Medullary Thyroid Carcinoma in Norway

Clinical, molecular-biological and follow-up studies

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Research environment, collaborators and funding

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The research is part of the MTC-NOR project; *“Clinical and molecularbiological studies on outcome of patients with medullary thyroid cancer in Norway”*. The studies were accomplished in cooperation between Oslo University Hospital and Haukeland University Hospital, St Olavs University Hospital and the University Hospital of Northern Norway, based on signed interinstitutional agreements. Molecular biological analysis were performed at the Centre for Cancer Biomarkers, CCBIO, University of Bergen. The Cancer Registry of Norway provided names and treatment centers on all patients recorded with MTC during 1994-2016.

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Abbreviations

AKT	V-akt murine thymoma viral oncogene
APC	Annual percent change
ATA	American Thyroid Association
BRAF	V-raf murine sarcoma viral oncogene homolog B1
CDC14B	Cell division cycle 14 homolog B
CDM	Cancer driver mutations
CCH	C-cell hyperplasia
CEA	Carcinoembryonic antigen
CgA	Chromogranin A
CT	Computer tomography
DNA	Deoxyribonucleid acid
DSR	Desmoplastic stromal reaction
DSS	Disease specific survival
EDTA	Ethylene Diamine-Tetra-acetic Acid
ERK	Extracellular signal-regulated kinases
ETA	European Thyroid Association
FDG	2-[¹⁸ F-fluoro-2-deoxy-D-glucose
F-DOPA	¹⁸ F-dihydroxyphenyl-alanine
FFPE	Formalin fixed paraffin embedded
FMTC	Familiar medullary thyroid carcinoma
FNB	Fine needle biopsy
FTC	Follicular thyroid carcinoma
HD	Hirshsprung disease
HE	Hematoxylin eosin
HPT	Hyperparathyroidism
HUH	Haukeland University Hospital
IARC	International Agency for Research on Cancer
IGNM	Intestinal ganglionevromatosis
IHC	Immunohistochemistry

IONM	Intraoperative nerve monitoring
MAPK	Mitogen-activated protein kinase, also known as RAS/RAF/MEK
MEN	Multiple endocrine neoplasia
MRI	Magnet resonance imaging
MTC	Medullary thyroid carcinoma
MTC-NOR	The Norwegian Medullary Thyroid Carcinoma Project
mTOR	Rapamycin
NPNL	No disease-progression to next tumor level during follow-up
NPV	Negative predictive value
OS	Overall survival
OUH	Oslo University Hospital
PCC	Pheochromocytoma
PCR	Polymerase chain reaction
PET	Positron emission tomography
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PFS	Progression free survival
PPV	Positive predictive value
proGRP	Pro gastrin-releasing peptide
PTC	Papillary thyroid carcinoma
PTH	Parathyroid hormone
pTNM	Pathological Tumor Nodes Metastasis
RAS (H-, N-, K-)	RAt Sarcoma
REC	Regional Ethical Committee
RET	REarrange duringTtransfection
RLN	Recurrent laryngeal nerve
SEER	Surveillance, Epidemiology, and End Results Program
TKI	Tyrosine kinase inhibitor
TSH	Thyroid stimulated hormone
UICC	Union for International Cancer Control
WHO	World Health Organization

1. Thesis summary

“Research is not only to find new scientific truths, but to pave the way for further scientific questions.”
Annelin Eriksen, Vice Rector, University of Bergen

Medullary thyroid carcinoma (MTC) accounts for 1-10% of thyroid malignancy in major studies (1-4), occurs sporadically in 75% and hereditary in 25% of the patients. MTC develops from the parafollicular c-cells, developed from the neural crest (5, 6). The c-cells produce calcitonin, making the hormone a reliable tumor marker in pre-therapeutic staging and surgical strategy planning (7-9). Age at time of diagnosis and tumor stage are the most important prognostic factors regarding cure and survival in MTC (1, 10, 11).

