On incidence, diagnostic algorithms and in-depth characterization of thyroid cancer

Jakob Dahlberg

Department of Surgery

Institute of Clinical Sciences

Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

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jakob.dahlberg@vgregion.se

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To my family, my wife Pia and our children Lydia, Nils and Alice

"Everybody counts or nobody counts."

Harry Bosch

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Department of Surgery, Institute of Clinical Sciences
Sahlgrenska Academy, University of Gothenburg
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ABSTRACT

Background: Thyroid cancer (TC) incidence has increased dramatically. Overzealous detection of subclinical disease is the most common explanation. However, whether the higher rate of diagnosis of subclinical disease might conceal a true TC increase is unknown. EU-TIRADS has been developed to guide further management of nodules. Real-world data as well as controlled trials on effect and safety are largely missing. Although overall prognosis of TC is excellent, a subgroup of patients has limited treatment options and new ones need to be developed.

Aims: The objectives of Paper I were to investigate which tumor stages of TC were increasing and the modes of detection. Paper II and II aimed at assessing the impact and safety of EU-TIRADS on nodule management. Paper IV aimed to establish PDX model of TC development and drug testing.

Methods: Paper I was a population-based study. Paper II was a single center retrospective cohort study and Paper III was a regional randomized controlled trial. Paper IV was a prospective trial collecting tumor tissues from patients with advanced TC, subsequently transplanted to immunodeficient mice.

Results: TC increased threefold during 2001-2014 in Western Sweden. The increase comprised stages T1a to T3 and the most common mode of detection was clinical symptoms, mainly a palpable tumor. Imaging did not contribute to increased TC incidence. EU-TIRADS reduced cytology by 7% without missing TC diagnosis. Comparing selective

and non-selective FNA revealed that EU-TIRADS significantly reduced the frequency of unnecessary FNA. Among fresh tumor tissue samples obtained from advanced TC only squamous cell carcinoma (SCC) was successfully transplanted to immunodeficient mice. Targeted therapy based on mutation profile inhibited PDX growth.
Conclusion: Increased TC incidence in Western Sweden was due to other factors than imaging. A true increase in TC incidence cannot be ruled out. EU-TIRADS does not miss TC diagnosis but has limited impact on the clinical management of thyroid nodules. A thyroid origin of SCC was documented. Novel therapeutic options were suggested arguing for global mutation analysis of all patients with anaplastic TC.

Keywords: thyroid cancer, thyroid nodule, ultrasound, ultrasonography, SCC, PDX

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SAMMANFATTNING PÅ SVENSKA

Bakgrund: Sköldkörtelcancer har diagnosticerats i allt högre utsträckning i världen, inklusive Sverige. Flertalet studier rapporterar att ökningen består av tumörer utan klinisk betydelse till följd av ökad användning av ultraljud. Det är inte uppenbart att samma orsaker orsakar incidensökning i Sverige. EU-TIRADS används för att med ultraljud riskvärdera knölar i sköldkörteln. Prognosen för sköldkörtelcancer är oftast god. Enstaka tumörformer har dock ett mer aggressivt förlopp där det idag saknas effektiv behandling.

Syfte: Delarbete I syftade till att undersöka vilka tumörstadier som har ökat samt hur tumörerna har upptäckts i Västra Götaland. Delarbete II och III syftade till att undersöka hur EU-TIRADS påverkade handläggning av knölar i sköldkörteln. Delarbete IV syftade till att etablera en djurmodell för avancerad sköldkörtelcancer för ytterligare karakterisering och läkemedels-testning.

Metod: Delarbete I var en populationsbaserad kohortstudie. Delarbete II var en retrospektiv kohortstudie och Delarbete III en regional randomiserad studie. Delarbete IV var en experimentell prospektiv studie som inkluderade patienter opererade för avancerad sköldkörtelcancer. Tumörvävnad transplanterades till immunbristiga möss varpå mutationsanalyser och läkemedelstestning genomfördes.

Resultat och slutsatser: Sköldkörtelcancer ökade trefaldigt i Västra Götaland under perioden 2001–2014 och omfattade tumörstadier T1a-T3. Kliniska symtom, oftast en palpabel knöl, var den vanligaste orsaken till tumörupptäckt och incidensökningen berodde inte på ökad bilddiagnostik. Reell ökning av sköldkörtelcancer kunde inte uteslutas. EU-TIRADS minskade frekvensen av cytologisk undersökning med 7%. Vid en direkt jämförelse mellan icke selektiv och selektiv cytologi enligt EU-TIRADS minskade frekvensen av onödig cytologi signifikant utan tecken till att missa cancer. EU-TIRADS är säkert att använda men påverkar frekvensen av cytologi endast måttligt. Tre patienter med skivepitelcancer i sköldkörteln undersöktes varav tumörvävnad från en patient lyckosamt fördes över till och växte på immunbristiga möss. Mutation i bland annat NFEL2L-genen påvisades. Riktad behandling visade hämmad tumörväxt. Studien stödjer hypotesen att skivepitelcancer i sköldkörteln är en undergrupp till anaplastisk sköldkörtelcancer. Molekylärgenetisk testning av anaplastisk tyreoideacancer kan identifiera nya behandlingsmöjligheter.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Dahlberg J, Adok C, Bümming P, Demir A, Hedbäck G, Nilsson B, Nilsson M, Jansson S. **Incidence, detection and outcome of differentiated thyroid cancer in Western Sweden**. *BJS Open* 2021 Oct; 5(5): zrab099.

II. Dahlberg J, Carlqvist J, Larsson E, Elias E, Muth A. **Effects of implementation of EU-TIRADS risk stratification in a thyroid cancer programme in Western Sweden – a retrospective cohort study**. *Submitted.*

III. Dahlberg J, Carlqvist J, Örtoft A, Hammarstedt L, Aula E, Hellström M, Elias E, Muth A. **A randomized controlled trial comparing non-selective vs selective cytology using EU-TIRADS – the Ultracyt study**. *Manuscript.*

IV. Schoultz E, Dahlberg J, Nilsson L, Karlsson J Carlsson T, Mohammad G, Fagman H, Muth A, Elias E, Sayin V, Nilsson J, Nilsson M. **Tumor cell origin and new target therapy options of squamous cell carcinoma in the thyroid**. *Manuscript.*

CONTENTS

ABBREVIATIONS

ASR	Age-standardized incidence rates per 100 000 person-years
ATA	American Thyroid Association
ATC	Anaplastic thyroid carcinoma
CRF	Case report form
c.i.	Confidence interval
DHGTC	Differentiated high-grade thyroid carcinoma
EAPC	Estimated annual percentage change
EMT	Epithelial-mesenchymal transition
ETA	European Thyroid Association
FA	Follicular adenoma
FNA	Fine needle aspiration cytology
FTC	Follicular thyroid carcinoma
MRI	Magnetic resonance imaging
MTC	Medullary thyroid carcinoma
PDX	Patient-derived xenograft
PTC	Papillary thyroid carcinoma
PDTC	Poorly differentiated thyroid carcinoma
RSS	Risk stratification system

RAI	Radioactive iodine
ROM	Rate of malignancy
SCC	Squamous cell carcinoma
SU	Sahlgrenska University Hospital
TBSRTC	The Bethesda System for Reporting Thyroid Cytology
TC	Thyroid cancer
TERT	Telomerase reverse transcriptase
Tg	Thyroglobulin
TIRADS	Thyroid Imaging Reporting and Data System
TKI	Tyrosine kinase inhibitor
US	Ultrasound

DEFINITIONS IN SHORT

Driver mutations	Mutations that promote cancer development.
Overdiagnosis	Diagnosis of a condition, *e.g.* cancer, that will never cause symptoms or death.
Thyroid incidentaloma	Previously undetected thyroid nodule discovered on imaging performed due to indication other than thyroid disorder.

1 INTRODUCTION

Thyroid cancer (TC) is the most common endocrine malignancy (1) and comprises a heterogenous group of tumors with large differences in treatment and prognosis. The present thesis addresses several questions related to TC incidence, diagnostic challenges as well as therapeutic options in advanced disease. To set the framework, the introduction will discuss subtypes of TC, introduce diagnostic tools in clinical work-up and briefly cover the challenges of treating advanced TC.

Paper I was a population-based registry and clinical cohort study, investigating the incidence of all TC types in Western Sweden, ranging from subclinical disease to lethal types like anaplastic TC. Paper II and III addresses management of thyroid nodules of which the majority are benign although these study populations also harbor patients with aggressive disease. Finally, Paper IV was an experimental study of patients with advanced TC with poor prognosis in which treatment options are limited. For these reasons the following sections will explain the diagnoses included in this thesis.

Today, diagnosis of TC is primarily based on histopathologic examinations with hematoxylin-eosin (HE) staining and molecular testing is not mandatory in routine diagnostic workup (2).

1.1 TYPES OF THYROID CANCER

TC is commonly categorized into three main types (3): (1) differentiated TC (DTC) which includes papillary TC (PTC), follicular TC (FTC) and onkocytic TC. DTC originate from the follicular epithelium and constitute approximately 80% of all cases of TC in Sweden; (2) medullary TC is a neuroendocrine tumor derived from the parafollicular C-cells in the thyroid; (3) anaplastic TC (ATC) derives from DTC but is undifferentiated.

Except these three types of TC, poorly differentiated thyroid carcinoma (PDTC) and high grade thyroid carcinoma are recognized as distinct tumor entities and categorized under the term “high grade follicular cell derived thyroid cancer”. Both tumors are clinically aggressive and may be considered as intermediate forms between DTC and ATC (2).

1.1.1 BORDERLINE

Borderline tumors are not classified as cancer, but they are not unequivocally benign. Clinically and morphologically they may be regarded as intermediate tumor stages between benign and malignant lesions; however, they have the potential to metastasize indicating malignant potential but do so very rarely (2). The 2022 WHO classification of thyroid tumors list the following thyroid borderline entities (4):

- Hyalinizing trabecular tumor
- Follicular tumor of uncertain malignant potential (FT-UMP)
- Well differentiated thyroid tumor of uncertain malignant potential
- Non-invasive follicular tumor with papillary like nuclei (NIFT-P)

NIFT-P was previously regarded malignant and a subtype of PTC. Due to its excellent prognosis it was suggested in 2016 to reclassify NIFT-P as borderline (5) which was accepted in the 2017 WHO classification (6). NIFT-P has a very low risk of recurrence (≈0.5%) and the purpose of reclassification was to reduce overtreatment (5). As a consequence surgical treatment of this condition has been de-escalated and tumor removal by hemi-thyroidectomy is considered sufficient (7). Morphologically, NIFT-P is resembling a follicular adenoma except it has nuclear features typical of PTC comprising nuclear pseudoinclusions, grooves and enlargement (5).

FT-UMP tumors rarely recur or metastasize. An exception is FT-UMP with *Telomerase reverse transcriptase* (*TERT*) promoter mutation, which has been associated with a highly malignant phenotype (8). That this mutation indeed is pathogenic is indicated by the fact that FT-UMP without *TERT* promoter mutation showed no signs of metastases or recurrence.

1.1.2 PAPILLARY THYROID CARCINOMA

Papillary thyroid carcinoma is the most common type of TC, accounting for approximately 70-75% of all TC cases registered in Sweden (9, 10). PTC is derived from follicular epithelial cells and is diagnosed by characteristic nuclear morphology such as enlargement, crowding, grooves and the pathognomonic ground glass nuclei.

Subclassification of PTC has become more important and should be considered in all stages of PTC when stratifying for risk of recurrence or persistent disease (11, 12). Subtypes associated with a poorer prognosis are tall cell, columnar cell, Hobnail and diffuse sclerosing PTC (2). Key molecular profiles of these tumors are *BRAF*^{V600E} mutation and *TERT* promoter mutation. PTC regularly spread to regional lymph nodes whereas distant metastases do not occur until advanced stage.

