are diagnostically challenging. On histologic evaluation, it can be difficult, resource-consuming, and observer-dependent to pinpoint the exact location of capsular or vascular invasion. In some cases, it is impossible to do so unequivocally – for those, the term "follicular tumor of uncertain malignant potential" (FT-UMP) was created. On cytologic evaluation, it is less challenging but rather hardly possible to distinguish follicular thyroid adenoma (FTA) from follicular thyroid carcinoma (FTC). With the advent of molecular analyses in clinical diagnostic settings, many mutational events have been associated with specific cancers. Amongst those, two point mutations in the *TERT* promoter region, named C228T and C250T have been of particular interest as they have been associated with malignant properties in thyroid tumors in general and a worse prognosis with a higher frequency of relapse in particular.

This thesis aims to improve the diagnostic accuracy for thyroid tumors in general and for follicular thyroid tumors in particular through the implementation of *TERT* promoter mutational screening.

Study I evaluates the role of *TERT* promoter mutational screening in a clinical series of FT-UMPs and how this analysis aids in detecting relapse-prone tumors. This could help alter adjuvant treatment modalities even in the absence of clearcut histopathological evidence of malignant potential.

Study II shows that digital droplet PCR (ddPCR) can improve the sensitivity for the detection of *TERT* promoter mutations in follicular thyroid tumors and can even detect *TERT* promoter mutations when they occur subclonal and are heterogeneously distributed in FT-UMPs.

Study III validates *TERT* promoter mutational testing on preoperative material in the form of frozen pellets from thyroid FNAC material. We were able to show that ddPCR is a reliable analysis for cytologic material and may help to identify high-risk cases and triage them to a more aggressive treatment plan up-front, underlining the markers' diagnostic and prognostic value.

Study IV tries to evaluate 5hmC immunoreactivity as an expressional analysis to pinpoint *TERT* promoter mutations in FTCs. Even though the study was able to show that the loss of 5hmC immunoreactivity may signify *TERT* promoter mutations in subsets of FTCs, we could not prove its clinical value to predict the *TERT* promoter mutational status. Further studies are therefore warranted.

In summary, the findings in this thesis highlight the clinical importance of *TERT* promoter mutational screening in follicular thyroid neoplasms. Furthermore, we were able to show that ddPCR is a reliable technique for interrogating specific mutations of the *TERT* promoter.

# List of scientific papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I       **Hysek M**, Paulsson JO, Jatta K, Shabo I, Stenman A, Höög A, Larsson C, Zedenius J, Juhlin CC.
        Clinical Routine *TERT* Promoter Mutational Screening of Follicular Thyroid Tumors of Uncertain Malignant Potential (FT-UMPs): A Useful Predictor of Metastatic Disease.
        *Cancers (Basel).* 2019 Sep 26;11(10):1443. doi:10.3390/cancers11101443.

II      **Hysek M**, Jatta K, Hellgren LS, Stenman A, Larsson C, Zedenius J, Juhlin CC.
        Spatial Distribution Patterns of Clinically Relevant *TERT* Promoter Mutations in Follicular Thyroid Tumors of Uncertain Malignant Potential: Advantages of the Digital Droplet PCR Technique.
        *J Mol Diagn.* 2021 Feb;23(2):212-222. doi:10.1016/j.jmoldx.2020.10.016. Epub 2020 Nov 14.

III     **Hysek M.,** Hellgren LS, Stenman A, Darai-Ramqvist E, Ljung E, Schliemann I, Condello V, Larsson C, Zedenius J, Jatta K, & Juhlin CC.
        Digital droplet PCR *TERT* promoter mutational screening in fine needle aspiration cytology of thyroid lesions: A highly specific technique for pre-operative identification of high-risk cases.
        *Diagnostic cytopathology*, 2023, Advance online publication. doi:10.1002/dc.25120.

IV      **Hysek M,** Hellgren LS, Condello V, Larsson C, Zedenius J, Juhlin CC.
        5hmC immunohistochemistry: a predictor of *TERT* promoter mutational status in follicular thyroid carcinoma?
        Manuscript submitted to *Endocrine*, February 2023, PREPRINT (Version 1) available at Research Square, https://doi.org/10.21203/rs.3.rs-2642987/v1.

# Contents

# List of abbreviations

| | |
|---|---|
| 5hmC | 5-hydroxymethylcytosine |
| ALK | Anaplastic Lymphoma Kinase |
| AP | Alkaline Phosphatase |
| ATC | Anaplastic Thyroid Carcinoma |
| AUS | Atypia of Undetermined Significance |
| BRAF | v-Raf murine sarcoma viral oncogene homolog B |
| CC-PTC | Clear Cell Papillary Thyroid Carcinoma |
| CN | Copy Number |
| DAB | 3,3'-Diaminobenzidine |
| ddPCR | Digital Droplet Polymerase Chain Reaction |
| ddNTP | Di-Deoxynucleotide Triphosphate |
| DHGTC | Differentiated High-Grade Thyroid Carcinoma |
| DNA | Deoxyribonucleic Acid |
| eaiFTC | Encapsulated Angio-Invasive Follicular Thyroid Carcinoma |
| EIF1AX | Eukaryotic Translation Initiation Factor 1A, X-linked |
| EZH1 | Enhancer of Zeste Homolog 1 |
| FLUS | Follicular Lesion of Undetermined Significance |
| FNAC | Fine-Needle Aspiration Cytology |
| FND | Thyroid Follicular Nodular Disease |
| FTA | Follicular Thyroid Adenoma |
| FTC | Follicular Thyroid Carcinoma |
| FT-UMP | Follicular Thyroid Tumor of Uncertain Malignant Potential |
| FVPTC | Follicular Variant of Papillary Thyroid Carcinoma |
| HRAS | Harvey Rat Sarcoma viral oncogene homolog |
| HRP | Horseradish Peroxidase |
| IDH1 | Isocitrate Dehydrogenase 1 |
| IHC | Immunohistochemistry |
| HN-PTC | Hobnail variant Papillary Thyroid Carcinoma |
| KRAS | Kirsten Rat Sarcoma viral oncogene homolog |
| MAP kinase | Mitogen-Activated Protein Kinase |
| miFTC | Minimally Invasive Follicular Thyroid Carcinoma |
| MNG | Multinodular Goiter |
| MTC | Medullary Thyroid Carcinoma |
| NGS | Next-Generation Sequencing |
| NIFTP | Non-Invasive Follicular Tumor with Papillary Features |
| NRAS | Neuroblastoma Rat Sarcoma viral oncogene homolog |
| NTRK1/3 | Neurotrophic Tyrosine Kinase Receptor 1/3 |
| PAX8 | Paired box 8 |
| PCR | Polymerase Chain Reaction |
| PDTC | Poorly Differentiated Thyroid Carcinoma |
| PPARG | Peroxisome Proliferator Activated Receptor Gamma |
| PTC | Papillary Thyroid Carcinoma |
| PTEN | Phosphate and Tensin homolog |
| qRT-PCR | Quantitative Real Time Polymerase Chain Reaction |
| RAS | Rat Sarcoma viral oncogene homolog |
| RET | Rearranged during Transfection |
| RNA | Ribonucleic Acid |
| ssDNA | Single-Strand DNA |
| ST-PTC | Solid or Trabecular Variant of Papillary Thyroid Carcinoma |
| T3 | Triiodothyronine |
| T4 | Thyroxine |
| TBSRTC | The Bethesda System for Reporting Thyroid Cytopathology |
| TC-PTC | Tall Cell Variant of Papillary Thyroid Carcinoma |
| TERC | Telomerase RNA Component |
| TERT | Telomerase Reverse Transcriptase |
| TET | Ten-Eleven Translocation Family |
| TR | Telomerase RNA template |
| TRH | Thyrotropin-Releasing Hormone |
| TSH | Thyroid Stimulating Hormone |

wiFTC            Widely Invasive Follicular Thyroid Carcinoma

# 1  Introduction

For cytologists, thyroid lesions are – in theory – straightforward. In clinical routine, the Bethesda classification system is usually used to grade lesions. Bethesda I is reserved for insufficient material where a diagnosis cannot be made, Bethesda II are benign lesions and Bethesda VI are malignant tumors such as primary carcinomas, but can also be sarcomas, lymphomas, or metastasis to the thyroid. However, the real challenges for both surgeons and pathologists, lie in lesions classified as Bethesda groups III, IV, and V. These are lesions that exhibit atypia or constitute a follicular type of lesion, be it tumorous or not (Bethesda III), are suspicious for a follicular thyroid tumor (Bethesda IV) or suspicious for malignancy (Bethesda V). Bethesda IV lesions often lead to a diagnostic lobectomy of the affected thyroid lobe, and the pathologist is tasked with the difficult job to assess whether this lesion is a benign follicular thyroid adenoma (FTA), a malignant follicular thyroid carcinoma (FTC) or – in between – a follicular tumor of uncertain malignant potential (FT-UMP). FTAs require no further follow-up as they are benign, FT-UMPs are usually followed-up to a varying degree, and FTCs – especially locally more advanced cases – often require additional treatment with a contralateral lobectomy to achieve near-total thyroidectomy (1) followed by radioiodine therapy.

The goal of this thesis is to make this process of distinguishing malignant from benign cases easier for cytologists and pathologists which will give the surgeons better-defined clinical guidance and not least of all, make the process easier for the patients as well.

## 1.1    The Thyroid Gland

### 1.1.1    Anatomy

The thyroid gland consists of two lobes and a central part called the isthmus. It is situated in front of the trachea just underneath the thyroidal cartilage. The gland is surrounded by a bi-layered fascia consisting of an internal and an external capsule. The internal capsule joins the glandular tissue and contains the blood vessels which grow into the parenchyma, thus creating the organ's lobular structure. The external capsule, also referred to as the surgical capsule, is rougher and surrounds both the thyroid gland and the dorsally situated parathyroid glands as well as the blood vessels. Blood is supplied mainly by the superior thyroid artery originating from the external carotid artery and to a minor part by the inferior thyroid artery originating in the thyrocervical artery coming from the subclavian artery. The venous drainage is mainly ensured by the plexus thyroideus impar flowing through a singular inferior thyroid vein into the left brachiocephalic vein. To a lesser part, the venous drainage follows the superior thyroid veins into the internal jugular vein.

Of anatomic importance is the positioning of the laryngeal recurrent nerve posterior to the thyroid gland. With surgical resection of the thyroid, this nerve is in danger of being damaged, and even more so in the case of a secondary surgery where the area has developed scar tissue (2).

### 1.1.2    Histology and physiology

The above-mentioned fibrous capsule gives the organ its' histologic structure. Septae extend from the capsule inward, further subdividing the organ into lobules. These lobules consist of multiple follicles, all usually of similar size, and the surrounding connective tissue. The follicle is the functional unit of the thyroid. It is a round structure surrounded by a single cell layer of follicular thyroid cells and the lumen is filled with colloid. Colloid is the inactive form of thyroid hormone and is produced by the follicular cells (Figure 1). When the hypothalamus registers a lack of Thyroxine (T4) and triiodothyronine (T3) in circulation, Thyrotropin-releasing hormone (TRH) is secreted and stimulates the release of Thyroid Stimulating Hormone (TSH) from the pituitary gland. TSH binds to TSH receptors on the follicular cells and stimulates iodine uptake and T3/T4 synthesis. The active hormone gets released into the blood vessels within the surrounding connective tissue. T4 is the main form of the available hormone in our body, whereas T3 is more potent. T4 can be converted into T3 by deiodinases. T3 and T4 interact with virtually every cell in the body to control the basal metabolic rate and affect growth and sensitivity to catecholamines.
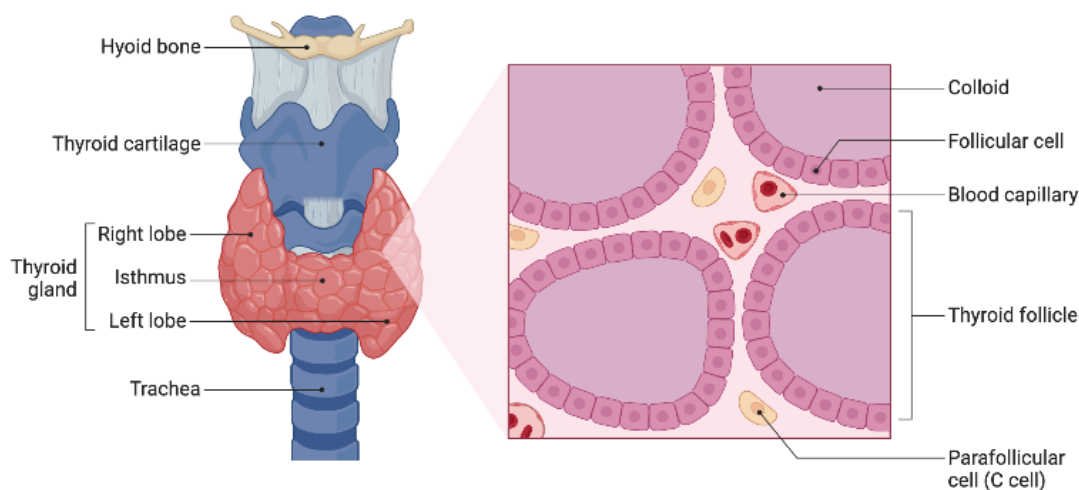


*Figure 1 – Anatomy and Histology of the Thyroid Gland.* *Created using BioRender.com*

## 1.2    Classification of follicular cell–derived neoplasms

Thyroid cancer is the most common form of endocrine malignancy and has a rising incidence rate (3,4), accounting for 1.4-4.6% of newly diagnosed cancers per year which amounts to 600-700 new cases in Sweden every year (5). Follicular cell-derived carcinomas are the most common and consist of papillary thyroid carcinoma (PTC) (80% of cases), FTC (15% of cases), and follicular cell-derived carcinoma, high-grade (5%).