During the last decades, preoperative diagnostics have improved due to better sensitivity as well as increased use of biochemical-, radiological- and cytological examinations, hence making MTC diagnosis possible at a lower tumor-stage (3, 11, 12). High-resolution ultrasound is important in disease staging, enabling the detection of even very small metastatic lymph nodes, although not micro-metastasis.

Surgery is the only means of potential cure for MTC, and should be performed at an early stage of the disease and to a sufficient extent (3, 4, 8, 13). Total thyroidectomy and dissection of lymph node compartments in the central neck are standard treatment, as is therapeutic dissection in the lateral neck compartments at clinical disease (3, 14). Time related trends in diagnostics and surgical treatment in Norway during 1994-2016, as well as the importance of sufficient dissection of the lymph node compartments are shown in **paper II**. Furthermore, studies have analyzed diagnostic predictors for the necessity of prophylactic lateral neck dissection, considering tumor features at ultrasound (15), calcitonin level (8, 9) or metastatic lymph node load in the central neck compartments (14, 16). Preoperative basal serum calcitonin level as a predictor for the necessity of prophylactic dissection in the lateral neck compartments were evaluated and the results are presented in **paper III**.

Hereditary MTC is part of multiple endocrine neoplasia type 2A (MEN2A) (90-95%) and 2B (MEN2B) (5-10%), autosomal dominant syndromes caused by germline mutation in the *RET* proto-oncogene (1, 4, 17-20) and with a life time risk of more than 95% for MTC penetrance.

In MEN2A, the age-dependent penetrance of MTC differs according to the genotype (21-26). Indicators predicting optimal timing and extent of prophylactic thyroid surgery have been studied (27, 28), in which treatment recommendations are based on (3, 21, 29). **Paper I** presents evaluation and results for the Norwegian MEN2A patients in the period from 1974 to 2015 according to optimal timing and extent of thyroid surgery related to preoperative basal serum calcitonin level.

Patients with MEN2B predominantly have p.M918T mutation (> 90%), and 75% are *de novo* mutation (3, 19, 30). MTC might develop already during the first year of life (23). The cure rate drops dramatically in patients treated later than 4th year of life (31, 32), mostly related to advanced disease stage at diagnosis. Somatic cancer driver mutations (CDMs), are well studied in sporadic MTC (33-45), but only few MEN2B patients have been included in the studies (40-42). Mutation analysis of CDMs in the Norwegian MEN2B patients were performed, and the results are presented and discussed in **paper IV**.

The regionalization of treatment of MTC in Norway, as well as mandatory reporting since 1953 to the Cancer Registry of Norway of all information concerning cancer diagnostics and treatment, allow for population-based cohort studies. The four papers on which the dissertation is based, including all patients diagnosed with sporadic and hereditary MTC, are the first nationwide population-based studies concerning MTC in Norway during the last decades. These multicenter population-based epidemiological studies cover a lack of knowledge in Norway in recent times by giving scientific updates in diagnostics, treatment and outcome in Norwegian patients with MTC, and to an extent that results can be generalized.