1.1.3 FOLLICULAR THYROID CARCINOMA

Follicular thyroid carcinoma is the second most common type of TC, accounting for approximately 10% of patients (10). Like PTC, FTC also derives from follicular cells, but the tumor lacks nuclear morphologic changes typical of PTC and it also shows signs of invasiveness. Depending on the type and extent of invasion, FTC is divided into (1) minimally invasive FTC (miFTC) in which the tumor infiltrates and penetrates the capsule surrounding the tumor; (2) angio-invasive FTC invades blood vessels within or outside the capsule; and (3) widely invasive FTC (wiFTC) that shows extensive capsular infiltration and vascular invasion. Extrathyroidal extension and distant metastases, *e.g.* to the lung and skeleton are frequent.

1.1.4 ONCOCYTIC THYROID CARCINOMA

Oncocytic thyroid carcinoma (earlier referred to as “Hürthle cell” but the term is discouraged in the 2022 WHO classification) was previously a subtype of FTC but since the 2017 WHO classification it is recognized as a distinct TC type due to unique genomic alterations in the mitochondrial DNA (13) or in the related GRIM19 (NDUFA13) gene (14). However, in clinical practice diagnosis is not made by genetic testing but rather by histology. If the cells in the tumor show more than 75% oncocytic differentiation it may be classified as oncocytic cancer (2). In addition, the tumor must have the same type of signs of invasiveness, *i.e.* capsular invasion or vascular invasion or both, as FTC.

1.1.5 MEDULLARY THYROID CARCINOMA

Medullary thyroid carcinoma (MTC) is a neuroendocrine tumor that derives from parafollicular C-cells. C-cells produce the hormone calcitonin which may serve as a tumor marker both pre- and postoperatively. It is also possible

to diagnose MTC by immuno-histochemistry (IHC) analysis of calcitonin. Since it is a neuroendocrine tumor, it may also stain positive for chromogranin A and synaptofysin which may be useful in dedifferentiated tumors where the expression of calcitonin is lower.

Germline *RET*-mutations are found in virtually all hereditary MTCs and somatic *RET*-mutations are found in approximately 50% of sporadic MTCs (15-17).

1.1.6 HIGH-GRADE FOLLICULAR-DERIVED CARCINOMA

Two separate entities of high-grade follicular-derived carcinoma are listed in the 2022 WHO classification: differentiated high-grade thyroid carcinoma (DHGTC) and poorly differentiated thyroid carcinoma (PDTC). Both tumor types are rare, usually occurring in patients >50 years of age, develop as a rapidly growing mass in the neck and are often large at first presentation. Lymph node metastases are common, especially in DHGTC which more often are associated with *BRAF*-mutations (18). Prognostically both PDTC and DHGTC have long-term survival intermediate of DTC and ATC: 10-year disease-specific of 60% and 56% respectively (18).

1.1.6.1 DIFFERENTIATED HIGH-GRADE THYROID CARCINOMA

DHGTC is an invasive tumor that is well-differentiated due to its growth pattern and, in most cases, displays features that are characteristic of PTC or more seldom a follicular growth pattern (*i.e.* derived from FTC). The diagnosis is confirmed if necrosis is present and/or mitotic activity is elevated. DHGTC has a worse prognosis than DTC but better than undifferentiated TC (2).

1.1.6.2 POORLY DIFFERENTIATED THYROID CARCINOMA

PDTC is derived from DTC but morphologically and clinically it is positioned between DTC and ATC. PDTC is often difficult to diagnose and categorize and is even less common than ATC. The diagnostic criteria for this tumor are established by the Turin consensus criteria (19) and the tumor is characterized by invasiveness in combination with one of three patterns of growth: solid, trabecular or insular. Furthermore, the tumor has either necrosis, elevated mitotic activity or convoluted nuclei.

The MSKCC (Memorial Sloan-Kettering Cancer Center) criteria for diagnosing PDTC has also been proposed, the main difference is that diagnosis is based on mitosis and necrosis rather than growth pattern (20).

1.1.6.3 GUIDELINE ASPECTS OF PDTC

According to Swedish national guidelines, treatment recommendations for PDTC follow those of DTC but the diagnosis and treatment is sparsely described (7). Neither American thyroid association (ATA) nor European thyroid association (ETA) provide guidelines specifically for PDTC. A review from 2022 stated that it is difficult to draw conclusions about uniform recommendations although surgery should be performed if feasible, radioactive iodine (RAI) might have some but usually limited effect and external beam radiation may be considered (21). The forthcoming revised Swedish national guidelines will however be updated on PDTC and contain recommendations about molecular testing and targeted therapy.

1.1.7 ANAPLASTIC THYROID CARCINOMA

ATC is a rare and highly malignant disease that affects mainly elderly patients. In Sweden, approximately 15-20 cases of ATC are diagnosed yearly and the incidence has been stable (10). The true incidence is possibly slightly higher due to missed reporting because ATC might not be correctly diagnosed with fine needle aspiration cytology (FNA), and the patients do not always undergo thyroid surgery. ATC is one of the most aggressive types of human cancer and most patients (> 90%) die within 12 months from diagnosis (22). The tumor arises from DTC by tumor progression, and there is a complete or close to complete loss of differentiation which is the reason why ATC sometimes is also referred to as undifferentiated TC. Upon histopathologic examination, a varying degree of mitotic activity is seen as well as invasive growth, necrosis and nuclear pleomorphism (23). A coexisting DTC may also be seen, which often is of an aggressive subtype (24). Immunohistochemistry (IHC) is usually negative for thyroglobulin (Tg) and TTF-1/NKX2-1 (positive in DTC) but is positive for PAX8 in up to 70% (25) (PAX8 is a transcription factor and is important in cell-cycle process and survival of differentiated thyroid epithelial cells (26)). A number of histological subtypes of ATC are described, including rhabdoid, spindle cell, giant cell and squamous cell carcinoma (SCC) (23).

Thyroid SCC was previously classified as unique entity but interestingly recognized in the latest WHO classification as a subtype of ATC (4). Thyroid SCC, its origin and tumor progression was investigated in Paper IV.

ATC may be diagnosed by FNA in most cases (>60%) (27). However, if ATC is clinically suspected, core needle biopsy is recommended (28). ATCs that are derived from PTC are often *BRAF* mutated and initial work-up should include analysis of *BRAF* mutation by IHC and next-generation sequencing (28).

The identification of driver mutations leading to targeted therapy of ATC has led to a paradigm shift and a bit of hope for patients with this diagnosis (22). The main signaling pathways are JAK-STAT, MAPK and PI3K/AKT/MTOR and include targets such as RET, EGFR and KIT (29). Tumor progression from DTC to ATC may be described as a multistep process with an accumulation of genetic changes (30). Frequently, a DTC component of an aggressive subtype, for example tall cell PTC, is observed adjacent to the ATC tumor. The theory of progression from DTC to ATC is further supported by the observation that mutations like *BRAF* and *RAS* are common in both tumor types and *p53* mutation is common in ATC but not DTC (31).

1.2 THYROID TUMOR PROGRESSION

As already mentioned, there is ample evidence both histologically and genetically of transition of one TC type to another more malignant type. The mutational burden of DTC is usually very low (32) and the somatic mutations found in PTC are nearly always mutually exclusive (33), *e.g.* only one pathogenic driver mutation is present in DTC. In FTC the most common genetic alterations are point mutations in *RAS* and rearrangements in PAX8-PPARγ (34, 35). The most common mutations in PTC are $BRAF^{V600E}$, *RAS* and rearrangements in *RET/PTC* (33).

The progression of DTC to more aggressive types of TC like PDTC and ATC, is a stepwise process involving an increased number of pathogenic mutations. As an example, *TERT* promoter mutations increase in frequency from 9% in PTC, 40% in PDTC to 73% in ATC (33). Another observation is that when present, *TERT* promoter mutations are subclonal in PTC, but clonal in PDTC and ATC suggesting that the *TERT* promoter mutation is highly oncogenic in the tumor evolution (36). Likewise, the tumor-suppressor gene TP53 is never

mutated in DTC (37) but is increasingly mutated in PDTC (8%) and in ATC (73%) (36).

Epithelial-mesenchymal transition (EMT) is a process in which carcinoma cells transform to a mesenchymal phenotype, allowing tumor cells to migrate and metastasize. In the multi-step model of TC progression, tumor stem cells supposedly fueling tumor growth are suggested to be generated through both genetic mutations and EMT (38). EMT entails changes promoting invasiveness and metastasizing features by upregulation of the Snail family of transcriptions factors (39, 40). This leads to downregulation of CDH1/E-cadherin which in normal epithelial cells is important for cell-cell adhesion and maintained epithelial cohesiveness and integrity. Thus, loss of CDH1-based adhesion renders the cancer cells more prone to leave its primary site and metastasize (41). CDH2/N-cadherin, another classical cadherin that is normally expressed in neuronal and mesenchymal tissues in embryos, is often upregulated during EMT. EMT biomarkers were analyzed in the patient-derived xenograft (PDX) tumor investigated in Paper IV.

Dedifferentiation comprising diminished expression of thyroid-specific genes and impaired thyroid function may be considered as another facet of TC tumor progression. For example, ATC has lost all thyroid biomarkers that designate the normal thyroid phenotype. Another example is *BRAF* mutated PTCs. They are often dedifferentiated of which one effect is the loss of NIS expression, making these tumors RAI resistant (42). This is a clinical issue of concern and further discussed in section 1.5.3 Iodine refractory DTC.

1.3 EPIDEMIOLOGY OF THYROID CANCER

TC incidence has increased worldwide for decades (43, 44). According to most studies this observation can mainly be attributed to more frequent detection of early stages of papillary thyroid carcinoma (PTC) of which the majority are unlikely to be of clinical importance (45), by some authors referred to as overdiagnosis (46, 47). The existence of a reservoir of subclinical TC was demonstrated as early as 1985 by Harach *et al*. in a still today frequently cited article (48). Although this study might not adhere to contemporary scientific standards, the notion of PTC as an indolent condition that in many cases will never cause symptoms has been supported by more recent publications (49, 50).

Increased access to health care in general (51, 52) and more specifically the use of diagnostic procedures such as ultrasound (US) of the neck has been considered to be a major contributing factor to increased detection (53). An outstanding example comes from South Korea where the incidence of TC rose dramatically when US screening of the neck of women also undergoing breast cancer screening was introduced (54) and it was estimated that 90% of all women in South Korea diagnosed with TC between 2008 and 2012 were "overdiagnosed" (55). The increased incidence was attributed mainly to PTC in patients lacking symptoms of TC and without observed effect on TC specific mortality rates, and it was concluded that the regimen of TC screening needed careful evaluation (56). Furthermore, there has been an ongoing debate and an increasing awareness of overdiagnosis and perhaps as a result of this, international guidelines have been updated with improved risk stratification, resulting in a plateau effect of TC incidence worldwide (57).

However, although most authors attribute increased incidence of TC world-wide to increased detection of subclinical disease or over-diagnosis of TC, other reports have been published arguing that increased detection cannot fully explain increased incidence (58, 59). The conceptualization of Paper I came from the observation that TC incidence was rising also in Sweden, but the explanations given by most authors were not obviously applicable to Swedish or regional conditions. In Sweden the mainstay of health care is provided by public third-party payer care givers, with relatively uniform access to care across geographic regions and eco-sociologic classes. There has been no organized screening of TC in Sweden and clinicians have been reluctant to introduce neck US. Retrospectively, this has been correct for obvious reasons, although conservative traditions have probably been the main reason. Thus, the pronounced increase of TC observed in Sweden, see Figure 1 (60), and the Nordic countries cannot easily be explained by increased detection as described by authors mainly from the USA, South Korea and some central European countries.

Due to the accelerated TC diagnosis, it is conceivable that a true increase of less frequent but potentially dangerous cancers might be concealed by the great number of small and indolent thyroid tumors being detected. This was the fundamental incitement of conducting the population-based study of Paper I, correlating TC incidence to tumor type, stage and mode of detection.