### 1.2.1    Papillary thyroid carcinoma

PTC is the most common form of follicular cell-derived carcinoma. It has generally a favorable prognosis with a 5-year survival rate of above 95% even with the development of lymph node metastasis (3,4), as it is generally surgically removable and responds well to radioiodine treatment.

PTC usually presents with a papillary growth pattern and nuclear atypia such as nuclear enlargement, chromatin clearing, nuclear groves, and intranuclear cytoplasmic inclusions (6). In recent years, however, the description of several new subtypes, such as non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) (7) made a more defined subclassification necessary.

In the latest (5th) version of the WHO classification of endocrine neoplasms (8), more emphasis was put on the subtypes of PTC (9,10), especially the ones that are associated with a worse prognosis: tall-cell PTC (TC-PTC), columnar cell PTC (CC-PTC), and hobnail variant PTC (HN-PTC) have all been classified as at intermediate risk for "structural disease recurrence" by the ATA (11) even in small tumors measuring less than 1 centimeter. Other studies also include solid PTCs (S-PTC) (12).

From a strictly morphologic point of view, all of the above-mentioned share invasive growth and distinct nuclear atypia. They differ though in cellular appearance: TC-PTC consists of at least 30% cells with tall cell morphology, that is cells with elongated shape, three times higher than wide and abundant eosinophilic cytoplasm (13); CC-PTC also shows columnar cells similar to TC-PTC but the cytoplasm is paler to clear eosinophilic and the nuclei appear pseudostratified with subnuclear vacuoles (14); HN-PTC has large and apically bulging nuclei, generating the name giving hobnail-like appearance (15); and S-PTC is defined through its growth pattern of mainly solid, trabecular or nested patterns (16).

### 1.2.2 Follicular neoplasms

Follicular neoplasms are the most common group of tumors in the thyroid and include FTA, FTC, and FT-UMP. Approximately 80% of all follicular neoplasms are benign FTA. FTC are malignant tumors with an estimated relative survival rate of approximately 80-90%, and patients that succumb to the disease usually exhibit distant metastases to the lungs and bone. The distinction between FTA and FTC is challenging in the histologic evaluation and is based on invasive features (capsular or vascular invasion) only (Figure 2).
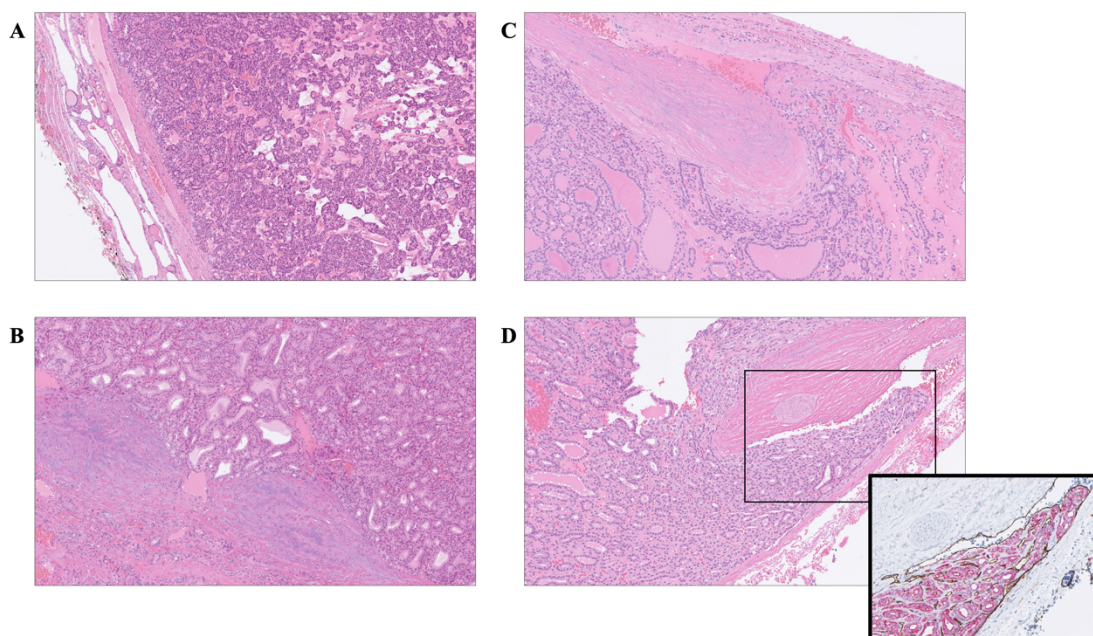


*Figure 2 – Histological aspects of follicular thyroid tumors. Depicted here are hematoxylin-eosin-stained sections of a follicular thyroid adenoma (A), a follicular tumor of uncertain malignant*

*potential (B), as well as two follicular thyroid carcinomas displaying capsular (C) and vascular invasion (D) respectively. The insert in D shows tumor cells marked in red (pan-cytokeratin stain) and endothelium is marked in brown (CD31 stain).*

All three tumor entities consist of a clonal proliferation of follicular epithelial cells with inconspicuous cellular morphology, usually exhibiting a micro- or macro-follicular growth pattern. Solid growth patterns are rare (6).

FTAs are surrounded by a fibrous capsule and show no signs of capsular penetration or vascular infiltration. FTCs are either penetrating through the capsule and/or exhibiting vascular infiltration. FT-UMPs further complicate the situation as this group features equivocal foci where a clear distinction between encapsulation or invasive growth and vascular infiltration cannot be made. The tumor might, for example, protrude into the capsular layer but a clear penetration into the surrounding tissue cannot be established (Figure 2, B). Likewise, these tumors may be seen in close relation to a blood vessel, but definitive infiltration into the vascular lumen cannot be unequivocally established (6).

Thus, establishing a definite diagnosis for follicular tumors requires extensive sampling of the tumor capsule and a thorough microscopic examination (1). This process is time-consuming and investigator dependent.

### 1.2.3 Follicular–derived carcinomas, high–grade and anaplastic thyroid carcinoma

These are the most aggressive follicular cell-derived carcinomas. They most commonly result from de-differentiation of FTC or PTC, but an alternate theory in which these tumors arise *de novo* also exists. These cancers are rare, accounting for 1-4% of all thyroid cancers (6). The prognosis is generally poor, with follicular-derived carcinomas high-grade having a reported mortality rate of 30-50% and anaplastic thyroid cancer (ATC) having a mortality rate of over 90% (6).

In the 2022 WHO classification, a new entity has been introduced, called differentiated high-grade thyroid carcinoma (DHGTC) which together with poorly differentiated thyroid carcinoma (PDTC) comprises the group of follicular-derived carcinomas, high-grade.

DHGTC was added as an entity to help distinguish a group of differentiated follicular cell-derived thyroid carcinomas (both PTC and FTC) that do not fulfill the Turin criteria for the diagnosis of PDTC but have a clinically comparable outcome. The Turin criteria for the diagnosis of PDTC state that the tumor needs to show solid, trabecular or insular growth patterns, usually small, round, uniform, and hyperchromatic nuclei without features typical for PTC and increased mitotic rate, necrosis, or convoluted nuclei (17). DHGTC on the other hand retains its differentiation in the form of growth pattern or cellular morphology but has an increased mitotic activity or necrosis present (18,19).

ATC presents microscopically with dedifferentiated cytomorphology such as sarcomatoid, epithelial, or giant cell phenotypes (6). Usually, these tumors are locally advanced with infiltration of the surrounding structures and exhibit necrotic areas as well as high mitotic activity.

## 1.3 Classification of thyroid lesions on cytology: The Bethesda Classification system

Before removing a thyroid nodule surgically, fine-needle aspiration cytology (FNAC) is performed. This is a fast and cost-effective way to identify the lesion. Through FNAC, unnecessary operations of benign lesions can be reduced (20,21). There are several national classification schemes for thyroid FNAC (22–24), the most commonly used one is the American Bethesda Classification System.

### 1.3.1 First and second edition of the Bethesda Classification System

In 2009, the Bethesda System for Reporting Thyroid Cytopathology (TBSRTC) was introduced to create a more standardized and thus, more easily reproducible classification system for thyroid lesions (25) and was updated in 2018 (2nd edition). It is a 6-tier classification distinguishing between non-diagnostic (Bethesda I), benign (Bethesda II), atypia/follicular lesion of undetermined significance (AUS/FLUS) (Bethesda III), follicular neoplasm/suspicious of follicular neoplasm (Bethesda IV), suspicious for malignancy (Bethesda V) and malignant (Bethesda VI). All of these tiers have associated diagnoses/subcategories, some of which are exemplified in Table 1 and shown in Figure 3.

| Bethesda Category | Examples of diagnoses | Usual Management |
|---|---|---|
| **I. NONDIAGNOSTIC or UNSATISFACTORY** | Cyst fluid<br>Virtually acellular specimen<br>Other (obscuring blood, clotting artifact, etc.) | Repeat FNA, preferably with ultrasound guidance |
| **II. BENIGN** | Benign follicular nodule (adenomatoid nodule, colloid nodule)<br>Consistent with lymphocytic thyroiditis<br>Consistent with granulomatous (subacute) thyroiditis | Clinical follow-up |
| **III. ATYPIA OF UNDETERMINED SIGNIFICANCE or FOLLICULAR LESION OF UNDETERMINED SIGNIFICANCE** | Difficult to classify, such as low cellularity that precludes a diagnosis of follicular neoplasm or<br>oncocytic cells only in a clinical context of lymphocytic thyroiditis or<br>focal features of PTC in otherwise benign background | Repeat FNA |
| **IV. FOLLICULAR NEOPLASM or SUSPICIOUS FOR A FOLLICULAR NEOPLASM** | Follicular Thyroid Adenoma<br>Follicular Thyroid Cancer<br>Oncocytic Tumor | Surgical diagnostic lobectomy |
| **V. SUSPICIOUS FOR MALIGNANCY** | Suspicious for papillary carcinoma<br>Suspicious for medullary carcinoma<br>Suspicious for metastatic carcinoma<br>Suspicious for lymphoma | Near total thyroidectomy or surgical diagnostic lobectomy |
| **VI. MALIGNANT** | Papillary thyroid carcinoma<br>Poorly differentiated carcinoma<br>Medullary thyroid carcinoma<br>Anaplastic carcinoma<br>Squamous cell carcinoma<br>Non-Hodgkin's lymphoma | Near-total thyroidectomy |