2. List of papers

- I:** **Opsahl EM**, Brauckhoff M, Schlichting E, Helset K, Svartberg J, Brauckhoff K, Maehle L, Engebretsen LF, Sigstad E, Grøholt KK, Akslen LA, Jorgensen LH, Varhaug JE, Bjoro T: A Nationwide Study of Multiple Endocrine Neoplasia Type 2A in Norway: Predictive and Prognostic Factors for the Clinical Course of Medullary Thyroid Carcinoma. *Thyroid* 2016;26:1225-1238. doi: 10.1089/thy.2015.0673. PMID 27400880
- II:** **Opsahl EM**, Akslen LA, Schlichting E, Aas T, Brauckhoff K, Hagen AI, Rosenlund AF, Sigstad E, Grøholt KK, Mæhle L, Engebretsen LF, Jørgensen LH, Varhaug JE, Bjørø T: Trends in Diagnostics, Surgical Treatment and Prognostic Factors for Outcome in Medullary Thyroid Carcinoma in Norway. A Nationwide Population-based Study. *European Thyroid Journal*, 2019;8(1):31-40. doi: 10.1159/000493977. PMID 30800639
- III:** **Opsahl EM**, Akslen LA, Schlichting E, Aas T, Brauckhoff K, Hagen AI, Rosenlund AF, Sigstad E, Grøholt KK, Jørgensen LH, Bjørø T: The Role of Calcitonin in Predicting Extent of Surgery in Medullary Thyroid Carcinoma. A Nationwide Population-based Study. *European Thyroid Journal*, April 29, 2019, doi: 10.1159/000499018.
[Epub ahead of print]
- IV:** **Opsahl EM**, Vintermyr O, Kalvenes MB, Schlichting E, Brauckhoff K, Sigstad E, Grøholt KK, Engebretsen LF, Jørgensen LH, Bjørø T, Akslen LA: Mutational Screening of *HRAS*, *KRAS*, *NRAS*, *PIK3CA* and *AKT1* in Medullary Thyroid Carcinoma in Multiple Endocrine Neoplasia Type 2B. *European Thyroid Journal*, 2019 Submitted paper for review, May 1, 2019.

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3. Introduction

3.1 Historical aspects

Already in 1844, Hawkins (46) described cases of carcinoma of the thyroid gland. In a review from 1940, Lahey et al. (47) described a patient diagnosed with papillary carcinoma in the thyroid in 1935. Meissner and McManus (48) studied the histological growth patterns of benign and malignant thyroid tumors, and classified the tumors as either papillary or follicular tumors. They also discovered that papillary carcinomas are far more frequent than follicular carcinomas. An earlier opinion was that malignant thyroid tumors developed from benign thyroid tumors. However, Meier et al. (49) reported in a study from 1959 that most of the thyroid cancers occurred in normal thyroid glands.

The entity of MTC was first described by Hazard et al. in 1959 (50) with the presence of amyloid in the stroma, high incidence of metastatic lymph nodes and an intermediate grade of malignancy. The parafollicular c-cells was first described by Williams et al. in 1966 (51). Combination of MTC and pheochromocytoma (PCC) in patients was first described by Stanbury in 1960 (52), followed by other reports (53, 54). Finally, in 1968 Steiner et al. (55) described a kindred with PCC, MTC, hyperparathyroidism (HPT) and Cushing's disease as multiple endocrine neoplasia 2. However, prior to this, in 1965, Shimke et al. (54) described two unrelated families with amyloid producing MTC and PCC in pattern consistent with autosomal dominant inheritance, as a genetic entity. In 1978 Carney et al. (56) described MEN2B as a disorder with unknown etiology with major involvement of the thyroid, the adrenal glands, the autonomic nervous system and connective tissue.

The *RET* proto-oncogene was discovered by Takahashi and associates in 1985 (57). The knowledge about the relation between the clinically described familiar multiple endocrine neoplasia and the germline mutation in the *RET* proto-oncogene, was first discovered in 1993 and described by Donis-Keller et al. and Mulligan et al. (17, 18). Genetic testing soon became a standardized part of the investigation of possible hereditary MTC, and family members with the gene- mutation were offered prophylactic thyroidectomy (3, 26, 29, 58).

Calcitonin was first demonstrated in 1962 by Copp et al. (59), and Foster et al. (60) described in 1964 that calcitonin was secreted from the para-follicular cells. The studies are referred to in the thesis by Normann in 1977 (61). Before genetic testing was available, stimulated calcitonin secretion of the relatives of the patients with MTC were used to differ between hereditary and sporadic MTC. Calcitonin stimulation tests with calcium and pentagastrine were first described by Melvin et al. in 1971 (62) and by Telenius-Berg in 1975 (63), respectively.