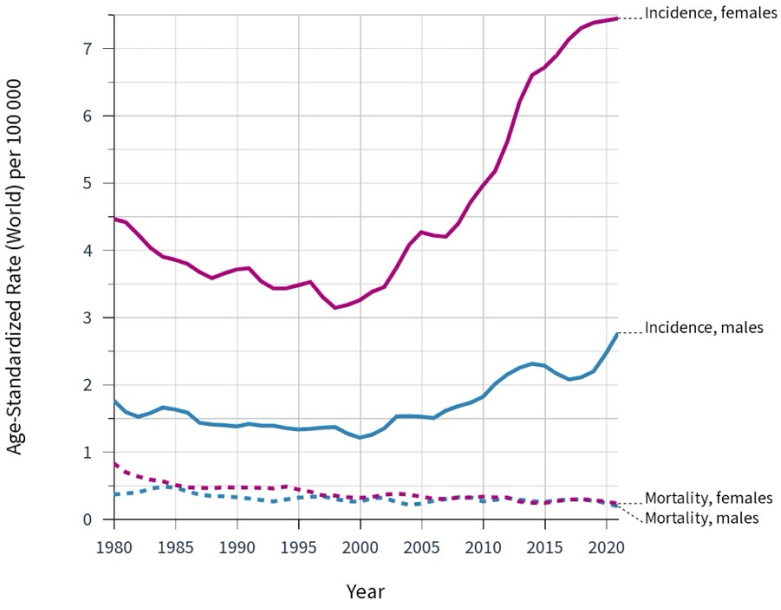

Figure 1 Age standardized rate (world) per 100 000. Incidence (solid lines) and mortality (dashed lines) for women (purple) and men (blue) in Sweden 1980 – 2021. Lines are smoothed using the LOESS regression algorithm (bandwidth 0.1). Obtained by nordcan.iarc.fr/en.

1.4 PREOPERATIVE DIAGNOSIS OF THYROID CANCER

The most common symptom leading to the diagnosis of TC is a palpable nodule in the neck. Incidental imaging findings ("thyroid incidentalomas", meaning thyroid nodules detected by imaging with indication other than thyroid disorders) are an increasing reason for assessing thyroid nodules for potential TC. In addition to this, a vast number of patients are referred to US of the neck due to symptoms that might or might not, be related to goiter such as discomfort in the neck or problems swallowing.

Starting in 2015, the Swedish government introduced a stepwise standardized care bundle (SCB) for different cancer diagnoses and for TC it was launched in April 2017 (61). The purpose was to ensure a uniform and rapid workup

process based on clinical symptoms that could indicate cancer. A "filter function" consisting of cervical US and FNA was established to estimate the initial risk of TC and guide further pre-operative examinations and/or lead-time of treatment (mostly surgery) if indicated. The patients included in Paper II are recruited solely from this "filter function" at SU.

1.4.1 CERVICAL ULTRASOUND

US is the primary radiologic modality for assessing thyroid nodules with respect to TC (62) and using high frequency scanner it is possible to detect thyroid nodules 2-3 mm and larger (63). Ultrasound is nowadays easily accessible, cheap, inflicts no pain on the patient, emits no ionizing radiation and can be used by radiologists with special interest in thyroid US and by clinicians with less experience. Disadvantages frequently mentioned refer to inter- and even intra-observer variability (64, 65). The development of US risk stratification systems (RSS) for TC is one way of addressing this issue.

1.4.1.1 ULTRASOUND RISK STRATIFICATION SYSTEMS

Ultrasonographic features of various types of thyroid nodules have been described since at least the 1980's (66, 67). Thyroid nodules may be divided into cystic, benign, follicular or malignant lesions. Specific US features of thyroid lesions such as microcalcifications, irregular borders and markedly hypoechoic appearance were proposed to be associated with an increased risk of TC and it was suggested that FNA should be based on the findings of the US examination (68, 69). Although entirely cystic or spongiform nodules strongly suggest benign lesions, no single characteristic has been shown to be predictive of TC which is why multiple ultrasonographic features must be considered (70).

In 2009, Horvath et al. published the first US RSS, named Thyroid Imaging Report and Data System (TIRADS) (71) inspired by BI-RADS (72) which was a RSS for evaluating cancer risk of nodules in the breast. Altogether almost 2000 nodules were included for evaluation. The underlying rationale for developing an RSS was to reduce the number of unnecessary FNA in an era of increased detection of thyroid nodules where most are benign (71). TIRADS was the first attempt to create an RSS that provided a clinical guide on how to manage thyroid nodules: FNA or follow the patient by US. It was

based on 10 different patterns, providing estimates of the TC risk correlated to each TIRADS category.

Furthermore, RSS serve as a checklist which in turn results in a more uniform US report, closer attention being paid to suspicious characteristics and less user dependent (73). Most RSSs provide recommendations to facilitate uniform management and minimize unnecessary procedures such as FNA (74).

Although the original TIRADS was accurate in terms of sensitivity and specificity, by many it was considered too complicated to implement in everyday clinical practice. Up to twenty different RSSs have been developed (75) and today there is no international consensus on which RSS to use or which is superior (76). Despite the existence of numerous RSSs, it may be pointed out that the differences are relatively small; RSSs are based on similar US features and thus it would theoretically be possible to agree on one common RSS (75). It is also important to recognize that most US RSSs are compromising between simplicity on one hand and specificity/sensitivity on the other hand, making it mainly a "rule-out" tool rather than "rule-in".

Before 2017 and the introduction of TIRADS in Western Sweden, US guided FNA had gradually replaced palpation guided FNA. However, no RSS was used to either omit FNA, perform FNA or to give recommendations on follow-up. With the possibility to "rule out" TC by US RSS, there was a concern among clinicians (*i.e.* surgeons at Sahlgrenska University Hospital) on the potential risk of missing TC by dismissing a thyroid nodule as benign and not perform FNA. In paper II and III we addressed these issues, both in terms of missed TC and the frequency of correctly omitted FNA, *i.e.* evaluating the effect and potential benefit of using US RSS. As further background it is relevant to recapitulate the currently used EU-TIRADS in some detail.

1.4.1.2 EU-TIRADS

When planning for the implementation of US RSS in Western Sweden in 2017, it was decided to use K-TIRADS (77). The decision to change US RSS to EU-TIRADS (78) after its publication was merely an attempt to adapt to an RSS that neighboring countries would use rather than believing that EU-TIRADS is superior to K-TIRADS. Like the original TIRADS, EU-TIRADS is a pattern-based system although

fewer US characteristics are weighted, making EU-TIRADS simpler to use. EU-TIRADS provides recommendations on further management of each nodule, *i.e.* FNA, follow-up or no follow-up, based on EU-TIRADS score and lesion size.

EU-TIRADS 1 indicates that there is no sonographically identifiable lesion within the thyroid (Figure 2), which is not necessarily equivalent to a "normal" thyroid. For example, echo-structural changes without focal lesions are common after Hashimoto thyroiditis and small (<5 mm) lesions might not be clinically meaningful to describe.

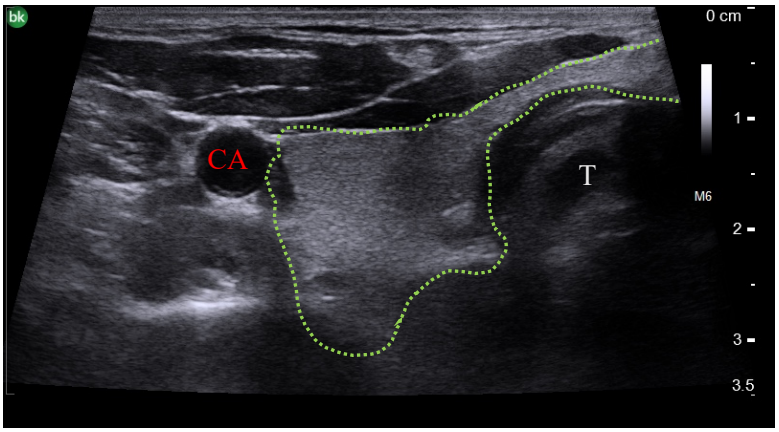

Figure 2. EU-TIRADS 1. Image of the author's right thyroid lobe, marked by surrounding green dotted line. Anterior to the thyroid is the strap muscle which is more hypoechoic compared to the thyroid. The carotid artery (CA) is completely anechoic. The trachea is seen to the right (T). No nodules are detected in the thyroid.

EU-TIRADS 2 is a non-solid lesion presenting as either a cyst (Figure 3) or what is referred to as spongiform lesion. A cyst is completely anechoic, contains only fluid and hence there is no doppler signal. The risk of malignancy is close to 0% and the only indication for FNA is to empty the cyst of fluid to relieve local symptoms. If the cyst contains solid parts, it should be referred to a higher EU-TIRADS classification that corresponds to the solid part. A spongelike nodule mainly contains fluid, but it is not possible to reduce the volume by draining the fluid since the fluid is separated by multiple septa. FNA is not indicated.

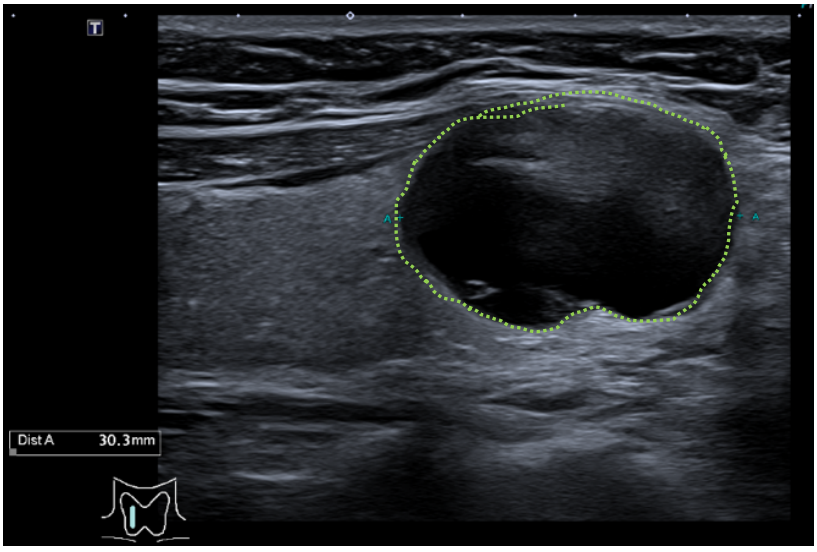

Figure 3. EU-TIRADS 2. Cystic lesion marked by surrounding dashed green line. Normal thyroid parenchyma is seen to the left (cranial of the cyst).

EU-TIRADS 3 is a solid nodule within the thyroid that is either iso- or hyperechoic when compared to the surrounding normal thyroid. Furthermore, no other signs of malignancy should be present (Figure 4). Partially cystic nodules may also be labeled in the EU-TIRADS 3 category. Generally speaking, the more cystic and less solid the nodule is, the lower the risk of malignancy (75). Smooth margins are present and sometimes a thin halo is seen. EU-TIRADS 3 are low-risk and according to accompanying recommendations, FNA should be performed only for lesions >2 cm.

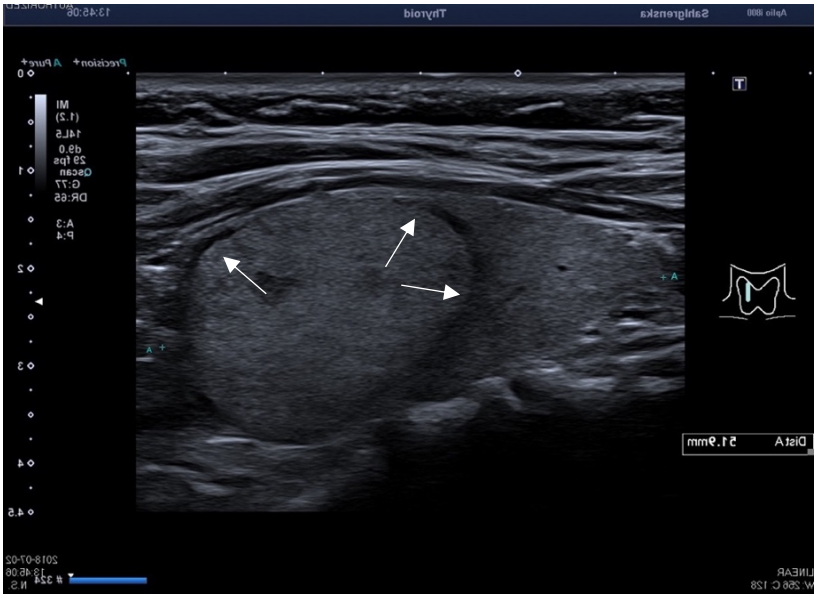

Figure 4. EU-TIRADS 3. Solid isoechoic lesion with smooth margins. A thin hypoechoic margin is surrounding the nodule, commonly referred to as a halo (white arrows).