*Table 1 – The Bethesda Classification of Thyroid Cytology, 2nd edition.*

The Bethesda classification has a defined risk of malignancy associated with each tier that ranges from 0-3% (Bethesda II) to 97-99% (Bethesda VI). These numbers were based on an American population (25), but similar results have been shown for other countries in retrospective analyses (26–31). Following the tiers and their risk of malignancies, different therapeutical approaches are suggested: re-biopsy for Bethesda I and III, clinical follow-up for Bethesda II, and surgery for Bethesda IV-VI. For tumors classified as Bethesda IV, a diagnostic lobectomy, and for Bethesda V and VI, a total thyroidectomy is recommended (6,25).
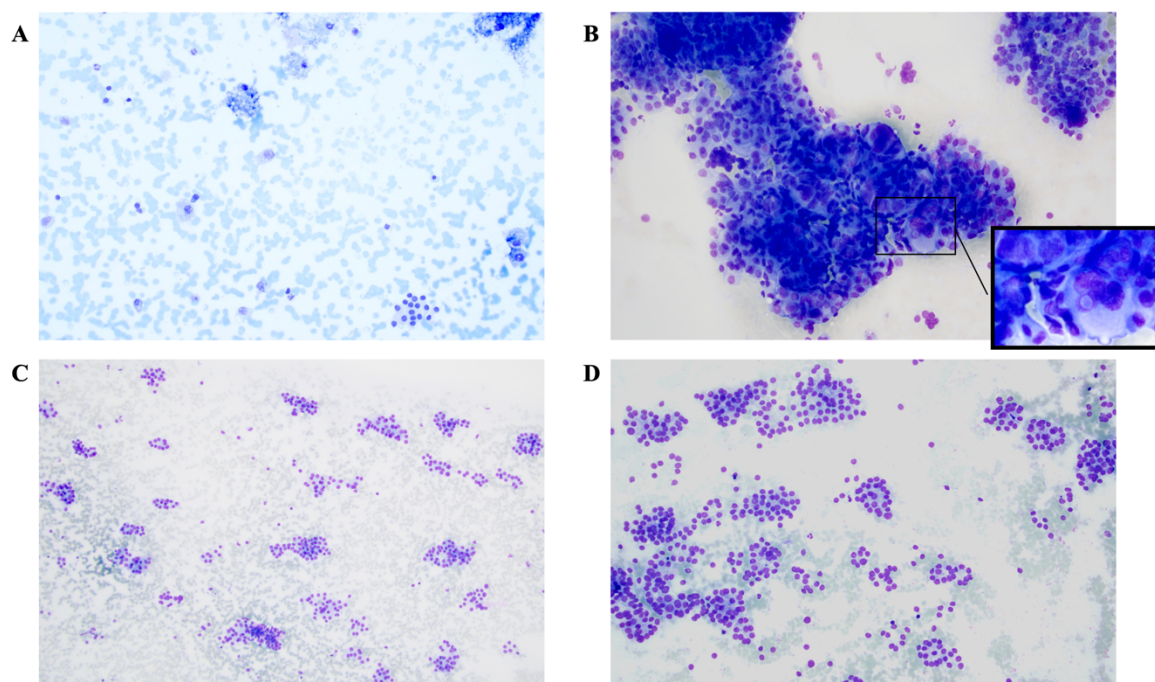


*Figure 3 – Examples of Different Bethesda Categories on Cytology. **Bethesda II (A)** Scarce groups of follicular epithelial cells, macrophages, and isolated cells with round nuclei. The blueish background is caused by colloid (100x magnification). **Bethesda VI (B)** densely packed 3-dimensional group of epithelial cells with smooth borders and visible nuclear pseudoinclusions (insert) (200x). **Bethesda IV (C&D)** follicular epithelial cells with a repetitive microfollicular or acinar pattern. Hardly any colloid is visible. (C 100x & D 200x).*

### 1.3.2 Overview of the changes coming in the 3rd edition

In the Spring of 2023, a third edition of TBSRTC is expected which will further develop the established categories and align them more closely with the 5th edition of the WHO classification of endocrine tumors (32). The diagnoses "Thyroid follicular nodular disease" and "DHGTC" will be included in the Bethesda classification. Furthermore, the unified nomenclature of oncocytic thyroid neoplasm instead of Hürthle cell neoplasm will be solidified. Category Bethesda III will be subdivided into only two groups namely Atypia of undetermined significance (AUS) with nuclear atypia, and AUS – other. The term follicular lesion with undetermined significance was dropped and is now subsumed in AUS-Other.

A chapter was added on clinical perspective and imaging studies to highlight the importance of an interdisciplinary approach to the treatment of patients with thyroid nodules. The risk of malignancy for each category will have been adapted to newly available data, and the management recommendation of nodules that were identified as Bethesda III – V now will include molecular testing (32).

The rising importance of molecular testing will be reflected in its own chapter as well, focusing on the purpose of molecular testing, different available platforms for molecular testing and the key molecular changes.

## 1.4 Key molecular changes in thyroid neoplasms

With the vast progress made in molecular analyses, attempts have been made – as with many other tumor groups – to help in classifying thyroid tumors according to their mutational profiles.

### 1.4.1 Molecular changes used for classification

The main research focus in thyroid cancers has been on mutations in genes belonging to the MAP kinase pathway – the activating *BRAF* V600E mutation being the most prevalent one – and RAS signaling pathways, mainly point mutations in *NRAS* codon 61 (33,34). *BRAF* V600E mutations have been found to occur in the majority of PTCs and are associated with worse prognostic features, such as more aggressive subtypes, lymph node metastasis, or recurrence (35–38). The point mutation activates the downstream MAPK signaling pathway and induces transcriptional programs related to proliferation, angiogenesis, and invasive properties (34,39).

*RAS* mutations are generally associated with more indolent tumors and predominantly in tumors belonging to the group of follicular thyroid neoplasms such as FTC, but also in FTA suggesting a role early in tumorigenesis, before true malignant transformation (33). In follicular thyroid neoplasia, *NRAS* is more common than the other isoforms *KRAS* and *HRAS* (40) and primarily activates the Pi3K-AKT pathway (41,42). The most common signaling pathways involved in thyroid neoplasms are shown in Figure 4.
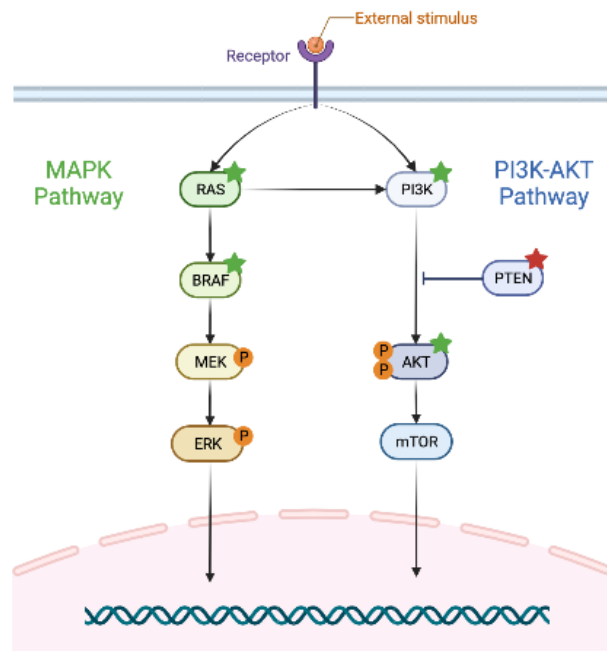
*Figure 4 – Most common signaling pathways involved in Thyroid neoplasm.* *Green stars indicate activating mutations and red stars inactivating mutations. Created using BioRender.com*

Other genetic aberrancies found in thyroid carcinomas are, amongst others, *DICER1* and *PTEN* mutations. Common driver mutations and fusions for the "RAS-like", follicular neoplasm, and "BRAF-like" papillary thyroid cancers are listed in Table 2 and Table 3 respectively.

| Follicular thyroid neoplasms | | |
|---|---|---|
| **Alteration** | **Frequency** | **Gene function** |
| *H/K/NRAS* | 20-57%^ | GTPase, activation of the MAP Kinase signaling pathway |
| *PAX-PPARG* **fusion** | 30 - 58%^ | PP fusion protein (43) |
| *EIF1AX* | 17%^ | A translation initiation factor (44) |
| *DICER1* | 5-8%* | Protein translational control through mRNA processing (45) |
| *EZH1* | 7.3% | Histone modification (46,47) |
| *PTEN* | 0-27%^ | PI3K pathway regulator |

*Table 2 – Most common mutations and fusions in follicular thyroid neoplasms.* *^numbers derived from the review "The genomic landscape of Thyroid Cancer Tumorigenesis and Implications for Immunotherapy" (48); *number derived from the review "Proceedings of the North American Society of Head and Neck Pathology, Los Angeles, CA, March 20, 2022: DICER1-Related Thyroid Tumors" (49).*

| Papillary thyroid cancer | | |
|---|---|---|
| **Alteration** | Frequency | Gene function |
| ***BRAF*** | Up to 62%^ | Activation of the MAP Kinase signaling pathway |
| ***H/K/NRAS*** | 13%^ | GTPase, activation of the MAP Kinase signaling pathway |
| ***RET/PTC* fusion** | 5-33%^ | Receptor tyrosine kinase |
| ***BRAF* fusion** | 3.9%^ | Activation of the MAP Kinase signaling pathway |
| ***NTRK1/3* fusion** | 1.3-26%^ | Tyrosine kinase regulated by nerve growth factors (50) |
| ***ALK* fusion** | 1%^ | Tyrosine kinase |

*Table 3 – Most common mutations and fusions in PTC. ^numbers derived from the review "The genomic landscape of Thyroid Cancer Tumorigenesis and Implications for Immunotherapy (48).*

### 1.4.2  Molecular changes used for targeted therapy

As of 2022, only BRAF inhibitors are suggested as a targeted treatment option for anaplastic thyroid carcinoma, therefore *BRAF* V600E reflex testing on this entity is recommended by the WHO classification (8) as well as by the Swedish national guidelines (1).

### 1.4.3  Molecular changes in disease progression

The division into the molecular groups *BRAF* V600E-like and *RAS*-like has not been able to significantly improve risk stratification models that could be beneficial for clinical routine practice (35) when compared to traditional histological grading in general. However, in cases with an uncertain malignant potential (such as FT-UMPs or encapsulated PTCs), molecular markers are of importance to acquire a better risk stratification (35,37,51). Markers that have been identified as "late event changes" and are more commonly found in high-grade tumors are amongst others, *TP53* mutations, cell cycle gene aberrations, and mutations in DNA mismatch repair genes.

More recently, mutations in the promoter region of the *telomerase reverse transcriptase* (*TERT*) gene have been studied and shown great promise as a diagnostic and prognostic marker in thyroid tumors (52–54).

## 1.5  Telomeres

The telomere is the terminal region of every chromosome and was first described by Barbara McClintock (55), who won the Nobel prize for her discovery in 1983. In human cells, the telomeric region is between 3000-10,000 base pairs long and consists of a repetitive 6-basepair long sequence (TTAGGG on the 3'strand) and protects the coding DNA from incomplete transcription. Incomplete transcription would occur on the (5'-) lagging strand as transcription only occurs in the 5'-3' direction and thus, the lagging strand needs primers to start transcribing sections of DNA (Figure 5). When the primers are removed, the very end of the strand - on which the last primer was bound – will not have been transcribed and will be lost. Telomeric DNA takes this "sacrificing role" to not expose coding DNA, and shortens with every mitosis. When telomeres become critically short, cells usually go into a

non-proliferative but still physiologically active state called senescence (56). This mechanism explains Hayflick's observation of a limited number of duplication cycles in cells (57) and could be considered the cell's internal clock.

When cells avoid senescence and telomeres erode after too many repetitive duplication cycles, the chromosomal ends get exposed and can end up fusing with other eroded telomeric ends – usually their sister chromatid. In anaphase, when the sister chromatids get drawn apart by the centromeres, the now fused chromosomal ends will break. These now uneven chromosomal ends are exposed and prone to fuse with other chromosomes whose telomere had been eroded and form a so-called breakage-fusion-bridges (58). This process leads to pronounced aneuploidy which is called "crisis" and usually results in p53-mediated apoptosis.