The thesis by Normann (61) presented the first population based studies concerning MTC in Norway (64-69), including morphological, clinical and experimental studies in patients with MTC during 1960-1974. Normann and Gautvik (65) screened 300 relatives of 43 Norwegian MTC-patients, by measuring serum calcitonin, a countrywide investigation in 1977. In 1994, Høie et al. (70) presented the experience with hereditary MTC at the Norwegian Radium Hospital, and in 2000 they presented the first results after prophylactic thyroidectomy for *RET* proto-oncogene mutation carriers (71).

On this background, it is fair to claim that the research performed in the present thesis stands on the shoulder of a steadily evolving tradition in the field of thyroid carcinoma research.

3.2 Epidemiology

The incidence of MTC in 1970-1985 in Norway was reported by Akslen et al. in 1990 to be 0.15:100,000 person years (2). Gatta and the *EUROCare* group reported in 2006 a world standardized incidence of 0.13 and a calculated incidence between 0.11 and 0.21 per 100,000 person years (72). In MTC related to the multi-endocrine syndrome MEN2, Machens et al. (58) calculated the minimum incidence of *RET* gene mutation (both MEN2A and MEN2B) in Germany raising from 1:200,000 to 1:100,000 live births per year in the period 1951-1960 and 1991-2000 respectively. Based on the 1991-2000 incidence, the prevalence was found to be approximately 1:80,000. The incidence of MEN2B isolated was 1:700,000.

The incidence of thyroid carcinoma, and especially papillary thyroid carcinoma (PTC), has increased during the last decades. In the US, the average annual percent change (APC) of

PTC was reported as 4.4% and 6.26% in the periods 1974-2013 and 1993-2012, respectively (73-75). There has also been increased incidence of follicular thyroid carcinoma (FTC), with APC 0.6 and 1.57%, and of MTC, with APC 0.7 and 1.87%, in the two periods referred to in the US (73-75). Whether the rise in incidence is due to more widespread use of diagnostics as ultrasound with FNB and other imaging as computer tomography (CT), magnet resonance imaging (MRI) and positron emission tomography (PET), leading to discovering more indolent papillary micro carcinomas, or if the raise in incidence is a real incidence raise are discussed (73, 76-80). Results from epidemiological studies suggest that a substantial proportion of PTC diagnosis (>40% in the US) could be attributable to environmental factors, such as radiation, obesity and cigarette smoking (73, 81-84).

The overall survival (OS) of MTC patients is inferior to patients with follicular cell derived thyroid carcinoma. Different studies have reported 5- and 10-year disease specific survival (DSS) of 76-99% and 70-87%, respectively (1, 10-12, 72, 85). In the study by Lim et al. (74), the annual incidence-based mortality rate in MTC has dropped during 1994-2013 in US (APC -0.7 % [95% CI, -3.2% to 1.9%]), whereas there was an annual increase in incidence-based mortality rates in PTC in the same period (APC 1.7% [95% CI, 0.6% to 2.9%]).

3.3 Germline mutations and hereditary MTC

Hereditary MTC, MEN2A and MEN2B, are autosomal dominant syndromes caused by germline mutation in the *RET* proto-oncogene (1, 4, 17-20). The earlier described familial medullary thyroid carcinoma (FMTC) is considered as a variant of the MEN2A disease spectrum rather than a freestanding syndrome (3).

The *RET* gene encodes the tyrosine kinase domain in a fused protein receptor, at the cell membrane with an extracellular and intracellular domain (86). The intracellular region contains two tyrosine kinase domains (TK1 and TK2). Upon ligand binding, the extracellular cysteine-rich domain facilitates receptor dimerization, leading to autophosphorylation and activation of the intracellular TK1 and TK2. This activates the downstream signal transducer pathways MAPK/ERK and PIK3CA/AKT/mTOR, regulating multiple cellular processes including proliferation, cell survival, differentiation and migration (86-88) (Figure 1).