EU-TIRADS 4 is different from EU-TIRADS 3 in echogenicity: a category 4 lesion is mildly hypoechoic with smooth margins and no other "worrisome" features (see next paragraph for description of "worrisome" features) (Figure 5). EU-TIRADS 4 is considered an intermediate-risk for cancer and FNA is indicated for lesions > 1.5 cm.

EU-TIRADS 5 is considered high-risk and has at least one of the following lesion features: taller than wide, irregular margins, micro-calcifications and/or being markedly hypoechoic. FNA is recommended for all lesions >1 cm and leaves an option of either follow-up or FNA for lesions ≤ 1 cm (78). However, both ATA and ETA advise against FNA for intrathyroidal lesions ≤1 cm with no signs of extrathyroidal growth or suspicious lymph node metastases (11, 75).

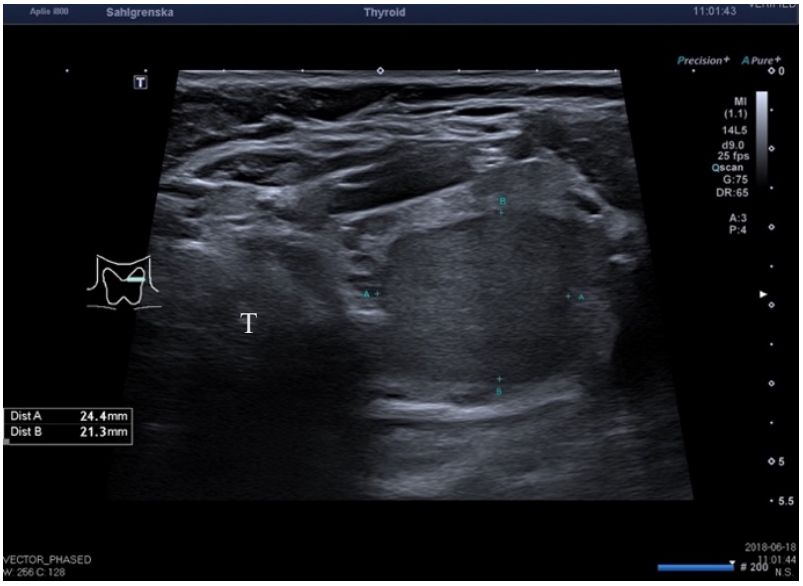

Figure 5. EU-TIRADS 4. Mildly hypoechoic with smooth margins and no other suspicious features. As indicated by the calipers the nodule measures 24x21 mm. A thin halo is also seen, at least anterior of the nodule. T – trachea.

The risk of TC is approximately 50% for each feature and increases with the number of encountered features designating a possible malignancy (78). A malignant tumor is generally more solid compared to a benign one and absorbs more energy from the ultrasound, resulting in a hypoechoic image. Markedly hypoechoic (Figure 6) means the nodule is less echogenic compared to strap muscles. Hypoechogenicity is associated with an increased risk of TC (79) although it has been shown to have the lowest sensitivity for TC of all suspicious features (80). In a study by Lee et al, nodules with moderate to marked hypo-echogenicity had a significantly higher frequency of TC compared to mildly hypoechoic (81). Heterogenous nodules are common and in these cases, the dominant echogenicity determines the degree of echogenicity (75).

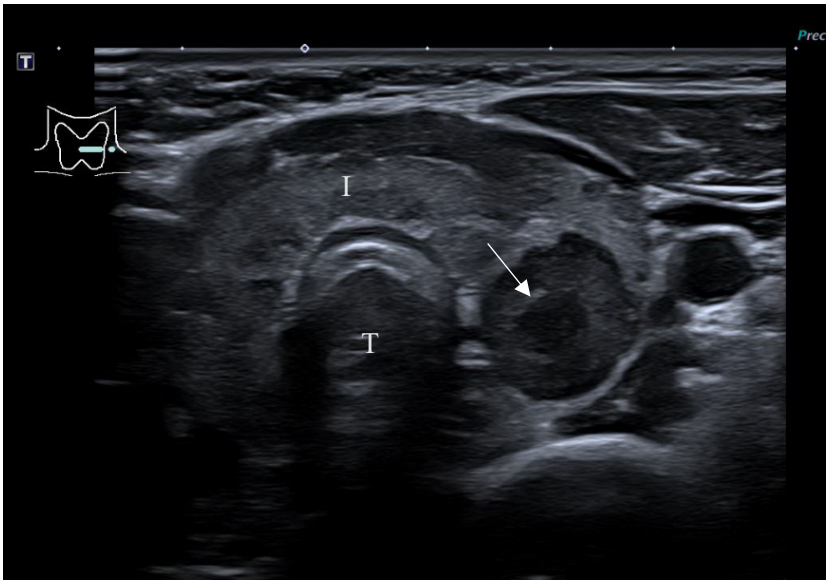

Figure 6. EU-TIRADS 5. Markedly hypoechoic in the center of a left sided nodule (white arrow). Irregular margin at the anterior part of the nodule. T – trachea, I - isthmus.

The margins of the nodule may be irregular, have microlobulations, spiculations or ill-defined margins (Figure 7). The interpretation of this feature may be that the tumor is invasive, growing outside the capsule.

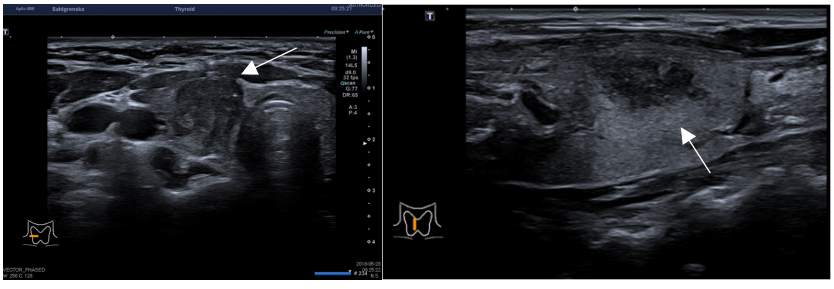

Figure 7. EU-TIRADS 5. Irregular margins (left image) marked by white arrow. Ill-defined margins (right): white arrow pointing at posterior part of ill-defined margins although margins are ill-defined all around (right image).

The term “taller than wide” is used when the anteroposterior measure is larger than the other directions, see Figure 8. There is no threshold for the ratio between “tall” and “wide”.

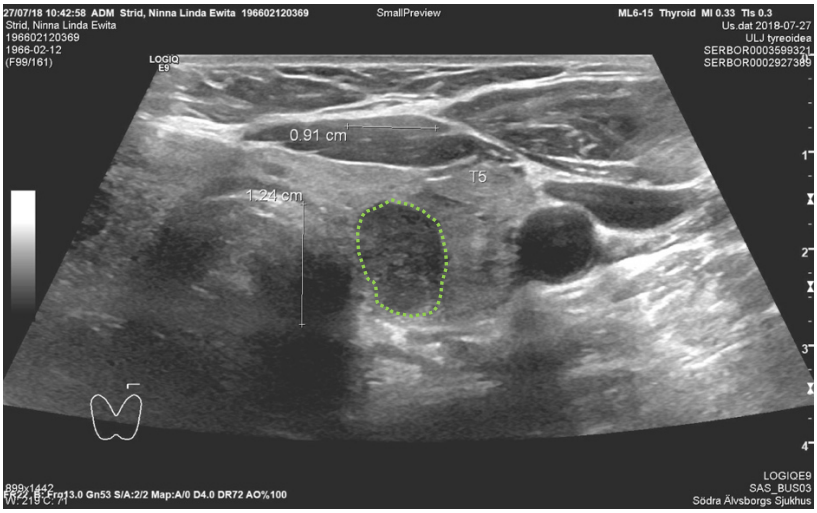

Figure 8. EU-TIRADS 5. Hypoechoic nodule indicated by green dots, "taller than wide" and therefore classified as high-risk. The radiologist has indicated by calipers "tall" – 1.24 cm and "wide" – 0.91 cm. The radiologist also indicated T5 – EU-TIRADS 5.

Calcifications are usually divided into macro- and microcalcifications and may be observed in both benign and malignant thyroid nodules (81). Generally speaking, calcifications, both macro and micro, are associated with an increased risk of malignancy (75). However, according to EU-TIRADS, microcalcifications are the only type of calcifications that affect classification, making the lesion high-risk and therefore EU-TIRADS 5 (78). Microcalcifications show no posterior shadowing and are defined as punctate hyperechoic foci ≤ 1 mm in size and are an independent ultrasonographic feature associated with an increased risk of TC (82, 83). Microcalcifications have been thought to represent psammoma bodies which in turn strongly suggests PTC (84). Although this may be true to some extent, microcalcifications have in different studies been shown to have highly variable positive predictive value of 17 – 78% (82, 83, 85, 86).

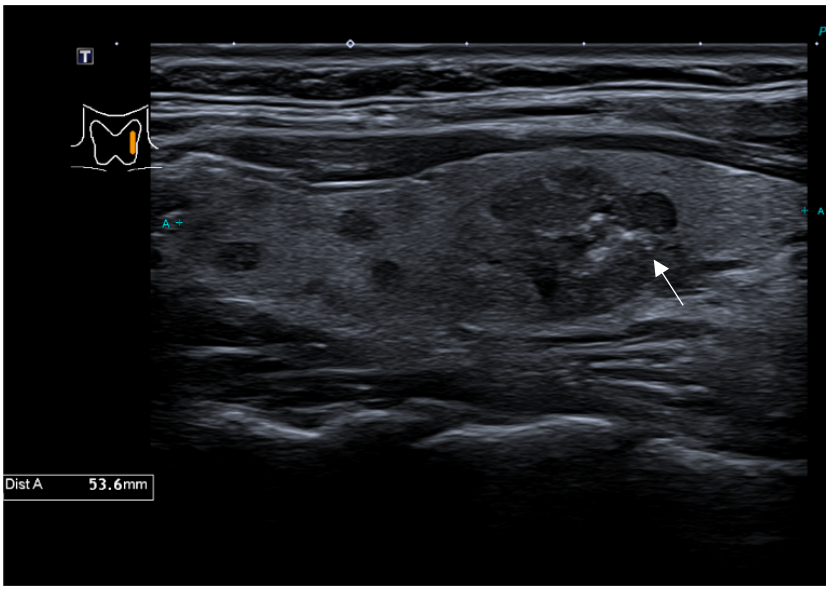

Figure 9. EU-TIRADS 5. Image of a left thyroid lobe with microcalcifications (white arrow). It could also be argued that this nodule has irregular and ill-defined margins as well as microlobulations.

1.4.2 FINE NEEDLE ASPIRATION CYTOLOGY

FNA is not the primary focus in any of the Papers included. Nevertheless, FNA is a crucial tool when discussing preoperative workup, a centerpiece of Papers II and III, which is why this section is relatively extensive.

1.4.2.1 HISTORICAL BACKGROUND

Cytology as a means of diagnosing cancer has been in clinical practice in Sweden since the 1950's. Sixten Franzén was a pioneer in prostate and bone marrow cytology and this work was later developed by Torsten Löwhagen in preoperative diagnostics on breast and thyroid (87-90). In the early 1980's, Bertil Hamberger and colleagues showed that thyroid surgery volumes decreased when FNA was introduced and at the same time the frequency of TC doubled (91). Today, FNA is fundamental in the assessment of thyroid nodules and according to Swedish guidelines, US guided FNA is recommended (7). Although the introduction of FNA analysis improved the management of thyroid nodules, concerns have been raised regarding the quality of FNA in Sweden. In 2008, data from local and Swedish national

quality registers showed that almost 20% of all TC cases had preoperative false benign FNA (92). Yet another problem at this time was that the cytologist was not encouraged to decide upon a definitive diagnosis which negatively affected the clinicians' ability to interpret the FNA report.