### 1.5.1 Telomerase

To counteract – or at least to slow down – telomeric shortening, immortalized cells can use a reverse transcriptase called telomerase. Telomerase consists of several subunits; the two most important ones are the catalytic subunit (TERT) and - unlike other polymerases - telomerase contains its own RNA template called TR (its gene is called *TERC*) (Figure 5). TR is a short RNA molecule (451 ribonucleases long) with a central "pseudoknot" containing a three-stranded helix around the actual template (59). This holoenzyme binds to the last 6 nucleotides of the 3' end of the G-rich strand of the chromosome and TERT reverse-transcribes the 6-nucleotide long sequence from the TR subunit (60). (Figure 5) This process then gets repeated at the newly formed "end". The complementary strand is filled in by a conventional DNA polymerase (61).
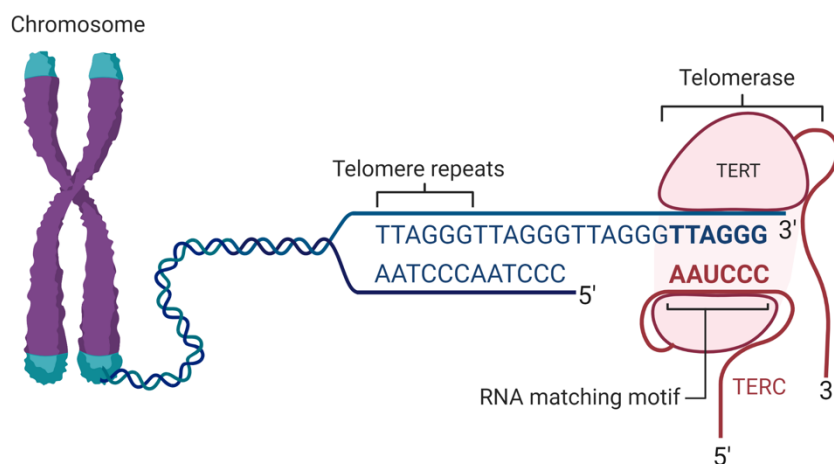


*Figure 5 – Schematic illustration of telomeric repeats and the role of telomerase in elongating telomeres. The action of the telomerase complex counteracts successive shortening of telomeres. Created using BioRender.com*

Hahn et al. demonstrated that ectopic expression of TERT, together with two oncogenes (the "*Simian Virus 40 Large-T oncoprotein*" to escape senescence and *HRAS* to avoid crisis) can transform both

mesenchymal cells such as fibroblasts and epithelial cells into malignant cells (62). To further prove this effect, it was shown that 85-90% of cancer cells show telomerase activity, highlighting the important role of preserving the telomere length during the replication (63) and making immortalization of cells one of the 16 hallmarks of cancer (63,64).

Healthy native human cells, except for lymphocytes, do not show telomerase activity and this is due to a lack of expression of the catalytic subunit TERT – although the other subunits are readily expressed (65). Activation of TERT can be achieved by de-repressing transcription of the *TERT* gene (66).

### 1.5.2   *TERT* gene

The *TERT* gene consists of 26 exons located on chromosome 5 (67,68). *TERT* upregulation in cancer can be achieved through gene amplification, rearrangements, and mutations or aberrant methylation within the promotor region (69) (Figure 6).

Two specific point mutations in the *TERT* promoter region have been proven as prominent mutations in cancers (69,70). As described in malignant melanoma (71,72), these point mutations occur as C>T transitions at Chr5:1295228 (-124 or C228T) and ch5:1295250 (-146 or C250T) respectively. These mutations create a new binding site for transcription factors from the ETS family that lead to an increased *TERT* gene expression (72). The same point mutations have been found in multiple cancer forms with varying frequency in different subtypes. Besides malignant melanoma, *TERT* promoter mutations have been reported in glioblastomas (73), urothelial cancer (74), basal cell- and squamous carcinoma of the skin (75), hepatocellular carcinoma (76) and thyroid carcinoma, where it is more frequently observed in advanced forms of the disease (53,54). Furthermore, other aberrancies of the *TERT* gene that lead to increased gene expression have been examined, namely copy number (CN) gain (77) and aberrant methylation in two different regions of the *TERT* promoter (78) (Figure 6). CN gains on both chromosome 5p15 (*TERT* gene) and chromosome 3q26 (*TR* gene) are among the most frequent chromosome arm gains in human cancers, found in 13,2% and 16,4% respectively (79).
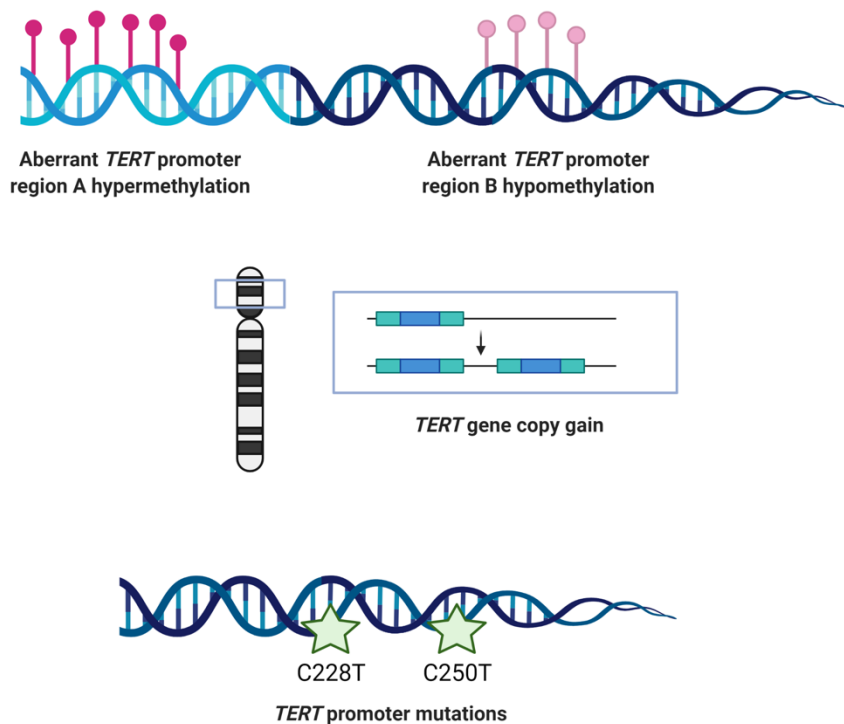
*Figure 6 – These genetic aberrancies are known to promote TERT gene expression in thyroid tumors. Created using BioRender.com*

Hypermethylation in the *TERT* promoter region entitled "Region A" (positions -578 to -541 base pairs) has been linked not only to the expression of *TERT* (80) but also to poor outcomes in patients with medullary thyroid carcinoma (81). Low methylation levels in a different region, designated "Region B" (at -162 to -100bp) proved to be necessary for *TERT* expression in vitro (82). Hypermethylation, as well as rearrangements (found in neuroblastomas), are generally rare in human cancers (83). However, in thyroid tumors, copy number gains and hypermethylation have been proven to occur at a higher frequency (84).

## 1.6    Telomeres and *TERT* in research outside of cancer diagnostics

Telomeres and *TERT* have not only found a lot of interest in cancer research but have also been found responsible for a plethora of other diseases, some of which are mentioned as exemplary for the field below.

### 1.6.1    Shortened telomeres as a cause of disease

Elizabeth Blackburn, who was awarded the Nobel Prize in Physiology and Medicine in 2009 for "how chromosomes are protected by telomeres and the enzyme telomerase" (85) and Mary Armanios summarize several diseases into the Telomere Syndromes (86). This group of diseases has in common, that the diseased cells have abnormally short telomeres. The telomere shortening in these diseases is

caused by different genetic mechanisms resulting in germline loss-of-function mutations in *TERT* or *TERC*. What they have in common, is that the telomere length seems to be inherited (87) and that the telomeres grow shorter from generation to generation which leads to an earlier onset of disease as well as more aggressive types of disease in later generations: A first-generation diseased person might develop pulmonary fibrosis (88), whereas his offspring might develop aplastic anemia (89) and the third degeneration might develop dyskeratosis congenita (90).

In the rare Hutchinson-Gilford Progeria Syndrome, a mutation in the *LMNA* gene leads to the expression of Progerin instead of Lamin A (91) which leads to, amongst other things, telomere dysfunction and shortened telomeres. The cells acquire DNA damage and chromosomal instability which leads to premature senescence of cells. Patients suffering from this disease will develop physiological changes usually experienced with old age such as alopecia, sclerodermatous skin changes, and arteriosclerosis, the latter may usually also contribute to the cause of death (92).

### 1.6.2 Telomeres as therapeutic targets

Telomerase activity is mainly limited to lymphocytes in healthy humans and is upregulated in many malignancies, which makes it an interesting therapeutic target. The general idea of telomerase inhibition is, that it will revert cells to normal telomere shortening with each cell division and through erosion of the telomeres lead to senescence or apoptosis again (93). Imetelstat® was one of the first telomerase inhibitors used in clinical trials. It is an oligonucleotide that binds to TR within the telomerase complex and thus inhibits attachment to the telomere (93,94). Imetelstat has been tested in various diseases and is currently completing Phase 3 trials for myelodysplastic syndrome (95) and myelofibrosis (96). A Phase 2 trial on brain tumors was terminated due to severe adverse effects such as brain hemorrhage (97), but other Phase 2 trials on solid cancers have been performed and completed, such as for non-small cell lung cancers (98) and breast cancer (95).

## 1.7 TERT aberrancies in thyroid cancer – potential markers to help in clinical dilemmas?

Regarding thyroid cancer, multiple studies have shown the importance of *TERT* promoter point mutations. *TERT* promoter mutations were found in 9-22% of PTC (53,54,99,100), 11-17% of FTC (54,100), and as high as 66% in aggressive variants of thyroid cancers and cancer cell lines (poorly differentiated and anaplastic thyroid cancer) (53,54,100,101). Both mutations (C228T and C250T) were found, with a predominance of the C228T variant.

Most importantly, *TERT* promoter mutations are associated with a worse prognosis. They occur significantly more often in advanced or spread disease and are significantly associated with disease-specific mortality (53,54,100). Furthermore, *TERT* promoter mutations are seen in FTC but virtually never in recurrence-free FTAs, which gives a *TERT* promoter screening a very high positive predictive value of close to 100% (51). This makes *TERT* promoter mutation not only a good candidate for clinical implementation as a screening tool for more aggressive forms of thyroid cancer but also as an aid in establishing a diagnosis (102).

*TERT* promoter mutations have been reported in subsets of FT-UMPs which later showed recurrence as metastatic FTCs, suggesting that screening for *TERT* promoter in FT-UMP could help in identifying a subset of tumors with a genetically established malignant potential (51).

One problem with the clinical application arises as isolated cases have shown a spatial heterogeneity (103) of *TERT* promoter mutations in thyroid cancer which makes sampling the correct section of the tumor for mutational analysis more difficult.

Regarding hypermethylation of Region A as well as CN alterations, a study on follicular thyroid tumors (FTA, FTC, and FT-UMPs), showed, that hypermethylation occurred significantly more often in FTCs and FT-UMPs than in FTAs and almost all cases expressing *TERT* exhibited either CN alterations, hypermethylation of Region A or *TERT* mutation (84).

In all, *TERT* aberrancies (either promoter mutations, copy number alterations, promoter methylation, and/or aberrant gene expression) are aggregated in 1) well-differentiated thyroid carcinomas with poor prognosis, and 2) found more frequently in recurrence-prone FT-UMPs than in benign FTAs. Therefore, there might be clinical implications to screening for subsets of these aberrancies in clinical material. As FT-UMP cases routinely are discharged as out-patients without further treatment, the detection of a *TERT* promoter mutation in postoperative analyses could in theory imply that the excised tumor is a misdiagnosed FTC and would warrant a different kind of follow-up or treatment. Moreover, the finding of a *TERT* gene aberrancy already on cytology would perhaps aid the clinician in choosing the treatment algorithm for the individual patient - for example, a more aggressive surgical procedure up-front.

## 1.8 Alternative methods to identify TERT alterations – can we find a protein that pinpoints TERT aberrancies?

Many, especially smaller, pathology laboratories don't have access to molecular analysis within their facilities yet. Several reasons for that can be speculated. Lack of clinical experience in evaluating the results may contribute, therefore the tests will not be requested. A second reason could be that smaller laboratories might not generate enough samples to run a significant number of tests, thus arguing against such an investment from a purely economic point of view.

The fact that the overexpression of *TERT* is so closely linked to a genetic aberrancy would make TERT an attractive protein target for detection with surrogate markers or protein expressions that can be identified with other techniques such as immunohistochemistry or immunofluorescence in-situ hybridization.

### 1.8.1 Immunohistochemistry for TERT

Traditional antibodies for immunohistochemical staining of TERT exist but have so far shown varying results. A study of borderline ovarian tumors found a good correlation between *TERT* promoter hypermethylation and nuclear TERT expression – although the study was small (104). A series of gastric cancer showed a good correlation between *TERT* mRNA expression and TERT immunohistochemistry, but the results showed no significant difference between precursor lesions and malignant tumors (105),

and a study on bladder-washing cytology was able to show a good correlation between positive staining for an anti-TERT antibody and increased risk for a high-grade urothelial carcinoma (106).