The *RET* gene mutations in hereditary MTC lead to gain of function with increased downstream signaling and dysregulation of multiple cellular processes (86, 89). However, in patients with Hirschsprung disease (HD) associated with *RET* mutation, there is a loss of function effect of the *RET* gene mutation causing HD (90). *RET* gene mutation is identified in 50% of patients with hereditary HD and in 15-20% of patients with sporadic HD (91). The *RET* gene also harbors normal genetic variants, common polymorphisms, which do not cause hereditary MTC (88).

Figure 1: The *RET* gene encodes the tyrosine kinase domain in a fusion protein, receptor, at the cell membrane, activating the downstream signal transducer pathways regulating multiple cellular processes, with the possibilities of targeted therapies.

Moura MM, Cavaco BM, Leite V. *RAS* proto-oncogene in medullary thyroid carcinoma. *Endocr Relat Cancer*. 2015;22(5):R235-52.

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RET comprises 20 exons located on chromosome 10 (10q11.2), and more than 100 pathogenic mutations in different codons have been identified in this gene (3, 17, 18, 55, 92, 93). The Utah University has by February 2019 registered 197 genetic variants in the *RET* gene (94).

Since 25% of patients with MTC have hereditary MTC, genetic mutation analyses of the *RET* proto-oncogene have become standard of care and should be offered to all patients with

diagnosed MTC (3, 89). *RET* mutation analysis are performed in blood cells and detect 92-98% of all mutation carriers (26, 29, 58, 92). In new families with hereditary MTC, and according to the revised American Thyroid Association (ATA) guidelines 2015 (3), the most common mutated *RET* cysteine codons in exon 10 (codon 609, 611, 618 and 620), exon 11 (codon 630 and 634) and *RET* codon mutations in exon 13, 14, 15 and 16, were previously first sequenced. Examination of exon 8 was also recommended. However, rare *RET* double or multiple mutations might be missed if the whole *RET* gene is not sequenced. In Norway today, all the exons are analyzed by whole genome sequencing using next generation sequencing technology.

If the *RET* mutation is found in the patient with MTC, the patient, which is the index patient, should be referred to medical genetics for counselling and control mutation analysis should be performed. According to ATA guidelines 2015 (3), the first-degree relatives of patients with hereditary MTC, MEN2A and MEN2B, and the parents of infants or young children with the classic MEN2B phenotype should be offered the *RET* mutation analysis. Furthermore, patients with cutaneous lichen amyloidosis (CLA), infants or young children with HD and exon 10 *RET* germline mutations, as well as adults with MEN2A and exon 10 mutations with symptoms related to HD, should be offered the analysis. Relatives with *RET* gene mutation (screening patients) are referred to a specialist center for diagnostics, prophylactic thyroidectomy or regular follow-up until optimal time for prophylactic thyroidectomy, according to current recommendations (3, 29). These recommendations are followed in Norway. Additionally, the first-degree relatives to each screening patient with *RET* gene mutation, are again offered the analysis for each family.

In MEN2A, MTC has a risk of penetrance of more than 95%, and can be accompanied with a 15-50% risk of bilateral PCC (21, 22, 55, 95, 96) and HPT at 5-30% risk (21, 22, 55, 95, 97). The age-dependent penetrance of MTC differs between the mutations, but MTC is generally the first manifestation. It becomes clinically apparent when patients are between 5 and 25 years old, or even later (21-26). Predictors for biochemical cure and survival of MTC are competently well described in many large studies that consider genotype, preoperative calcitonin level, age at thyroid surgery and tumor stage at diagnosis, including lymph node metastasis (1, 3, 4, 21, 22, 27, 98-102). Since MTC occurs in 95% of MEN2A patients and mutations in different codons

result in varying penetrance and disease manifestations, major studies have been carried out to identify indicators predicting the optimal timing and extent of prophylactic thyroid surgery (28, 103). Guidelines and treatment recommendations issued by the ATA 2009/2015 (3, 21) and the European Society of Endocrine Surgeons 2014 (29) were based on these and other major studies. A summary of ATA 2009/2015 is given in Table 1.