1.4.2.2 CLASSIFICATION SYSTEMS

With the intent to provide reproducible reports that are sufficient for the clinicians to apply in the clinical decision making, several different systems for reporting thyroid cytology have been suggested. In Sweden the Bethesda System for Reporting Thyroid Cytology (TBSRTC) (93, 94) is recommended and in Western Sweden it was gradually introduced from 2012 and completely implemented by 2013. Classification systems usually have three levels or categories for estimating TC risk: benign, follicular neoplasia and malignancy (*e.g.* Bethesda II, IV and VI). Apart from these three, there are usually additional categories to indicate either a non-diagnostic test (*e.g.* Bethesda I) or varying degree of uncertainty (*e.g.* Bethesda III or V). The Bethesda system thus provides an estimated risk of malignancy for each category (updated in 2017) and recommendations for action, *i.e.* follow-up, no follow-up or surgery. However, guidelines usually conclude that FNA alone is not sufficient to guide clinical decision making in the event of cytologically indeterminate results (11, 95). For example, family history, previous irradiation, co-morbidity and US features should also be considered (94).

1.4.2.3 FNA LIMITATIONS

FNA cannot distinguish follicular adenoma (FA) from follicular carcinoma because the diagnosis of the latter is based on tumor growth related to the capsule, as discussed in section 1.1.3 Follicular thyroid carcinoma. FNA shows follicular neoplasia in both FA and FTC and typically yields Bethesda IV. The estimated risk of TC for Bethesda IV is 10-40% (96) and the only definite diagnosis is achieved by histopathologic evaluation of the entire tumor. Furthermore, the definite diagnoses are not restricted to FA or FTC. For example, a follicular variant of PTC may also be classified as Bethesda IV. The majority of patients undergoing diagnostic surgical procedures, most commonly hemithyroidectomy, will end up going through an unnecessary operation. Furthermore, in cases of malignant histopathology, completion surgery might be warranted which means that the initial surgical procedure was incorrect irrespective of diagnostic outcome. A recent study from Sweden showed that up to 15% of patients diagnosed with TC after undergoing surgery

with less than total thyroidectomy, had completion surgery due to inadequate preoperative FNA (97). Attempts have been made to come closer to a definite diagnosis with cytologic samples in cases of follicular neoplasia. In a retrospective study from the Karolinska Institute, FNA from FTC had significantly higher Ki-67 index compared to FA. The authors concluded that Ki-67 index may be helpful in predicting FTC but there is a substantial overlap (98).

Nodules yielding Bethesda IV are usually considered for diagnostic surgery in Sweden (7), as is the recommendation by TBSRTC (94). Nodules yielding Bethesda I or III usually undergo repeat US and FNA and the outcome of repeat examination at Sahlgrenska University Hospital (SU) was briefly described in Paper II and III. According to the TBSRTC (94), the risk of TC is 5-10% and 6-18% for Bethesda I and III respectively which is not negligible, and repeat FNA is recommended. However, in a retrospective study from Canada in 2019, only 40% of nodules with Bethesda I or III came to a conclusive diagnosis after repeat FNA (99), making this approach somewhat limited. ATA suggests that US features should be weighed into risk assessment and diagnostic hemithyroidectomy is a valid option (11). Surgery is however costly and is also associated with adverse events (100, 101). Others have found that US RSS is of limited additional value in clinical decision making when facing cytologically indeterminate nodules (102). For this reason, in cases of Bethesda III and also Bethesda IV, molecular testing may be an alternative (94) although presently not in routine use in Sweden.

1.4.2.4 FNA MOLECULAR ANALYSIS

As discussed in the previous section, FNA gives accurate information to guide further clinical management in most cases. However, in up to 30%, cytology is indeterminate which may be challenging and some argue that this prompts for molecular analysis (96). In cases of indeterminate FNA (Bethesda III and IV) it is potentially possible to either diagnose (rule in) or alternatively rule out TC using molecular profiling from FNA samples.

Molecular approaches may either be directed to nuclear or mitochondrial DNA, patterns of RNA expression or the detection of under- or overexpressed proteins. *BRAF*^{V600E} mutation, *RET/PTC* rearrangement and *RAS* mutations are examples of some of the most common mutations in TC (33). Not all mutations drive tumor to malignant transformation and some mutations exist

in both benign and malignant tumors. A nodule with a *BRAF*^{V600E} mutation is most likely malignant and prompts for surgery while a mutation in *RAS* has a 40-60% risk of TC (96), only marginally changing the risk assessment compared to the initial Bethesda III or IV. In fact, two studies by Noureldine *et al*., showed that molecular testing had no significant effect on pre-operative decision-making (103, 104). However, a more recent but retrospective publication using Thyroseq® v2 (commercial kit analyzing 14 TC related DNA mutations and 42 fusions from RNA (105)) concluded that molecular testing of nodules with Bethesda III (80% of included nodules) or IV (20%) was helpful and surgery was adequately avoided in close to half of the cases (106). Today, the most widely used commercial systems used for thyroid molecular testing are Afirma® (RNA based) and Thyroseq® v3 (compared to previous version of Thyroseq®, v3 is extended to include more genes). In a randomized controlled trial comparing the two panels, 346 patients with 372 nodules were included in two arms. No significant difference in performance was found between Afirma® and Thyroseq® v3 and like the study by Steinmetz *et al*. (106), approximately half of the patients avoided a diagnostic surgical procedure (107).

The cost-effectiveness of molecular testing has been studied widely but with conflicting results (108-114). The fact that different studies reach different conclusions largely depends on differences in included parameters that affect the overall costs in the analyses. Not only is the price of the test itself of importance. Another factor to consider is the frequency of malignancy in the group that is tested. The higher the frequency the more expensive routine testing of indeterminate nodules will be since more patients will undergo both testing and surgery. Compared to molecular testing performed by commercial companies, FNA is less expensive, and it might be more cost-effective to improve the quality of FNA.

1.4.2.5 FNA QUALITY DIFFERENCES

The quality of FNA in clinical routine has been debated (115). In 2014, some 50% of non-follicular TC (most are PTCs) with stage ≥T1b (mostly tumors >1 cm) had preoperative FNA showing malignancy (Bethesda VI) or suspicious for malignancy (Bethesda V) (Figure 10). Corresponding figures in Western Sweden were slightly lower. In Sweden, the only region that contrasted was Stockholm-Gotland where corresponding figures are constantly 80% or higher

during the same period (data not shown but available at https://statistik.incanet.se/Tyreoidea/).

For Western Sweden, the figures have gradually improved from 2014 to 2022 (2022 is presently the latest available year of diagnosis) for reasons that not yet have been explored. However, it is probably safe to say that the quality of FNA depends on experience, and that it is important for the clinician "to know" their own cytologist (92).

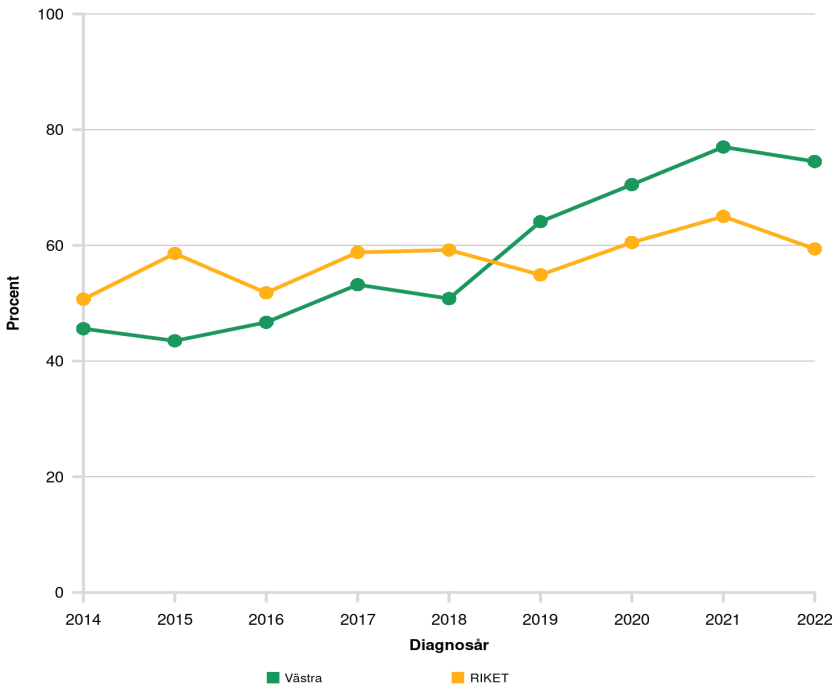

Figure 10. Frequency (y-axis) of non-follicular thyroid cancer stage ≥T1b with preoperative FNA category Bethesda V or VI. Year of diagnosis 2014 – 2022 (x-axis). Green dots/line Western Sweden, yellow dots/line Sweden. Figure obtained from Regionalt Cancercentrum (https://statistik.incanet.se/Tyreoidea/).

1.5 TREATMENT OF DIFFERENTIATED THYROID CANCER

1.5.1 SURGERY

The goal of the surgical treatment of DTC is to entirely remove the primary tumor and regional lymph node metastases if present with as low morbidity as possible. However, surgical goals, strategies, techniques and technical adjuncts are constantly evolving. The overall trend over the last 20 years has been to de-escalate surgery, in favor of less extensive surgical procedures. Even active surveillance omitting primary surgery has been introduced as an option in select cases (116). Consider for instance local guidelines at SU in 2005, recommending total thyroidectomy and bilateral central lymph node dissection even for PTC <1 cm without clinically suspicion of lymph node metastases. In 2012 the first Swedish national guideline for treatment of TC was published. The recommendation for PTC >1 cm was total thyroidectomy and prophylactic central lymph node dissection. At this time, ATA also recommended total thyroidectomy for PTC >1cm (117), based on large studies showing that PTC patients with primary tumor between 1 and 4 cm treated with total thyroidectomy compared to hemithyroidectomy had a slightly decreased risk of recurrence (118, 119). These studies did not consider more aggressive subtypes of DTC such as tall cell, hobnail or columnar cell type. With the recognition that DTC is a heterogenous type of cancer, the treatment guidelines have changed to a more individualized recommendation that considers more than just tumor type, size and metastatic spread. According to the latest ATA guideline from 2015, the recommendation changed to opt for hemithyroidectomy for DTC <4 cm in the absence of risk factors (11). Swedish national guidelines have not yet fully adapted to the ATA guidelines and according to the latest version from 2021, total thyroidectomy is still recommended for PTC between 1 and 4 cm. However, prophylactic central lymph node dissection was no longer recommended after revision in 2017 despite that lymph node metastases in the central compartment are common in clinically node-negative patients (120). Prophylactic lymph node dissection in these patients has not been shown to be superior in terms of biochemical response, local recurrence or survival (121-123). At present, no randomized studies have been performed and there are no ongoing randomized controlled trials registered at ClinicalTrials.gov exploring the potential benefits of

prophylactic central neck lymph node dissection, as it is not feasible due to too many patients needed to be included.

1.5.2 RADIOACTIVE IODINE

Thyroid follicular cells have a unique ability of concentrating and organification of iodide. Iodide is transported into the cell by the sodium-iodide symporter (NIS), it binds to thyroglobulin and is used in the synthesis of thyroid hormones: triiodothyronine and thyroxin. This can be utilized for both therapeutic and diagnostic purposes by administration of RAI, mostly the isotope ^{131}I (diagnostic and therapeutic) but also ^{123}I (diagnostic). Although the definitions of the therapeutic goals of RAI may be a source of frustration and conflict (12), the three main purposes of RAI treatment may be divided into: (1) ablation of remnant benign thyroid tissue, (2) adjuvant treatment of regional lymph node metastases and, (3) treatment of distant metastases.