However, in well-differentiated thyroid cancers, these antibodies were not able to provide reliable results. In a cohort of follicular thyroid cancers studied within our group at Karolinska, no correlation between TERT immunoreactivity and *TERT* promoter mutational status or *TERT* mRNA expression was shown (107). Furthermore, attempts to stain for TERT on thyroid specimens showed a cytoplasmic expression instead of the expected nuclear staining pattern (107,108). Whether these surprising results on immunohistochemistry can be explained by chemical reactions during formalin fixation or paraffin embedding or whether this might be due to a yet unknown function of TERT remains to be examined.

## 1.8.2 In-situ hybridization for TERT mRNA

Considering the results above regarding TERT protein expression in thyroid tumors, another surrogate technique could be examined: In-situ hybridization, a technique that attaches fluorescent dyes to nucleic acid probes which are then hybridized to the complementary target region. The fluorescent markers can be identified under a microscope. The nucleic acid sequence in question is *TERT* mRNA as it has shown a good correlation to *TERT* promoter mutational status (84). A study on follicular thyroid tumors showed a good correlation between mRNA expression (in-situ hybridization) and *TERT* promoter mutational status (109) but surprisingly enough, the mRNA seems to accumulate in the nucleus instead of in the cytoplasm.

## 1.8.3 Immunohistochemistry for 5-hydroxymethylcytosine (5hmC)

As in-situ hybridization is also a technique not as widely available as immunohistochemistry, another immunohistochemical surrogate marker for *TERT* promoter mutations that warrants further exploration is 5-hydroxymethylcytosine (5hmC). This marker is a measurement for the hydroxymethylation of cytosine, which is a process dependent on the Ten-eleven translocation (TET) family of 5-methylcytosine hydroxylases. It has been shown to be diminished in different types of malignant tumors, such as gliomas and melanomas in association with *TERT* promoter mutations (110,111). Two studies have evaluated 5hmC immunohistochemistry with PTCs (112,113), but for follicular thyroid tumors, no studies are published yet.

To summarize, the current gold standard for the diagnostic work-up of thyroid nodules is still 1) fine-needle aspiration cytology (preoperative), followed by 2) histologic (postoperative) evaluation and 3) molecular analyses only for cases of particular interest.

# 2 Research aims

This thesis aims to improve the diagnostic accuracy for thyroid tumors in general and follicular thyroid tumors in particular.

This goal shall be achieved through the following research topics:

   I. Evaluate the role of *TERT* promoter mutational screening in a clinical series of FT-UMPs

  II. Improve the sensitivity for detection of *TERT* promoter mutations in thyroid neoplasia with a special focus on follicular tumors

 III. Preoperatively determine *TERT* promoter mutational status in cytological preparations from thyroid tumors

 IV. Evaluate alternative means of identifying *TERT* promoter mutations in a cohort of follicular thyroid tumors

# 3 Materials and methods

## 3.1 Tissues

All materials used in studies I, II, and IV were gathered through a search of the clinical pathology database for cases fitting the inclusion criteria. Material for study III was gathered prospectively in the Karolinska University Hospitals' cytology clinic at the judgment of the cytologist regarding feasibility and inclusion criteria.

### 3.1.1 Fresh frozen material from fine–needle aspiration cytology

Fresh frozen material for DNA sequencing was gathered through the process of needle washing. In this process, the needle that was used for fine-needle aspiration cytology will be rinsed with 1,2 ml phosphate buffered saline solution (PBS-Solution, Apotek Produktion & Laboratorier – APL, Kungens Kurva, Sweden), after all the smear slides for clinical diagnostic purposes are produced. From this solution, we create a control stain by pipetting 50-90µl into a cytospin cuvette and have it transferred onto a slide through centrifugation (3 min, 700 rpm). If the slide shows sufficient cells, the remaining cell solution will be sedimented (10 min, 2500 rpm), and the excess liquid removed. The remaining cell pellet is frozen (-20°C) and stored for further analysis.

### 3.1.2 Formalin–fixed paraffin–embedded material

All formalin-fixed paraffin-embedded material was prepared and stored following the clinical routine at our pathology department. The material required for our research was requested through a biobank application. Immunohistochemistry was performed in part at the university's core facility and in part at our clinical pathology laboratory. The molecular analyses were performed in our clinical molecular pathology department.

## 3.2 Immunohistochemistry

Immunohistochemistry is by now one of the most readily available ancillary testing methods for morphologic examination used in clinical routine (114,115). It is used to stain proteins or antigens expressed in a cell. These proteins can be contained within the nucleus, such as the proliferation index marker Ki67, in the cytoplasm – for example, thyroglobulin expression – or in the cell membrane – such as E-cadherin.

In 1941, Dr. Coons first developed fluorescent antibody labels which were then further developed into the modern-day immunohistochemistry (116,117). Nowadays, immunohistochemistry consists of three steps: A primary antibody that binds to a specific target antigen, a secondary antibody that binds specifically to the first antibody, and usually has a horseradish peroxidase (HRP) polymer at the end.

And thirdly a dye, usually 3.3'Diaminobenzidine (DAB) for brown color or substrates metabolized by alkaline phosphatase (AP) for red color (Figure 7).
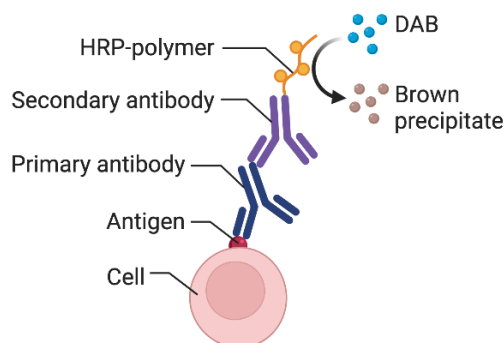


*Figure 7 – Schematic antibody binding in immunohistochemistry. Created using BioRender.com*

Antibodies used for immunohistochemistry in clinical practice can be derived from a single B-cell parental clone (monoclonal antibody) which only recognizes one epitope of the antigen and is highly specific. Alternatively, they can be derived from several B-cells resulting in a polyclonal antibody that recognizes several epitopes of the antigen and is thus more sensitive but usually less specific in its staining pattern (118).

## 3.3  DNA and Gene Expression Studies

### 3.3.1  Sanger sequencing

Sanger sequencing is a relatively old technique developed by its namesake Frederick Sanger in 1977 (119). The DNA gets denaturized into single-strand DNA (ssDNA) and di-deoxynucleotide triphosphates (ddNTPs) are added which get incorporated into the DNA and stop DNA strand elongation. These ddNTPs can be labeled with dyes – usually fluorescent dyes which can later be used for detection. The material is split into four parts, each containing one of the di-deoxynucleotides (ddATP, ddGTP, ddCTP, and ddTTP) and normal deoxynucleotides that will lead to a variation in strand length of the DNA upon polymerase-elongation from primers. The different length fragments are sorted by gel electrophoresis and the fluorescent colors allow for a continuous read-out from the shortest to the longest fragments rebuilding a readable copy of the original DNA sequence (Figure 8).
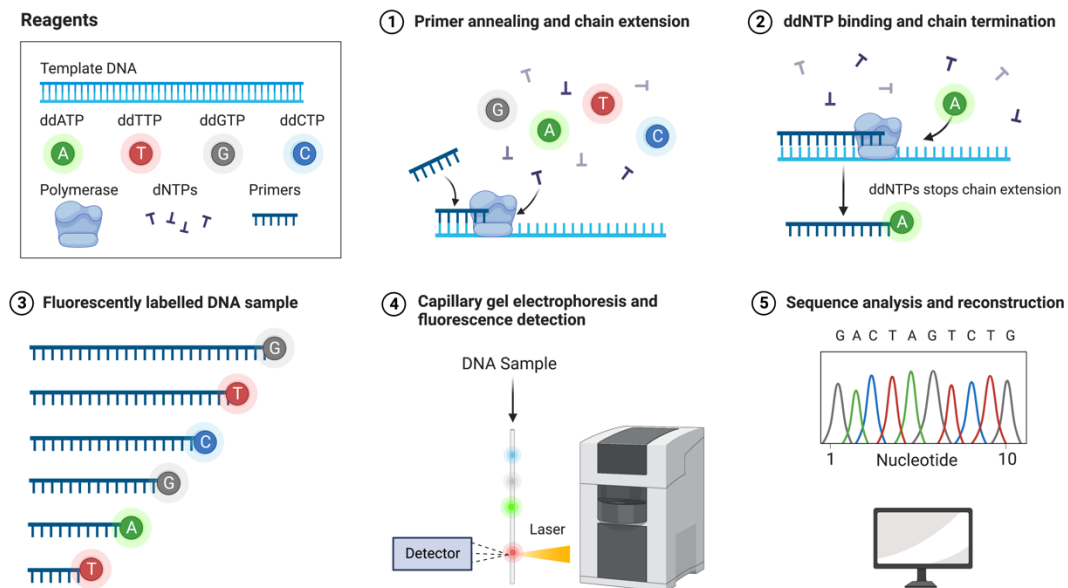
***Figure 8 – Schematic illustration of Sanger sequencing.*** *Created using BioRender.com*

Sanger sequencing was used to establish *TERT* promoter mutational status in study I. The analysis was performed at the clinical Department for Molecular Pathology at the Karolinska University Laboratories.

### 3.3.2 Polymerase chain Reaction (PCR) and Digital Droplet PCR (ddPCR)

PCR, which was first described by Kary Mullis (120), can be considered the backbone of modern molecular analysis (121). In PCR, target DNA gets amplified through repeated cycles of denaturation (to create ssDNA), annealing (adding primer to the ssDNA), and elongation (synthesis of new DNA). The steps are temperature regulated and usually, 35 cycles are applied (121) and the result is read out through gel electrophoresis.

In digital droplet PCR (ddPCR), which was first described as digital PCR in 1999 (122), the sensitivity gets improved by dilution of DNA and separating the material into multiple wells in which the PCR reaction will be supported by nanoliter-sized water in oil droplets. After adding fluorescents referring to either a wild-type template or a mutated template, the results are read out and plotted on a graph (Figure 9). A threshold for positive droplets is defined and the fraction of positive read-outs vs negative read-outs gets mathematically compared to the DNA concentration which allows for a calculation of allele frequency (122–126).
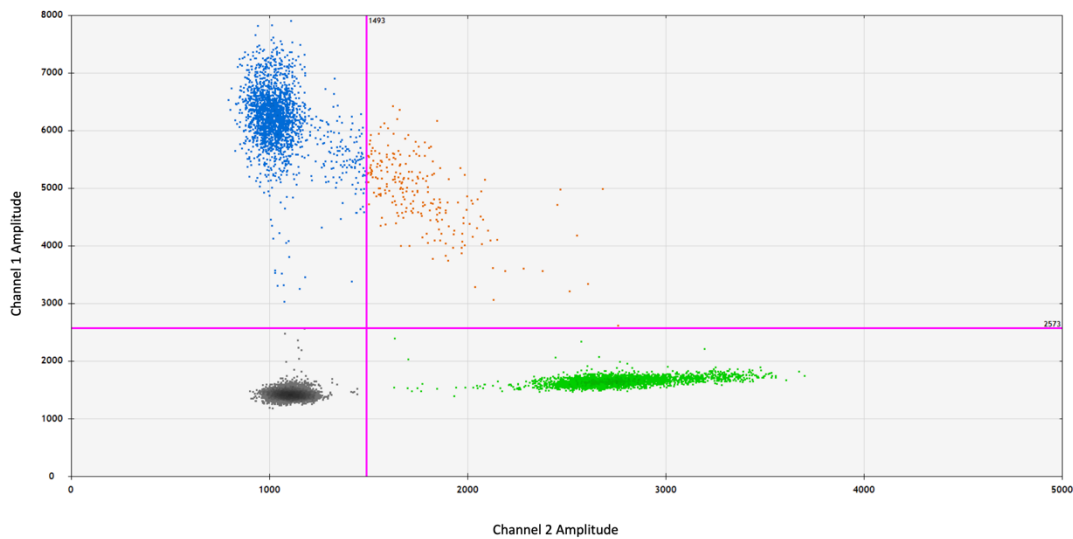
*Figure 9 – Read-out ddPCR. The upper left quadrant shows mutation-positive droplets, the upper right quadrant shows droplets containing both wild-type and mutated alleles, the bottom right quadrant shows wild-type droplets and the bottom left quadrant has droplets without the template. (Reprinted and adapted with permission from Wiley).*

ddPCR was used to identify *TERT* promoter mutations in studies II-IV. The analyses were performed at the clinical Department for Molecular Pathology at the Karolinska University Laboratories.