Table 1: Risk classification in MEN 2A according to American Thyroid Association Guidelines from 2009 (21) and 2015 (3) including recommended management and age at thyroid surgery in children and adults with confirmed *RET* germline mutation.

ATA risk class 2009	Codon mutated ¹	Recommended age at thyroid surgery and follow-up ²	ATA risk class 2015	Codon mutated ¹	Recommended age at thyroid surgery and follow-up ²	
					Child	Adult
A	531	May delay surgery beyond age 5 years if stringent criteria are met ³	MOD (moderate)	531	Thyroidectomy to be performed when the serum calcitonin level becomes elevated or in childhood if the parents do not wish to embark on a lengthy period of evaluation which might last for years or decades.	<u>At normal serum calcitonin level:</u> Annual testing of calcitonin. <u>If calcitonin becomes elevated:</u> Thyroidectomy and dissection of lymph node compartments depending on ultrasound findings and preoperative calcitonin levels
	533					
	635					
	649					
	768					
	790					
	791					
	804					
	891					
	912					
B	609	Consider surgery before age 5. May delay surgery beyond age 5 years if stringent criteria are met ³		768		
	611			790		
	618			791		
	620			804		
	630			891		
	633			912		
C	634	Before age 5 years	H (high)	634	Thyroidectomy at or before 5 years of age based on serum calcitonin levels	

¹Mutations based on limited families are not included in the table (3), ²Screening for PCC is recommended from 11-16 years of age independent of calcitonin level. PCC must always be excluded before surgery. ³A normal annual basal +/- stimulated serum calcitonin, normal neck ultrasound, less aggressive MTC family history, and family preference. PCC: Pheochromocytoma

MEN2B is caused by activating germline mutation in high-risk codons of the *RET*-proto oncogene, predominantly codon 918 in > 90% (3, 19, 30). Approximately 75% of the patients

have *de novo* mutation (3). Mutations in codon 883 (p.A883F) or present as tandem mutations including codon 804 (p.V804M/p.E805K, p.V804M/p.Y806C, or p.V804M/p.S904C) are significantly less frequent (104-107). Different mechanisms of *RET* activation induced by the mutations, trigger different patterns of phosphorylation and cell signaling, and the p.M918T codon mutation in MEN2B is associated with increased PIK3CA/AKT pathway activation, compared with MEN2A-associated mutations (88, 108).

The phenotype in MEN2B includes the combination of early onset medullary thyroid carcinoma (MTC), bilateral PCC, and clinical abnormalities such as orofacial and conjunctive neuromas (Figure 2), thickened corneal nerves, musculoskeletal abnormalities called marfanoid habitus, intermittent constipation and diarrhea due to intestinal ganglioneuromatosis (IGNM) and tearless crying (56, 95, 109, 110).

Figure 2:
a) Oral nevrioma
b) Conjunctival nevrioma

*Informed
written consent
is available*

As MTC might develop already during the first year of life in MEN2B patients (23), and the cure rate dramatically drops in patients treated later than 4 years of age (31, 32), thyroidectomy is recommended as early as possible and during the first year of life (3). Most often, the MEN2B patients have advanced disease stage at diagnosis with corresponding higher mortality rate (4, 21). However, earlier diagnosis has improved during the last years (32).

3.4 Pathology

MTC derives from the parafollicular C-cells, are neuroectodermal in origin and belong to the amine precursor uptake decarboxylation cell family developed from the neural crest (5, 6).

The c-cells which accounts for 1% of the thyroid cells, are located at the posterior upper third of the gland, and secrete the hormone calcitonin involved in the calcium hemostasis (111-113).