In Sweden, patients with low-risk DTC (according to ATA risk-evaluation) have been treated with RAI ablation with the purpose of facilitating follow-up and eventually terminate active tumor surveillance (7). ETA states that the benefit of RAI in low-risk DTC patients is a matter of controversy and they recommend that the RAI should be based on individual risk factors such as elevated postoperative levels of serum thyroglobulin (Tg) or the presence of structural disease identified by US (124). The ESTIMABL 2 study, a randomized controlled trial, compared patients with low-risk DTC, treated with either thyroidectomy alone in one arm or total thyroidectomy + RAI 1.1 GBq in the other arm. There was no difference in rate of recurrence or persistent disease between the two groups after three years of follow-up (125). Although three years of follow-up may seem short for drawing definite conclusions on outcome, it may be pointed out that most of these patients would have a follow-up time of two years according to Swedish guidelines. A British study that has not yet been published, the IoN trial (NCT01398085), also questions the need of RAI in low risk DTC and includes also larger tumors.

RAI treatment of intermediate-risk patients is also somewhat controversial but for different reasons. Theoretically, these patients are more likely to benefit from RAI therapy since the risk of recurrence by definition is higher compared to low-risk patients. However, these tumors often display aggressive histology

and as such, they generally have poor iodine uptake due to impaired expression of NIS (12). In such a situation one may argue either that (1) these tumors often have poor iodine uptake and hence a limited activity of 1.1 GBq should be administered due to lower toxicity, or (2) since these tumors have such poor uptake it is necessary to give a higher activity, *i.e.* 3,7 GBq. In the forthcoming Swedish guidelines on TC, there was no consensus on which is the ideal activity of RAI for these patients.

1.5.3 IODINE REFRACTORY DTC

RAI refractory DTC refers to tumors that lack the ability to concentrate and retain iodide but there is no universally accepted definition (126). However, the following clinical situations may predict the likelihood of RAI refractory (RAI non-avid) tumors: (1) patients who previously had RAI uptake and on post treatment scans have no uptake in lesions that previously showed uptake. These patients usually have large metastatic burdens, and it can be speculated that well differentiated clones of TC cells were effectively treated but more poorly differentiated clones survived. (2) a mixed uptake, *i.e.* some lesions have uptake whereas some do not, and in these cases, progress is likely to occur in lesions without RAI uptake. (3) metastatic TC lesions resistant to iodine at the time of diagnosis and when the patient is treated with RAI for the first time. These patients do not benefit from RAI and should be considered RAI refractory. Finally, a fourth situation may be mentioned: if the thyroid is not resectable, RAI treatment is not possible, and the tumor should be regarded as RAI refractory whether this is physiologically true or not (127). Furthermore, aggressive histologic types and molecular profiles such as *BRAF* or *TERT* promoter mutations also predict RAI resistance (128-131).

Systemic targeted drug treatment should be considered if the patient is assessed RAI resistance, harbors a significant tumor burden and progressive disease is observed. Before the era of tyrosine kinase inhibitors (TKI), no active oncologic treatment for patients with RAI refractory DTC was available (3) and at the start of the work with this thesis, we wanted to understand more about these patients with aggressive types of TC (relapsing DTC, PDTC and ATC). The idea was to develop tumor platforms based on *in vitro* tumor tissue models and PDX models to allow experimental studies, which generated data presented in Paper IV.

First line treatment for patients with progressive or symptomatic RAI refractory DTC are lenvatinib or sorafenib (7). Although not tested prospectively in direct comparison, the SELECT trial showed that lenvatinib had a median progression free survival of 18 months compared to 3 months in placebo (132) and sorafenib showed 5 months of improvement in the DECISION trial (133). A more recent but retrospective study comparing lenvatinib and sorafenib, showed that lenvatinib had superior progression free survival (134).

TC with *BRAF* mutation regularly have decreased iodine uptake due to decreased expression of NIS. The concept of restoring RAI avidity by MAPK-inhibition (135) is interesting but has not yet been approved. Phase II studies showed that treatment with BRAF/MEK-inhibitors was safe and restored iodine uptake in patients with previously RAI-refractory and unresectable TC (136, 137). A review article suggested that select patients with metastatic RAI refractory DTC may benefit (138).

1.6 TREATMENT OF ANAPLASTIC THYROID CANCER

1.6.1 CHEMO-RADIOTHERAPY AND SURGERY

The Swedish approach to ATC treatment has been strictly palliative. The goal of the treatment has been local tumor control to prevent the patient from dying of suffocation. This has been achieved by pre-operative radio-sensitizing chemotherapy followed by surgery if the patient is operable and the tumor resectable (7, 139). Tracheostomy is discouraged but sometimes necessary due to risk of acute airway obstruction (28).

1.6.2 TARGETED THERAPY AND SURGERY

With new therapeutic systemic agents available, surgery may be considered without preoperative radio-chemotherapy if complete tumor resection is possible (28). These cases are restricted to patients with tumor confined to the neck, stage IVa, and some cases of stage IVb.

The introduction of molecular analyses and targeted therapy has meant a paradigm shift in the treatment of ATC, offering patients with locally/regionally limited disease a chance of treatment with curative intent

(22). In cases of tumors with mutations in "druggable" targets, a more efficient systemic treatment is available compared to radiochemo-therapy described above. Treatment of ATC tumors with *BRAF* mutation is the most studied which is not surprising considering that approximately 45% of all ATC tumors have this mutation (23). *BRAF* mutated ATC is treated with a combination of BRAF and MEK inhibitors, following the same protocol as for other *BRAF* mutated tumors, for example metastasized malignant melanoma. The combination of *BRAF* and MEK inhibitors has been shown to delay drug resistance (140). Treatment with BRAF/MEK inhibitors have rapid tumor response and immediate testing of *BRAF* mutation (IHC preferably because result is reliable and can be obtained within 2 days) is important since untreated ATC usually progresses quickly with risk of threatening local symptoms, *e.g.* loss of airway. A non-randomized phase 2 study showed overall response rate of 56% in 36 patients with *BRAF* mutated ATC (141).

Novel treatment options for ATC patients with *BRAF* wild-type tumors, are less studied. As discussed in section 1.1.7 Anaplastic thyroid carcinoma, ATC has a higher mutational burden compared to DTC implying that immunotherapy could be an effective treatment. A phase I/II study included 42 patients with progressive ATC and showed that 19% of patients treated with the PD-L1-inhibitor spartlizumab responded (142). A subgroup analysis showed that patients with tumors expressing PD-L1 $\geq$ 50% had higher response rate (35%) compared to those who lacked PD-L1 expression (0%). Treatments with combinations of PD-L1-inhibitor and TKI has also been studied. A retrospective study including eight ATC patients treated with a combination of PD-L1-inhibitor pembrolizumab and lenvatinib showed promising results (143).

2 AIM

The primary aims of this thesis were to investigate the patterns of the increasing incidence of TC In Western Sweden, the performance of US RSS (specifically EU-TIRADS) and finally to establish an experimental platform for advanced TC.

Paper I: The purpose of this study was to investigate changes in TC incidence, the mode of TC detection, and the outcome of patients diagnosed with TC 2001-2014 in Western Sweden.

Paper II: This study aimed to investigate the magnitude of reduction in unnecessary FNA when implementing US RSS EU-TIRADS in a TC programme at Sahlgrenska University Hospital.

Paper III: The purpose was to evaluate the safety of selective FNA using EU-TIRADS with non-selective FNA as a reference regarding missed TC.

Paper IV: The aim was to establish a PDX model for TC development and evaluation of drug therapy in advanced TC.

3 PATIENTS AND METHODS

The four studies included in this thesis are summarized in Table 1.

Table 1.Thesis at a glance.

Study	Design	Aim/ Hypothesis	Subjects/Study period	Main findings
I	Population-based	Exploring changes in TC incidence and patterns of detection	Patients diagnosed with TC in Western Sweden 2001-2014 n=1230	Threefold increase of TC, included stages ≤T3 Clinical signs, not imaging, was the most common mode of detection.
II	Cohort	Investigate the clinical impact of introducing EU-TIRADS as an RSS to reduce FNA rates	Patients referred to SU for US 2018-2022 n=990	EU-TIRADS omitted FNA in 7% of patients. EU-TIRADS is safe but its impact limited.
III	Multicenter RCT	Rate of suspicious FNA higher in selective group No difference in ROM	Patients undergoing US in Western Sweden 2022-2023 n=195 in 2 arms	Rate of Bethesda IV-VI higher in selective group. No difference in ROM.
IV	Prospective experimental cohort	Establish TC PDX model Drug testing	Patients undergoing surgery for advanced TC at SU 2018-2020 n=8	PDX of advanced TC rarely successful. Support of thyroid SCC being a subtype of ATC. New targeted drug option, based on molecular characterization

TC – thyroid cancer, SU – Sahlgrenska University Hospital, US – ultrasound, FNA – Fine needle aspiration cytology, ROM – Rate of malignancy, SCC – Squamous Cell Carcinoma, PDX - Patient derived xenograft.

3.1 PAPER I

Paper I was a population-based regional cohort study. All patients in Western Sweden diagnosed with primary TC (ICD code C73.9) 2001 - 2014 were included in the initial analysis. Data on diagnosis and personal identity number was retrieved from the National Cancer Register (144). Local medical records were searched to ensure complete inclusion of patients. Local medical records were also used for retrieval of data on tumor type, stage, mode of detection and follow-up.

3.2 PAPER II

Paper II was a retrospective single center study. The study included patients referred to the department of neuroradiology at SU due to signs or symptoms according to the standardized care bundle for TC (61) (Swedish: filterfunktion SVF sköldkörtelcancer) between March 2018 and January 2022. Patients with a previous history of TC and patients with non-thyroid disease (lymphoma, n=3) were excluded. Patients with no focal lesions on US (EU-TIRADS 1) were also excluded.

In addition to basic patient characteristics, nodule size, distributions of EU-TIRADS and Bethesda category, repeat examination, frequency and outcome of surgery were studied.

3.3 PAPER III

Paper III was a regional randomized controlled interventional study, including four hospitals in Western Sweden. Patients referred to US of the thyroid due to symptoms that could indicate TC were eligible for inclusion. Patients ≥18 years old and no previous history of TC were included. Participating radiology departments were SU, Norra Älvsborg Hospital, Kungälv Hospital and Södra Älvsborg Hospital. A care report form (CRF) was used by the radiologist and collected by a research coordinator.

Primary endpoint was the frequency of Bethesda IV-VI and it was hypothesized that this would be higher in the intervention group. The secondary outcome was rate of malignancy (ROM), and the hypothesis was that there would be no significant difference between the two groups. Sample

size calculation estimated at power 0.8 and significance level 0.05 that approximately 150 observations were required for the primary outcome. Sample size for the secondary outcome was calculated using a method for a non-inferiority trial. With the same levels of power and significance, it was estimated that between 619 and 10 142 observations were needed depending on difference in ROM accepted between the groups and depending on ROM in the control group. It was considered not possible to include enough patients to answer the secondary outcome with statistical significance. For these reasons, it was decided to include 200 patients.

Patients were randomized 1:1 in random permutated blocks to either US-guided non-selective FNA (control) or selective FNA according to EU-TIRADS criteria (intervention). Patients in the control group were also assessed according to EU-TIRADS and FNA was performed in all EU-TIRADS 3 and 4 lesions >1 cm if no more than five were identified. For EU-TIRADS 5 the indication for FNA was identical to the intervention group.

All FNA specimens were evaluated using routine assessment according to the Bethesda system (94).

3.4 PAPER IV

Patients referred to SU for surgery of locally advanced TC were eligible for inclusion. Patients were identified according to a pre-specified study protocol. Clinical data such as preoperative imaging, FNA and routine histo-pathology was retrieved from medical records.

Directly after surgical removal, fresh tumor tissue was excised with assistance of the pathologist. Samples were implanted subcutaneously into the flank of immunodeficient mice. Tumor size was repeatedly estimated macroscopically with a caliper in 3 dimensions to calculate tumor volume.

In parallel, tumor tissue was fixed in 4% formalin, subject to routine hematoxylin-eosin and IHC. IHC antibodies were labeled against NKX2-1/TTF-1, CDH1/E-cadherin, CDH2/N-cadherin, SLUG, Ki67 and NQO1.