## 3.4 Statistical analyses

The Pearson's chi-squared test and Fisher's exact test were used to examine differences (usually regarding *TERT* promoter mutations) between two groups regarding categorical variables (e.g., tumor type or presence of metastasis), and Mann-Whitney U test and Kruskal Wallis test were used to examine differences between groups regarding continuous variables (e.g., Ki67-index, or tumor size) (Studies I – IV).

Kaplan–Meier survival analysis and log-rank test were used to calculate differences in disease-free survival (study I).

A p-value of < 0.05 was considered significant in all analyses.

All calculations were performed in SPSS Statistics software, in studies I, II, and IV Version 25 (IBM, Armonk, North Castle, NY) and in study III IBM SPSS Statistics, version 27 (SPSS Inc, Chicago, IL). The Kaplan-Meier graphs in Study I were drawn using GraphPad Prism 8 software (GraphPad Software Inc, San Diego, CA).

## 3.5  Ethical considerations

We have an existing ethical permit from the Swedish Ethical Review Authority; diarienummer: 2015/959-31 (Studies I – IV) with an added adaptation 2020 with diarienummer Dnr 202-00281 regarding GDPR (General Data Protection Regulation) and an updated information sheet for fine needle aspiration cytology (Study III).

**_TERT_ promoter mutational status had no clinical implication on patients with FT-UMPs or patients investigated through FNAC when data collection started**

For the first two sub-studies, the biggest issue was that we generated information about the malignant potential of tumors that was not yet fully understood and clinically implemented - but communicated to the responsible surgeon. Therefore, our studies created somewhat of an inconsistency in patient follow-up, as the decision on follow-up procedure in case of a _TERT_ promoter mutation was made at the patient's discretion. This problem was resolved thanks to _TERT_ promoter mutational status for FT-UMP becoming part of the Swedish thyroid cancer guidelines (1).

When testing for a _TERT_ promoter mutation preoperatively, we would have encountered a similar problem. This was avoided by testing the material only after surgical resection of the nodule. Thus, the standard treatment was offered to all patients and if a _TERT_ promoter mutation was encountered as part of the study, the findings were confirmed on FFPE material, and the patient was discussed anew at a multidisciplinary tumor conference.

**Acquiring consent of patients to perform studies on cytologic material**

Patients included in Study III have been classified as having Bethesda III (Atypia of unclear significance) and IV (follicular tumor) lesions on preoperative cytology – but as the definite diagnosis is only available after investigating all cases postoperatively, we must extensively save samples from all patients who present with a lesion that might be diagnosed as a follicular tumor. That means that basically, all patients with thyroid lesions that get referred to our center for cytology need to be informed about the study but ultimately many of them will not participate – as most diagnoses on cytologic preparations from the thyroid ended up being benign nodules which either will not be included in the study or not surgically removed at all.

For this reason, we updated the ethical permit and added a patient consent form for the cytology procedures, and ultimately informed the patient about study participation only after the decision for surgical resection was made, and retrieved the stored cytological material only after the surgical specimen was analyzed.

Still, many patients will have given their consent to a study that they didn't participate in the end, but this was unavoidable as we could not discriminate these cases until after surgery.

# 4  Results and Discussion

## 4.1  Study I – Clinical Routine TERT Promoter Mutational Screening of Follicular Thyroid Tumors of Uncertain Malignant Potential (FT–UMPs): A Useful Predictor of Metastatic Disease

The goal of this study was to prove that *TERT* promoter mutational screening in FT-UMPs can identify a group of tumors with distinct and elevated malignant potential. FT-UMPs are generally considered benign entities and are – regarding surgery and follow-up – treated the same way as adenomas, although a small percentage of these are known to develop recurrence and distant metastases. At the Karolinska Pathology Department, we started screening all FT-UMPs in 2014 for *TERT* promoter mutations and the cases have subsequently been discussed in a multidisciplinary therapy conference. This study summarizes all 51 cases of FT-UMP in regards to their *TERT* promoter mutational status clinical outcome and compares them to a cohort of 40 miFTCs from a previous study (84). Of these, 8 cases in the FT-UMP group harbored a *TERT* promoter mutation and 3 developed distant metastases, whereas none of the cases without *TERT* promoter mutation showed a relapse. The distribution of these 8 cases regarding their further treatment after established mutational status can be seen in Figure 10.
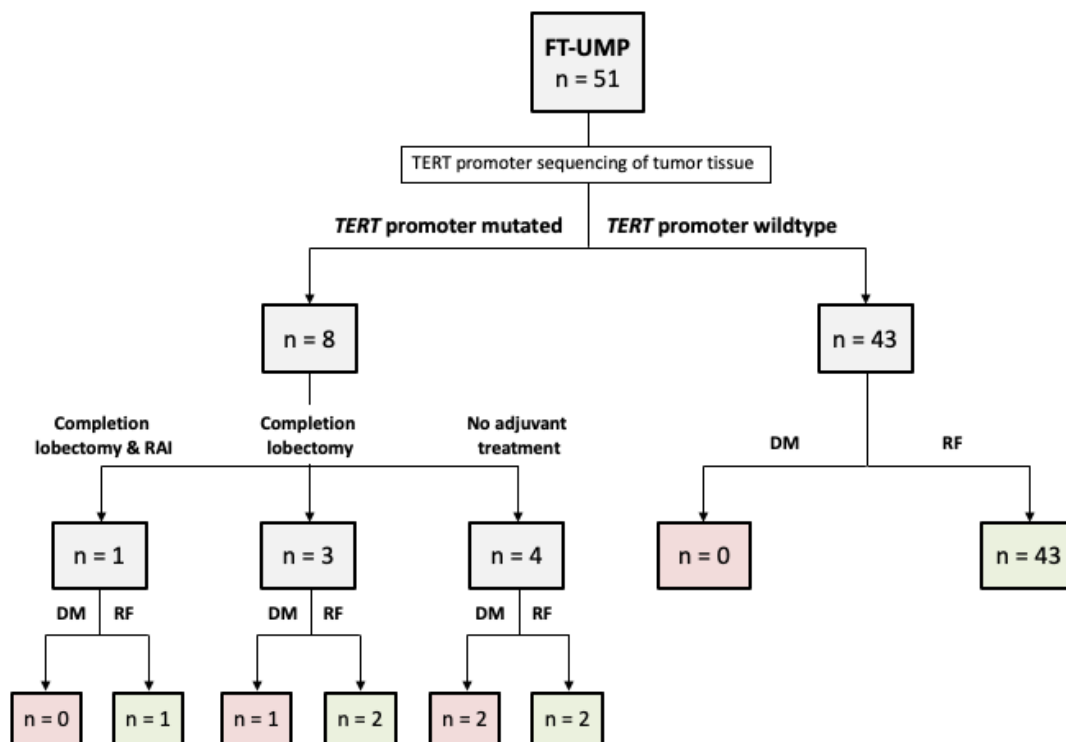


*Figure 10 – Schematic distribution of treatment and recurrence after establishing TERT promoter mutational status. Of the 51 clinically screened tumors, 8 were found to carry a TERT promoter mutation, whereas the remaining 43 cases were wild-type – of which two cases were lost to follow-up. While no recurrences have yet been recorded among the non-mutated FT-UMP cases, three recurrences (occurrence of distant metastases) have been noted among the mutated FT-UMP patients, all recorded in patients who did not receive adjuvant radioiodine therapy. DM: distant metastases, RF: recurrence-free. (Reprinted with permission from MDPI).*

In the control cohort of miFTC cases, 7 harbored a *TERT* promoter mutation. Of these, 2 cases with *TERT* promoter mutation and 2 without developed distant metastases. There was not just a significantly worse prognosis for FT-UMP cases with *TERT* promoter mutation compared to wild-type FT-UMP cases (p = 0.016) but also when compared to miFTC cases without *TERT* promoter mutations (p < 0.0001). Moreover, no significant difference was observed when comparing the groups of miFTCs and FT-UMPs with *TERT* promoter mutations (Figure 11).
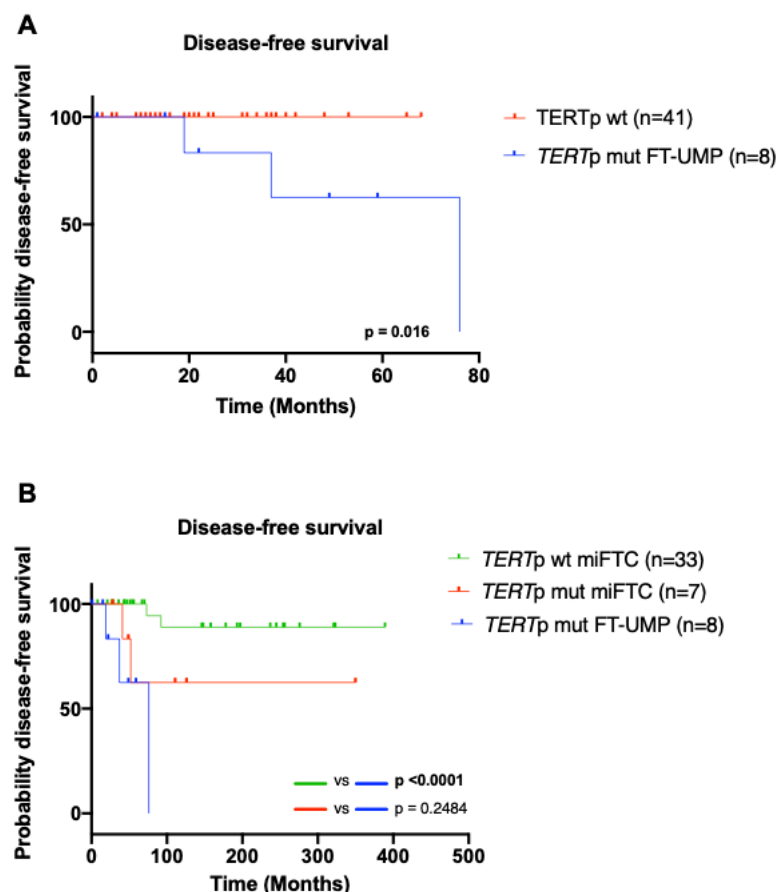


*Figure 11 - Kaplan Meier Plots illustrating disease-free survival with and without TERT promoter mutation. A. Time to detection of distant metastasis in TERT promoter mutated FT-UMP cases compared to wild-type FT-UMP cases. B. Time to metastasis/recurrence in TERT promoter mutated FT-UMP cases compared to mutated and wild-type (wt) miFTC cases. P values were calculated using the Log-rank test. Significant P values are in bold. (Reprinted with permission from MDPI).*

These findings required a thorough re-investigation of all cases to make sure no areas of malignancy (i.e., vascular invasion or infiltration into the surrounding tissue) were overlooked. No case revealed areas of unequivocal invasion and the sampling of the capsule was deemed sufficient in all cases. Furthermore, our cohort showed a significant association between *TERT* promoter mutation and the presence of areas suspicious for vascular invasion as well as greater tumor size and patient age. In this cohort, no association to Ki67 index was shown.

Although the follow-up time was limited in this study, it was interesting to observe such a high recurrence rate in cases with *TERT* promoter mutations highlighting the malignant potential even further. The fact that their recurrence-free survival was even worse than that of miFTC without *TERT* promoter mutations led to a national discussion regarding the treatment of FT-UMP resulting in an

addition to the National guidelines, which now acknowledge the potential role of testing for *TERT* promoter mutation in FT-UMP (127).

## 4.2 Study II – Spatial Distribution Patterns of Clinically Relevant TERT Promoter Mutations in Follicular Thyroid Tumors of Uncertain Malignant Potential: Advantages of the Digital Droplet PCR Technique

After establishing the clinical significance of *TERT* promoter mutations in FT-UMP in the first project, this study aimed to investigate if *TERT* promoter mutations could appear as subclonal and spatially heterogenous events in FT-UMPs, as previously highlighted in a case report of an FTC (103).

In a total of 16 FT-UMPs and 10 miFTCs, different areas of the tumor were evaluated by selecting 3 different FFPE blocks from each case for *TERT* promoter mutational analysis with both techniques – conventional Sanger sequencing, and ddPCR. On Sanger sequencing, four of seven cases with mutation showed heterogeneity. On ddPCR three cases retained this heterogeneity (Figure 12, Cases 2, 4, and 6), the fourth one (Figure 12, Case 5) showed a subclonal *TERT* promoter mutation (i.e., a very low allele frequency of the mutation – 7.2%) – less than the threshold for Sanger sequencing (10%). Additionally, a fifth case showed a congruently appearing subclonal C250T mutation. This case was on Sanger sequencing only positive for C228T (Figure 12, Case 1).