Whole exome sequencing and transcriptional profiling by RNA sequencing were performed on DNA/RNA extracted from xenografted tumor material

originating from case 3 (described in Paper IV) to assess possibly targetable mutations.

NOG mice carrying tumor third generation tumor transplant from case 3 in Paper IV were randomized for drug treatment with cabozantinib, GDC-0236 (specific PI3Kα-inhibitor) or CB-893 (glutaminase inhibitor) based on mutational analysis.

3.5 STATISTICS

Paper I: Age-standardized incidence rates per 100 000 person-years (ASR) were calculated with correlation to the world standard population. ASR was calculated separately for means of detection, tumor type, T-stage. ASR differences at 5% significance level between groups was considered significant by calculating standardized rate ratio with 95% confidence interval.

Paper II: Chi-squared test was used for comparison between groups. Rate of malignancy was defined as number of patients diagnosed with TC divided by the number of patients operated. Sensitivity, specificity, positive predictive value and negative predictive value was calculated for EU-TIRADS.

Paper III: For comparisons between groups chi-squared test, Wilcoxon rank sum test and Students t-test was used for comparison between groups. A p-value <0.05 was considered significant.

Paper IV: Two-sided t-test was used when comparing tumor sizes. Statistical significance was set at $p<0.05$.

3.6 ETHICAL CONSIDERATIONS

All studies were approved by the regional ethical board in Göteborg, Lund or Uppsala (paper I: diary number 261-11 and complementary diary number T131-18, paper II: diary number 2022-03136-02, paper III: diary number 2021-01490, paper IV: diary number 822-15 and complementary diary number 2023-03923-02). For papers III and IV patients received written and oral information and provided a written consent.

4 RESULTS

4.1 PAPER I

According to the Swedish cancer registry, a total of 1230 patients were diagnosed with TC in Western Sweden during the study period 2001 – 2014. The age-standardized incidence rates per 100 000 person years (ASR) increased from 1.1 (men) and 3.1 (women) in 2001 to 3.8 (men) and 10.7 (women) in 2014. This corresponds to an estimated annual percentage change (EAPC) of +8.0% for men (95% c.i. 4.3 to 11.7) and +10.4% for women (95% c.i. 8.1 - 12.8). The incidence of TC was higher in Western Sweden compared to the entire Swedish population.

A cohort of all 736 patients diagnosed with TC in Western Sweden during three time periods 2001-02, 2006-07 and 2011-2014 were analyzed with respect to type of TC, mode of tumor detection and tumor stage. ASR for MTC and ATC did not change during the study period. PTC increased threefold and FTC increased fivefold when comparing the first and the last study periods. For DTC all tumor stages T1a – T3 increased significantly and of similar magnitude across T-stages. DTC stage T4 doubled in ASR but this increase was not statistically significant.

The most common mode of tumor detection was palpation (64%), *i.e.* signs or symptoms that led to pre-operative diagnostic workup showing TC or suspicious TC. The ASR of this group increased approximately three times comparing the first and last study periods. The second most common mode of detection was incidental diagnosis of TC (30%), *i.e.* patients who underwent surgery due to benign indications and where the postoperative histopathology report showed TC. The incidental group increased more than three times during the study period. Mean size of incidentally detected tumors did not change during the study period and was significantly smaller compared to TC detected by palpation. Some 65% of incidentally detected tumors were microcarcinomas, where 21% were >2 cm in diameter, and 10% were >4 cm. No TC was diagnosed due to imaging, *i.e.* thyroid incidentaloma (see definition page vii) during the first study period (2001-02). Altogether, 37 patients (5%) were diagnosed due to thyroid incidentaloma.

4.2 PAPER II

Some 990 patients referred to the department of neuroradiology for cervical US and FNA according to SCB criteria between March 2018 and January 2022 were included.

FNA was omitted in 7% of the patients due to EU-TIRADS recommendations. Some 121 (10%) nodules underwent FNA despite no indication (lesions smaller than cut-off levels and PET-positive findings or patients with lymph nodes suspicious for metastatic spread excluded). Of these, 19 nodules underwent surgery of which 3 (2%, in three patients) were malignant.

Some 282 (28%) patients underwent thyroid surgery. The indication for surgery was malignancy or excluding malignancy in 200 patients (71%), benign non-toxic goiter in 74 patients (26%) and hyperthyroidism in 7 patients (2%). ROM was 33% compared to 26% in the historical cohort.

4.3 PAPER III

After having prolonged the inclusion period by three months, a total of 195 patients with 294 nodules were included, 93 patients (48%) in the non-selective arm and 102 (52%) in the selective. Five patients less than planned were included and inclusion was closed due to slow recruitment rate. Four centers in Western Sweden participated (distribution of patients): SU (44%), Norra Älvsborg Hospital (30%), Kungälv Hospital (25%) and Södra Älvsborg Hospital (1%).

The mean number of FNA performed was significantly higher in the non-selective group (1.53 nodules) compared to the selective (0.94) ($p= 0.0016$).

The frequency of Bethesda IV-VI in patients randomized to intervention was 13% (counting the highest yielding Bethesda category if multiple nodules) vs 7% in the control group ($p=0.039$) (primary endpoint.

Altogether 41patients (21%) underwent surgery at latest follow up 8 January 2024. TC (*i.e.* microcarcinomas) that had not been recorded in the CRF were excluded. Five patients (5%) in the control group and six patients (6%) in the intervention group were diagnosed with TC (secondary outcome).

4.4 PAPER IV

Tumor tissue was collected from eight patients with locally advanced TC and subsequently xenografted to immunomodulated mice as described in the methods section. See Table 2 for details. Of the eight tumors collected, one tumor (referred to as case 3 in Paper IV) was successfully grafted in which routine histo-pathology showed squamous cell carcinoma (SCC). Additionally, two patients (case 1 and 2) diagnosed with SCC but not included preoperatively were analyzed with routine histo-pathology, enhanced IHC and molecular profiling.

Case 1 was diagnosed with follicular adenoma plus an incidental finding of a 5 mm lesion of mixed PTC/SCC. The tumor was radically removed, and the patient was considered free of disease.

Case 2 was diagnosed with a mixed PTC/SCC tumor, stage pT3N1a, and at the same time diagnosed with vulvar SCC. The patient received RAI (3.7 GBq) and external beam radiation due to incomplete resection of the thyroid tumor. Post RAI whole body scan showed uptake in the neck consistent with persistent disease but no signs of distant metastases. Post radiation magnetic resonance imaging (MRI) showed no sign of tumor progression.

Histology could not rule out the possibility of collision tumor between PTC and SCC in the thyroid. However, IHC in a subset of deviant PTC cells revealed expression of CK7 and CK19 in accordance with adjacent SCC tumor cells. These PTC cells were CK5/6 negative, as opposed to SCC cells, but displayed weak CD10 expression, which was completely absent in the PTC bulk tumor but strongly expressed in SCC cells. Target capture sequencing confirmed identical pathogenic mutations in both thyroid tumors but unique signature in the vulval SCC, strongly suggesting a transition of PTC to SCC by transdifferentiation.

Histopathology reports showed SCC of the same growth pattern in the thyroid and the vulva.

Table 2. Clinical data corresponding to patient-derived xenografts in Paper IV.

Age/sex[1]	Diagnosis[2]	Stage[3]	Adjuvant Treatment[4]	Survival[5]	PDX[6]
40/F	PTC	T2(m)N1bM0	RAI 3.7 GBq	NED	-
47/F	ATC	T4N1bM0	XTR 45 Gy + paclitaxel	DOD (6)	-
89/F	PDTC	T4N1bM1	RAI 7.4 GBq	DOD (8)	-
70/F	FTC	T3NxM0	RAI 3.7x2 + redo surgery	AWD	-
85/F	PTC	T1bN1bM0	RAI 3.7 GBq	NED	-
69/F	FTC/NET*	T1bNxM1	temozolamid/kapecitabin	DOD (18)	-
69/M	SCC	T3N1bM0	See Paper IV	DOD (14)	40
35/M	PTC	T3N1bM0	RAI 3.7 GBq	NED	-

[1] Years; F – female; M – male.

[2] Based on routine histopathology: PTC - papillary thyroid carcinoma; PDTC - poorly differentiated thyroid carcinoma; ATC - anaplastic thyroid carcinoma; FTC – follicular thyroid carcinoma; NET – neuroendocrine carcinoma; SCC - squamous cell carcinoma.

[3]Tumor staging according to 7th edition of the TNM Staging System of the American Joint Committee on Cancer (AJCC).

[4] XTR - External beam radiation; RAI – radioiodine.

[5] NED - No evidence of disease; DOD - Dead of disease; AWD - Alive with disease; mo - Months survival time after diagnosis.

[6] Observation time (days) until successful growth of transplant. Negative transplants were observed for at least 12 months.

*calcitonin negative, RET negative, neuroendocrine tumor primary in thyroid.

Case 3, SCC, was successfully enrolled in the PDX-study, with evident tumor growth 40 days post-implantation and grafts propagated for three generations. Primary tumor and grafts displayed high similarity in tumor cell morphology and specific staining, with evident signs of EMT and thyroid precursor origin. Whole exome sequencing of PDX-grafts revealed possible targetable mutations including *PIK3CA* and *NFE2L2*. PDX-mice treatment experiment with cabozantinib, GDC-0326 (PIK3a-inhibitor) or CB-893 (glutaminase inhibitor) showed that single treatment with CB-893 had largest successful impact on tumor progression (*i.e.* preventing growth) followed by a combination of cabozantinib and GDC-0326.

5 DISCUSSION

This thesis explores different diagnostic aspects of TC. Paper I investigated incidence changes of TC in Western Sweden, not only in overall frequency but also types of TC, tumor stages, how tumors were detected and outcome. Paper II and III evaluated the initial work-up for patients with clinical/radiological symptoms or signs that could indicate TC. In conclusion these papers showed that EU-TIRADS adequately omits FNA for benign nodules without missing TC but the frequency of omitting benign nodules is rather low. Paper IV addressed TC patients with advanced disease where treatment options are limited. It supported the hypothesis that thyroid SCC is a subtype of ATC. Paper IV also suggested treatment with novel targeted therapy after molecular analysis.

5.1 THE SCOPE OF THYROID CANCER INCIDENCE

There has been an ongoing debate the last two decades or so on the mechanisms behind the worldwide increase in TC and there are an overwhelming number of reports agreeing that the largest contribution to the increase in TC is detection of clinically insignificant PTC identified by US (3, 145, 146). Some authors argue that because of this debate, awareness of the problems with potential overdiagnosis led to adjustments of guidelines which in turn has led to a reduced increase of TC incidence (147, 148). However, in Sweden it is not likely that the same mechanisms have caused TC incidence as described by American, South Korean, French and Italian authors (56, 57, 145, 146, 149, 150). In Sweden, health care is almost entirely funded by public means and practice guidelines are uniform throughout the country. The use of office-based US is uncommon among physicians in primary care and endocrinologists which means that this modality is not as easily accessible as in many other countries. The threshold to perform cervical US was most likely higher in Western Sweden in 2001-2014 compared to many other countries at that time and probably also compared to the situation in Sweden today. This is supported in Paper I by the finding that the overall frequency of imaging findings (thyroid incidentaloma) was low (5%), but it increased from 0% in the first period to 7% in the last period. If Paper I was to be carried out again

today, this number would probably be even higher but still not comparable to what is observed in many other countries.

The prerequisite for the increased TC detection to be driven by increased and unmotivated cervical US is the existence of a subclinical reservoir of TC. In Paper I, the subclinical reservoir was exposed by increased surgical procedures rather than preoperative diagnostic procedures, confirming the notion of such a reservoir. Patients who underwent thyroid surgery due to benign indication with TC as an incidental finding, contributed significantly to the observed increase. This was confirmed by the finding that 65% of these were microPTC (PTC ≤1 cm). As described in Paper I, the total number of total thyroidectomies increased five-fold during the study period although the reason for this dramatic increase was not investigated and is unknown.

Although Paper I showed a threefold rise in TC incidence in Sweden, the overall incidence is of a significantly lower magnitude compared to South Korea, the United States and Italy (145).

The study showed that increased TC incidence was not restricted to clinically insignificant small PTC but also comprised more advanced TC. Apart from analyzing patterns of TC incidence in Western Sweden, follow-up was also recorded. In the two first cohorts 11% (first cohort) and 7% (second cohort) of the patients with DTC or PDTC died of disease. The extent of surgery was the only treatment that was recorded in the study but at this time systemic treatment with TKI was not available. We do not know to what extent these patients were treated with RAI or how they responded to RAI but most likely, the majority were either RAI refractory at the time of diagnosis or developed RAI resistance.

The relatively high number patients with poor outcome is partly due to the decision to include also PDTC in the analysis but provides further support to the conclusion that most patients in this study had symptomatic tumors, not indolent tumors detected by overzealous US examinations.

5.2 THYROID NODULE MANAGEMENT

Paper II aimed to evaluate the (1) impact and (2) safety of selective FNA using EU-TIRADS after four years of clinical practice at SU. The effect size of EU-

TIRADS, or any other US RSS, has on the frequency of FNA varies in the literature from having no significant impact to 50% FNA omitted (151, 152), which places the finding of a 7% reduction of FNA in the present study in the lower range. All US RSS are based on similar features although each feature may be weighted slightly different and cut-off diameter for performing FNA also vary slightly (75). Therefore, the difference in overall performance is most likely not decisive. A more important factor that influences the frequency of omitted FNA is the selection of patients being referred to US RSS. EU-TIRADS can only affect nodules between 1 and 2 cm concerning FNA or no FNA. Mean size of nodules in Paper II was 27 mm (±15) which means that most solid nodules underwent FNA regardless of US features, *i.e.* EU-TIRADS score. This relatively large mean nodule size might be due to the selection of patients according to the SCB criteria. Nevertheless, the effect on patients that did not undergo FNA was only 7% which is less than expected.

Considering that 9% of the included patients had TC, > 80% underwent FNA despite benign outcome meaning that the EU-TIRADS could not sufficiently rule out malignancy. The results also showed that the frequency of omitted FNA could have been higher (17%) if strict criteria had been followed. This discrepancy cannot be explained since this was a retrospective study. However, a large proportion of nodules undergoing FNA despite not recommended by EU-TIRADS, were ≤ 3 mm smaller in diameter than recommended cut-off size. It may be speculated that the radiologists were more comfortable performing FNA than to abstain, especially considering that according to regional guidelines there was no structured follow-up of nodules (except for EU-TIRADS 5) if FNA was omitted.

Out of 121 nodules undergoing FNA without fulfilling criteria for FNA according to guidelines, only three were malignant (2%). The reasons for not following strict criteria are not known but it could be speculated that the radiologist in borderline cases preferred to perform FNA. It should also be mentioned that patients not undergoing FNA are not subject to follow-up which might contribute to the decision to perform FNA.

Paper III also assessed the impact on FNA frequency. The proportion of patients undergoing FNA was significantly reduced in the intervention group compared to the control group. This is not a surprise considering that omitting FNA is a major concept of EU-TIRADS and any other US RSS. In the current

study, FNA was omitted in 30% of the patients in the intervention group compared to 8% in the control group if all cases are included. If only solid lesions are considered, FNA was omitted in 9% of patients. This figure is similar to the frequency of omitted FNA in Paper II but with the important difference that strict criteria for FNA was kept in Paper III but not in Paper II. In Paper II, 17% of patients would have avoided FNA if strict criteria had been applied which illustrates that the effect of EU-TIRADS on avoiding FNA depends highly on what patients are examined.

The primary endpoint of Paper III was the rate of Bethesda IV-VI. This endpoint was assessed both for nodules and patients (counting the highest yielding nodules for each patient). The intervention groups had significantly higher proportion of Bethesda IV-VI when comparing patients with the highest yielding nodule (11% vs 7%, p=0.039). This is to the authors' knowledge, the first randomized controlled trial that investigates the ability of an US RSS to select nodules with increased risk for TC.

The secondary outcome was the rate of malignancy, and it was hypothesized that there would be no difference. Paper III showed that 7% in the intervention group were diagnosed with TC and 6% in the control group. The statistical significance was not tested because we did not include enough patients to show a non-inferiority of selecting nodules using EU-TIRADS. Using a non-inferiority design demands more patients compared to a hypothesis in which superiority is tested. In Paper III we aimed at including 200 patients but had to extend the inclusion period by three months due to slow inclusion rate. In retrospect it was therefore correct not to aim for statistical significance for this endpoint. However, the rate of malignancy was one percentage unit higher in the intervention group and there was no indication that EU-TIRADS dismissed malignant nodules.

5.3 ADVANCED THYROID CANCER

One important aim of the work that eventually led to Paper IV was to establish tumor platforms that could be used for further characterization of advanced TC. The two models were based on (1) in vitro 3D tumor biopsy and (2) PDX model in immunomodulated mice. In conclusion, these models were difficult to establish. The *in vitro* 3D tumor biopsy platform showed that tumor tissues were viable for weeks to months and signs of tumor infiltration was seen

although it was difficult to distinguish cancer cells from stroma cells (data not published), thus making tumor progression difficult to examine.

Already in the early 1980's, tissue from a patients with Graves´ disease was successfully transplanted to athymic nude mice (153). ATC tumors have previously been transplanted to immunomodulated mice and *in vivo* models have been proven useful in the evaluation of novel drugs (154, 155). In our series of eight cases of advanced TC transplanted to immunomodulated mice, only one case successfully progressed. The preoperative diagnosis in this case was not known and the histopathologic report showed SCC, at the time of diagnosis not recognized as a subtype of ATC. Considering that adjacent organs such as the esophagus are known to harbor tumors of SCC type it is not farfetched to believe that a tumor of SCC in the thyroid in fact is not primary from the thyroid but rather from the esophagus. In the case described in Paper IV, clinical investigations could not confirm origin other than the thyroid. CT showed that there was no clear layer between the thyroid tumor and the esophagus but trachea-esophagoscopy showed no signs of tumor. Biopsies from the esophagus and trachea showed normal epithelium. In addition to the above-described case (case 3 in Paper IV), two other patients were identified being diagnosed with SCC. Case 1 was an early-stage carcinoma with tumor diameter of only 5 mm and surrounded by normal thyroid parenchyma. Histopathology showed a gradual shift from PTC to squamous metaplasia and further to SCC, providing proof of concept that SCC may arise primarily in the thyroid. A gradual tumor transformation from PTC to SCC is more likely than a collision tumor.

Case 2 in Paper IV was also a compound PTC/SCC tumor, although larger (38 mm in diameter). The tumor had a small PTC component in the center with a surrounding larger dominating SCC differentiated component showing signs of infiltrative growth into the surrounding stroma. This tumor also showed signs of tumor transdifferentiation rather than colliding tumors. A small subset of PTC cells was clearly different compared to the main bulk of PTC. They were negative to CK5/6 (positive in SCC) while CD10 was strongly expressed in SCC, weak expression in the small subset of PTC and absent in the main bulk of PTC. Other markers showing the same type of pattern are TTF-1 and Tg which both were strongly positive in the bulk of the PTC with gradual weakening to not being expressed at all in the SCC part. This indicates a transdifferentiation of cancer cells from PTC to SCC. Like the tumor in case

1, the tumor in case 2 was surrounded by a capsule, indicating one single tumor rather than two merging tumors (colliding tumors).

Case 3 was in the first histopathologic report classified as entirely SCC. Further work-up revealed signs of small islets of SCC cells expressing NKX2-1/TTF-1 indicating thyroid origin.

Indeed, case 3 resembled ATC regarding tumor onset, infiltrative growth of the recurrent laryngeal nerve, R2 resection, poor response to postoperative oncologic treatment and finally outcome (dead of disease 14 months after diagnosis). The conclusion in Paper IV that SCC may progress from PTC to SCC is concordant with the 2022 WHO classification stating that SCC is regarded as a subtype of ATC (2).

Out of eight patients, case 3 was the only tumor that was successfully xenotransplanted. A whole exome sequencing of PDX biopsies was performed. The mutations that were likely pathogenic included *CDKN2A*, *NFE2L2*, *PIK3CA* and TP53 which is commonly found in SCC of other origin than thyroid. The patient was treated with radio-chemotherapy with curative intent but post radiation radiology showed residual tumor at the target of radiation and metastases that were previously undetected. This lack of response may be explained by the overexpression of NRF2 (encoded by *NFE2L2*) in esophageal SCC which has been shown to promote resistance to radiation (156, 157).

The *NFE2L2* gene is a “druggable” target although there are no drugs in clinical use today (158). This approach was tested in the PDX model, with significant tumor response in mice treated with selective glutaminase inhibitor CB-893 (GLSi) and in mice treated with a combination of cabozantinib and PI3K-inhibitor GDC-0326, strongly suggesting that the *NFE2L2* is a driver mutation. These results encourage further testing of this drug in ATC tumors with SCC subclassification.

6 CONCLUSION

- A threefold increase of TC was observed in Western Sweden during the study period, 2001 – 2014. Primary tumor stages T1a-T3 contributed equally to this increase. Stage T4 tumors also increased although not statistically significant.
- TC related symptoms like a palpable tumor was the most common mode of detection in all three time periods investigated. In contrast to most other reports, imaging did not contribute to the increase of TC incidence.
- The proportion of FNA was reduced by 7% in patients undergoing US with selective FNA according to EU-TIRADS. This proportion could have been higher if strict criteria had been applied. It was estimated that 2% of the omitted nodules were TC which was considered acceptable. Thus, it was concluded that EU-TIRADS has a limited effect on reducing unnecessary FNA although it is safe to use.
- In a randomized controlled trial setting EU-TIRADS reduced the proportion of unnecessary FNA compared to control group (FNA without applying US RSS). As hypothesized, the proportion of FNA requiring surgery (Bethesda IV-VI), was significantly higher in the intervention group. There was no difference in rate of malignancy between control and intervention groups.
- PDX of advanced TC was difficult to establish, only one out of eight transplants was established. Paper IV supports that SCC should be regarded as a subtype of ATC. Molecular analysis revealed a druggable target (*NFE2L2*) which was confirmed by tumor response in treated mice (selective glutaminase inhibitor CB-893).

7 FUTURE PERSPECTIVES

Paper I did not provide a hypothesis for the underlying mechanisms of TC increase. The only commonly accepted environmental risk factor is radiation It was discussed that increased radiation as a cause of increased TC was possible but not likely although no data was provided in support. Iodine imbalance was also discussed but contributed most likely even less. Obesity was not discussed although it has been inflicted as a risk factor for developing TC (159). This was not assessed in Paper I since data was not possible to retrieve and this could be studied further considering Sweden has good quality registries that are validated and have good coverage.

The conclusions in Paper II and III were that EU-TIRADS has some effect but rather limited. As mentioned in 1.4.1.1 Ultrasound risk stratification systems, up to twenty different US RSS have been developed and most of them share features that influence the performance. Perhaps the great number of US RSS is a symptom of their shortcomings and there might be more to explore.

Progress has been made in the preoperative diagnosis of TC since the introduction and development of FNA, US and RSS. However, from this thesis it is obvious that obstacles remain to be overcome. Molecular analysis is an area of rapid development and improvements in preoperative diagnosis has the potential to reduce costs and provide correct treatment for patients.

Besides KEAP1 and NRF2 mutations, which are associated with SCC tumor development in other locations *e.g*. head and neck, other mutations identified in particular in Case 2 (STK11, RB1) are new for TC and the potential pathogenic role(s) in squamous differentiation of tumor cells yet unknown. One way to address this issue would be screen for mutations retrospectively in formalin-fixed paraffin-embedded-banked tissue and employing the 560 target panel recently implemented at SU in a larger series of SCC tumors obtained from thyroid and other locations where squamous epithelia are normally absent. Another approach would be to experimentally monitor the possibility of SCC transdifferentiation after knockdown target genes of interest, a challenge would be to find a suitable, preferably thyroid-derived cell line.

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