To separate whether this heterogeneity was specific for *TERT* promoter mutations, we investigated the prevalence of *RAS* mutations in the cohort and found that even cases with heterogeneous *TERT* promoter mutations displayed identical *NRAS* mutational status in all interrogated blocks of a case irrespective of *TERT* promoter mutated and wild-type areas. When compared to the Ki67 labeling index, areas with a higher proliferation index (in our cohort >3,8%) had a significantly greater likelihood of harboring a *TERT* promoter mutation.

Our data showed that *TERT* promoter mutations occur heterogeneously and can be subclonal in FT-UMPs. Furthermore, we were able to show not only the presence or absence of *TERT* promoter mutations in different areas but also variations and even co-occurrence of C250T and C228T. This strengthens the argument to use more sensitive methods for interrogation such as ddPCR which can detect subclonal events with an allele frequency of less than 10%, as the reference DNA used in our institution records allele frequencies of 0.2% as background noise. The fact that mutations occur heterogeneously leads inevitably to the question of how to choose the right area of a tumor to interrogate as it might affect our interpretation of these lesions. Therefore, we need to generate algorithms that help choose the right sample for *TERT* testing. In this study, no correlation to growth patterns or areas with equivocal relation to the tumors capsule or blood vessels was found, which might also be explained by the small number of cases in this series as other studies were able to draw such conclusions in similar cohorts (128,129). All cases with *TERT* promoter mutation showed an increased proliferation rate (here always more than 3,8% in the Ki67 labeling index). Similar results were found in other studies as well (128,130) but the overall available data is still not convincing enough to draw general conclusions. In fact, the true biological consequences of finding subclonal *TERT* promoter mutations as well as "double" C228T and C250T mutations within the same lesion are not known. The first step, however, could be to switch from conventional Sanger sequencing to ddPCR when interrogating *TERT* promoter mutations, which our institution incorporated.
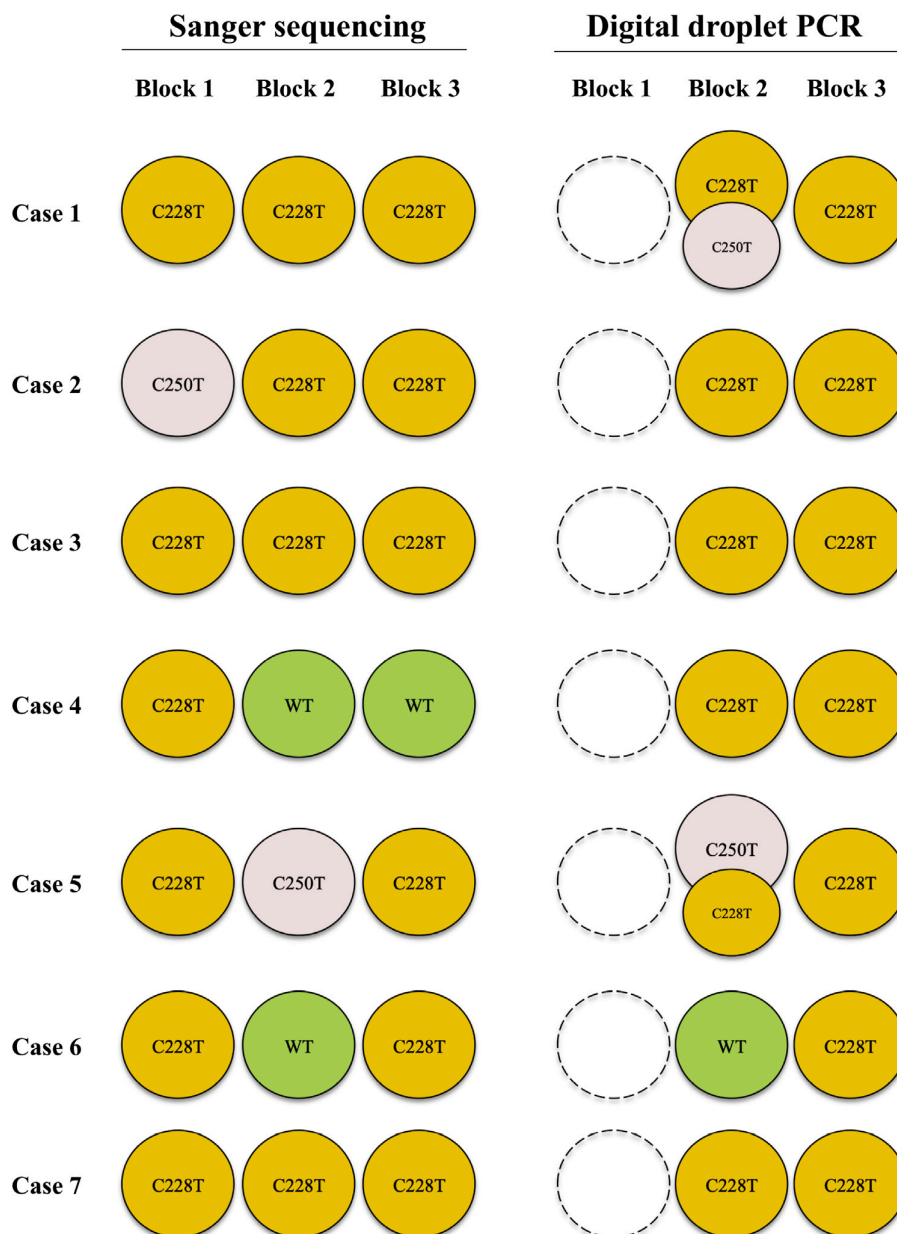
***Figure 12 – Schematic overview of TERT promoter mutation spatial heterogeneity in FT-UMPs.*** *Left-most columns denote the results of the conventional Sanger sequencing of all three formalin-fixated paraffin-embedded blocks (denoted blocks 1-3), while the right-most columns represent blocks 2 and 3 interrogated through digital droplet PCR (ddPCR). Orange circles represent the C228T mutation, circles with bright purple coloring denote the C250T mutation and green circles symbolize a wild-type (WT) sequence. For ddPCR results, allele frequencies were determined – and heterogeneity is represented by the smaller size circle in cases 1 and 5 with low-frequency C250T and C228T mutations respectively. Block 1 (from clinical routine) was not available for ddPCR testing and is therefore represented by a dashed line in the figure. (Reprint with permission from Elsevier).*

## 4.3 Study III – Digital droplet PCR TERT promoter mutational screening in fine needle aspiration cytology of thyroid lesions: A highly specific technique for pre-operative identification of high-risk cases

With the change to *TERT* promoter mutational testing through ddPCR on FFPE material, this study aimed to show that diagnostic and prognostic information can be increased by adding molecular analysis to fine needle aspiration cytology (FNAC) as well. We collected material from thyroid FNAC prospectively and performed *TERT* promoter mutational analysis (ddPCR) on both frozen pellets from FNAC material and on subsequently excised FFPE tumor tissue for comparison. We also investigated associations between mutational status and histopathology or clinical parameters.

In total, we evaluated 65 cases for *TERT* promoter mutations, consisting of 15 Bethesda III (23%), 26 Bethesda IV (40%), 1 Bethesda V (2%), and 23 Bethesda VI (35%) lesions according to the Bethesda System for Reporting Thyroid Cytopathology, 2nd edition. *TERT* promoter mutations were detected in 7 cases; 4 papillary thyroid carcinomas (all with preoperative Bethesda VI status), two follicular thyroid carcinomas (one Bethesda IV and one Bethesda V status), and one poorly differentiated thyroid carcinoma (with Bethesda VI status) (Figure 13). All mutated cases were verified by subsequent sequencing of tumor tissue derived from postoperative FFPE tissue, while all cases identified as wild-type on FNAC remained wild-type upon testing postoperative FFPE material.
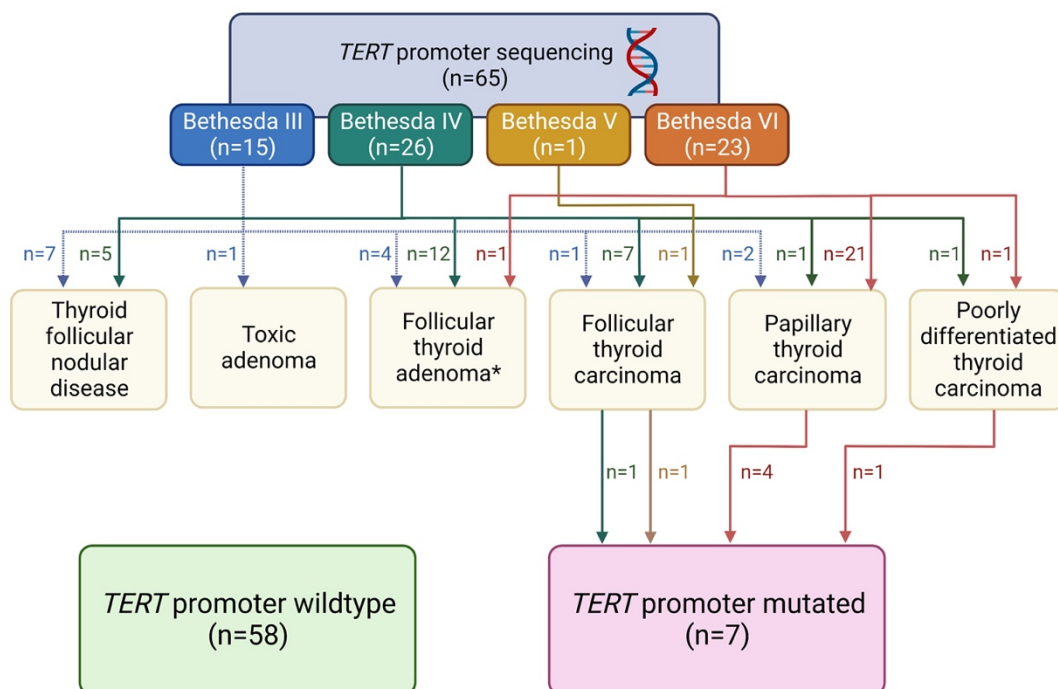


*Figure 13 – Overview of the results from the fine needle aspiration cytology (FNAC) analysis coupled with histologic diagnosis and TERT promoter mutational analysis. The Bethesda categories determined on FNAC for the 65 cases in the study are indicated at the top, and the corresponding diagnosis from the histological classification of surgical material is indicated below. The TERT promoter mutation status is summarized at the bottom. Arrows indicate the relationships between sample groups for the different analyses. *The follicular thyroid adenoma group also*

Furthermore, the occurrence of a *TERT* promoter mutation was significantly associated with malignant disease and – as in the previous study – higher Ki-67 proliferation indices.

The low frequency of detected mutations in this cohort is in line with previous results (54,100) and entirely in line with the general low occurrence of *TERT* promoter mutations in well-differentiated thyroid cancers. This leads to a low sensitivity for this analysis. However, the high specificity of the method makes it a great rule-in criterion as a diagnostic aid for equivocal Bethesda categories (Bethesda III – V). Our cohort's high sensitivity and specificity for identifying PTCs has two consequences: Firstly, the interrogation of this group with *TERT* promoter mutational analysis adds primarily a prognostic, rather than a diagnostic value as all the *TERT* promoter mutated cases in our cohort were identified as cancers with high-risk morphology and secondly, we opted not to add *BRAF* analysis to the study as a second marker. In other cohorts, ddPCR for *BRAF* mutations has been shown to improve diagnostic accuracy (131) as well, and analyses of those two mutations together could possibly improve the quality of cytology classifications in general and especially in equivocal categories such as Bethesda III – V.

Choosing material from fresh frozen pellets from FNAC for molecular testing has two advantages: One, DNA and RNA are presumably better preserved in fresh frozen material than in FFPE, and two, not having to formalin fixate and embed material saves time in the preanalytical process and can lead to a faster time to diagnosis. Our results showed some discrepancies in allele frequency between FNAC and FFPE material. These differences are difficult to assess but – if one does not assume chance as an explanation – might be due to spatial heterogeneity of the mutation and a different sampling mechanism, where the shearing movement of the FNAC needle can be seen as a more dynamic method compared to choosing a tumor section of FFPE material.

In conclusion, *TERT* genotyping using ddPCR from frozen pellets from thyroid FNAC material is a reliable technique for single gene analysis and might be a good alternative for laboratories that do not have access to multi-marker panels for genetic analysis of thyroid tumors.

## 4.4 Study IV – 5hmC immunohistochemistry: a predictor of TERT promoter mutational status in follicular thyroid carcinoma

In this manuscript, we evaluated a well-characterized cohort of thyroid tumors for 5-hydroxymethylcytosine (5hmC) immunohistochemistry, a marker of epigenetic demethylation events. In a previous study, loss of 5hmC was intimately associated with the presence of a *TERT* promoter mutation in PTCs (112,113). Therefore, we sought to investigate if 5hmC immunoreactivity was associated with *TERT* promoter mutations and/or *TERT* mRNA expression in a cohort of 29 follicular thyroid tumors.

We used two different anti-5hmC monoclonal antibodies (RM236 and 4D9), one stained using an automated system and one stained manually, and found that out of 10 cases with *TERT* promoter mutation and established *TERT* expression, only 1 stained negatively or very scarcely for 5hmC with both antibodies while the others showed either focally or diffusely positive staining patterns. Of the 19 *TERT* negative cases, none showed a lack of 5hmC expression using the automated system with the RM236 clone and two cases showed negative staining using the 4D9 clone (different staining patterns shown in Figure 14). The sensitivity and specificity for 5hmC IHC to detect *TERT* promoter mutated cases were 10% and 100% for RM236 and 20% and 89% for 4D9 respectively. The differences between the *TERT* promoter mutated and wild-type groups were non-significant (Fisher's Exact P=0.35 and 0.59 respectively The RM236 antibody was also used to stain three PTCs with a *TERT* promoter mutation from a different cohort (Study III) and these showed a complete lack of immunoreactivity, corroborating the results of the study we based our hypothesis on (113).
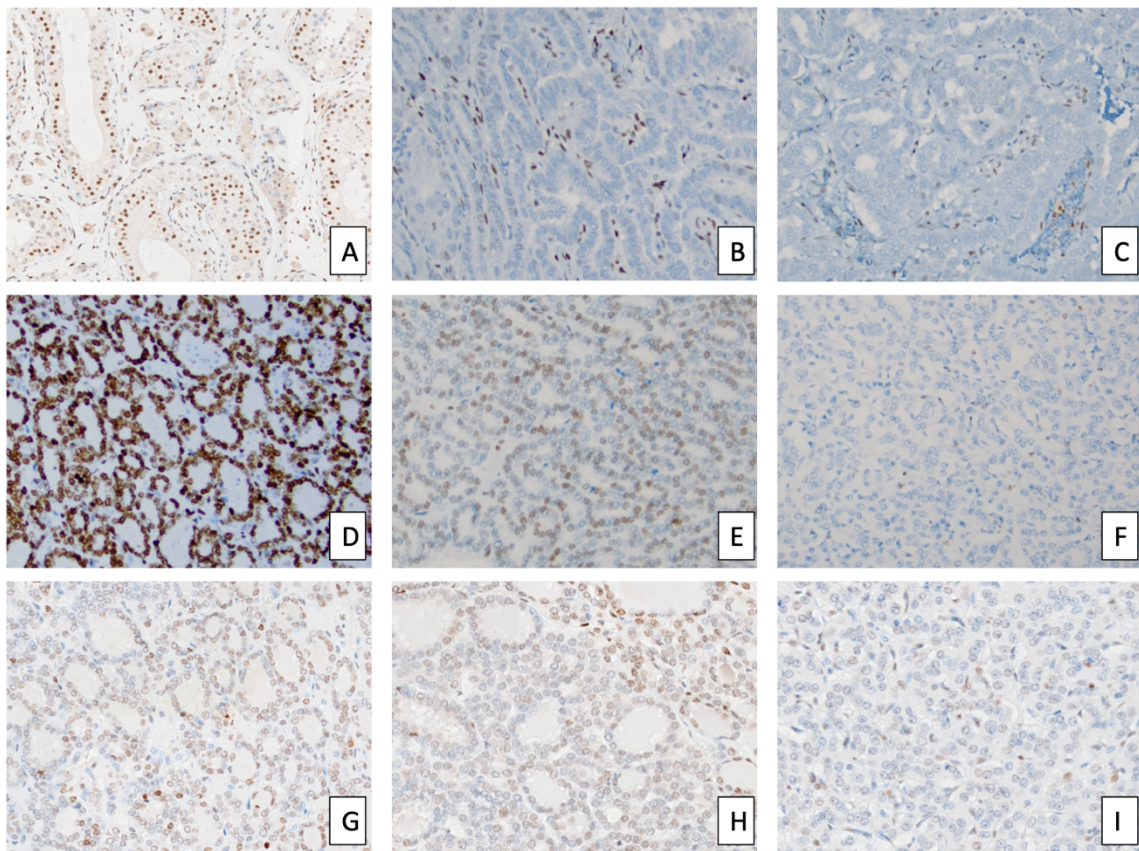


*Figure 14 – Different 5hmC staining patterns. (A-C) Control tissues: A – normal testicular tissue (200x magnification), manually stained with clone 4D6; B – Negative stain in a BRAF + TERT promoter mutated oncocytic papillary thyroid carcinoma (PTC), Ventana clone RM236 (200x); C – Negative stain in a BRAF + TERT promoter mutated tall cell PTC, Ventana clone RM236 (200x); (D-F) Clone RM236: D – Score 4 (Sample #28, a TERT promoter wild-type follicular thyroid carcinoma (FTC) without TERT mRNA expression, 200x); E – Score 3 (Sample #4, a TERT promoter mutated FTC with established TERT mRNA expression, 200x); F – Score 0 (Sample #8, a TERT promoter mutated follicular thyroid tumor of uncertain malignant potential (FT-UMP) with established TERT mRNA expression, 200x). (G-I) Clone 4D6: G – Score 4 (Sample #2, 200x) H – Score 3 (Sample #4, 200x); I – Score 1 (Sample #8, 200x). (Previously submitted with Study IV as Figure 1).*

Thus, we were unable to reproduce the loss of 5hmC expression in our cohort of *TERT* promoter mutated FTCs. Several reasons for these discrepant findings can be postulated. The most obvious explanation would be antibody malfunction which we bypassed by testing two different antibodies on different staining platforms with reliable internal control and correlation between antibodies. A second reason might be the usage of monoclonal antibodies instead of polyclonal antibodies. This seems to be an unlikely explanation as well, as the staining of PTC control cases has worked as expected with a monoclonal antibody. Less technical and more biological explanations include a possible heterogeneity for *TERT* promoter mutation as described in our previous projects (Study II). This would also corroborate the finding of heterogeneous staining patterns for 5hmC in circa half of the cases in our cohort and is in keeping with heterogeneity for *TERT* mRNA expression shown in another cohort (109). And finally, in our mind, the most likely explanation is that we still only have an incomplete understanding of the biology of *TERT* promoter mutations in thyroid tumors. The pathway linking TET and 5hmC to TERT in *BRAF* mutated PTCs is well described, however, for the heterogeneous group of non-*BRAF* associated tumors such as FTCs, this pathway is not yet fully established.

To conclude, 5hmC IHC only identified very small subsets of *TERT* promoter mutated follicular thyroid tumors in our cohort and was not deemed a highly sensitive marker for this purpose. The findings, therefore, indicate that *TERT* promoter mutational screening is still the gold standard to pinpoint clinically troublesome follicular thyroid tumors.

# 5 Concluding remarks and points of perspective

This thesis was planned in 2017 and since then a lot has changed as science is developing at an ever-increasing rate. Our studies were able to contribute to this development and managed to influence national guidelines.

However, the original premise of not every pathology lab having access to molecular techniques has changed, or better: is changing. We believe that through advances in technique, molecular analyses will more and more move from an auxiliary method towards becoming a crucial part of routine diagnostics on every level of pathologic diagnostics – from university laboratories to smaller private laboratories.

**The number and types of cases selected for molecular testing are growing.**

With the update of the Swedish national guidelines regarding *TERT* testing of FT-UMPs postoperatively, we saw the first group of thyroid tumors chosen for routine molecular testing. Now, a parallel can be seen for PTCs where small (sub-centimeter) carcinomas, are treated less aggressively. This creates a need to further scrutinize and subclassify them to identify the more aggressive histologic subtypes (i.e., TC-PTC, CC-PTC, and HN-PTC) (9) as these have even with a size of less than one centimeter a higher risk of recurrence. Especially in Study III, we saw an overrepresentation of these aggressive subtypes in the group of *TERT* promoter mutated PTCs, so it stands to reason that molecular testing will be recommended for those in the future as well. Regarding the selection of cases for molecular analysis in FNAC, it is expected that the upcoming 3rd edition of the Bethesda classification System for thyroid cytology will recommend molecular analyses like the ones we evaluated for equivocal lesions (Bethesda III – V), but in the future, they will most probably become part of the routine workup of all thyroid tumors.

**The selection of tumor areas subjected to molecular testing will have to become more standardized.**

Concerning our findings on spatial heterogeneity of *TERT* promoter mutations in FT-UMPs, we will have to find a better way to identify areas of interest for molecular analysis. Some studies were able to see associations between morphologic growth patterns, mitotic activity, and the probability for *TERT* promoter mutations (128), and even in our study (Study I) we saw a significant association between *TERT* promoter mutation and FT-UMPs with areas suspicious for vascular infiltration. In immunohistochemistry for example, the Ki-67 proliferation labeling index has been proven valuable when assessing thyroid tumors from a prognostic standpoint (132,133) and it can also help in identifying areas of interest for molecular analysis as shown in Study II. But we have not yet been able to identify immunohistochemical markers other than Ki67 that could help in identifying areas of interest for *TERT* promoter mutational analysis. Our experiment on 5hmC could unfortunately not show the same result as published for PTCs (112) and previous studies on immunohistochemistry for TERT were not successful either (107). So maybe the future lies in the hand of artificial intelligence and image analysis to help us in choosing the right area of tumor for analysis (129).

**The molecular markers to test for, need to be chosen carefully.**

The available, comprehensive multi-gene panels are not yet deemed cost-effective in the clinical routine (134), but smaller panels of several gene analyses will, especially with the emergence of techniques like ddPCR, facilitate this progress in the short term. One could for example consider testing a combination

of *BRAF* V600E and *TERT* on thyroid cytology as a complement to routine diagnostics to increase the sensitivity for detecting malignant cases or cases with a higher risk of recurrence at least. At the same time fusion analysis such as for *RET, NTRK1/3* or *ALK* can become a great addition to the above-mentioned mutations. *RET* fusions are found in PTCs and MTCs and lead to increased proliferation through activation of the MAPK pathway but can also be found in adenomas and thyroiditis (as reviewed in (48)). *NTRK1/3* and *ALK* fusions are only found rarely in adult differentiated thyroid carcinoma and induce proliferation through downstream signaling via receptor tyrosine kinase activation (9,48).

But the usefulness of molecular markers like these is not only prognostic and diagnostic but can also become therapeutic as targeted therapy becomes more readily available. Recently, BRAF inhibitors were implemented into the Swedish national guidelines for *BRAF* mutated ATCs (1). Other molecular targets – such as the above-mentioned fusions – are being investigated for their feasibility in the treatment of thyroid carcinomas as well: RET-inhibitors in medullary thyroid carcinoma (135), ALK inhibitors (136), and NTRK inhibitors (137) in Phase 1 & 2 trials. However, for successful treatment with TERT inhibitors, the way is still long as mentioned in the introduction.

Furthermore, immunotherapy is also evaluated on thyroid tumors – mainly ATC with partially promising results (138,139) and as the overall mutational burden has been proposed as a therapy indicator for immune checkpoint inhibition, this might be a good parameter to evaluate with a thyroid panel on FNAC as well.

To conclude, there is no way around molecular analyses in modern thyroid diagnostics, and a panel or several panels of molecular tests on thyroid specimens (surgical and preoperative FNAC) will be needed and all of them will have to include *TERT* promoter mutational analysis.

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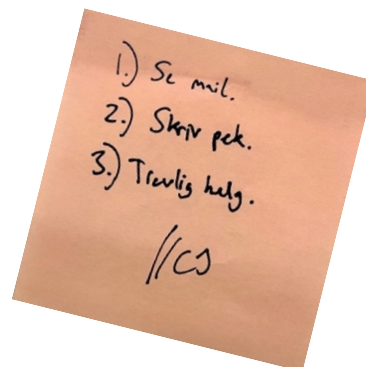
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Vivat, crescat, floreat,

Academia

Ad multos anos.

# 7  Legend of Figures and Tables

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