The MTC tumor is firm, rounded, grey-white and often gritty, laying within the thyroid lobe and sharply demarcated from the normal thyroid tissue (5, 50). The tumor is made up of sheets and nests of eosinophilic granular cells separated by fibrous stroma with irregular masses of amyloid in 80% of the tumors (51, 88). Microscopic features associated with reduced survival are tumor necrosis, presence of oxyphilic cells in the tumor, a squamous pattern and absence of cells with intermediate cytoplasm; i.e lack of the intermediate protein filaments giving structural framework in the cell-cytoplasm surrounding the cell nuclei, visible as pale cytoplasm in the microscope (114). Less than 50% of calcitonin immunoreactive cells in the tumor as well as increased Ki67 index in combination with high age, predict poorer survival (114, 115).

FNB is important in diagnostics, but cytological diagnosis might be difficult (116). The sensitivity in detecting MTC, by evaluating the morphological picture, is poor and reported to be 45-63% in different studies (7, 117). By adding immunocytochemistry (IHC), including calcitonin on slides processed by thin layer cytology, the sensitivity in diagnosing MTC highly improves (116, 118). Furthermore, FNB with IHC on cell blocks gives the MTC diagnosis with more than 95% sensitivity (119). A positive stain of calcitonin in IHC is diagnostic for MTC in thyroid tumors, as well as a positive stain for carcinoembryonic antigen (CEA), as they are negative in other thyroid tumors. However, calcitonin is not specific for MTC and can be positive in neuroendocrine carcinomas from another organ system (88).

C-cell hyperplasia (CCH) is a precursor lesion in MTC characterized by nodular aggregates of enlarged C-cells with pale pink to clear cytoplasm and granular chromatin, often encircling the thyroid follicles and confined by basement membrane (88). Neoplastic C-cell proliferation similar to CCH (“neoplastic CCH”) is commonly seen in hereditary MTC and occasionally in sporadic MTC (88, 120). When CCH is present on hematoxylin-eosin (HE)-stained sections, the patient should be offered genetic testing (3, 88). If CCH is not visible on routine HE sections, it should be considered reactive rather than neoplastic, although the distinction between reactive and neoplastic CCH may be difficult. CCH is not associated with

patient survival and disease progression (120). Histological specimens with HE stain and immunohistochemistry with calcitonin stain in CCH and MTC are shown in Figure 3.

Figure 3: C-cell hyperplasia (a and c) and micro MTC (b and d) Hematoxylin-eosin (a and b) and calcitonin stain (c and d) in a patient with MEN2A. *Informed written consent is available*

Histological diagnosis of MTC are implemented in line with applicable WHO/IARC tumor classifications (121). The tumor-nodal-metastatic (TNM) stage classification have changed over time with some changes for Endocrine tumors in the WHO 2010 version, the 7th revision (UICC 2010) (122), as well as in the 8th revision (UICC 2017) (123). The differences between the 7th and 8th TNM classification for MTC are shown in Table 2. The pT3 in the 7th edition is divided into pT3a and pT3b in the 8th edition, as pT3b is a tumor of any sizes with gross extrathyroidal extension. However, the tumor stage classification has not changed between the editions.

Table 2: TNM classification in MTC (UICC TNM 7th and 8th edition) (122,123)

TNM	7 th edition	TNM	8 th edition
pTX	Primary tumor not assessed	pTX	Primary tumor not assessed
pT0	No evidence of primary tumor	pT0	No evidence of primary tumor
pT1a	≤ 10 mm Limited to the thyroid	pT1a	≤ 10 mm Limited to the thyroid
pT1b	> 10 mm and ≤20mm Limited to the thyroid	pT1b	> 10 mm and ≤20mm Limited to the thyroid
pT2	> 20 mm and ≤ 40 mm Limited to the thyroid	pT2	> 20 mm and ≤ 40 mm Limited to the thyroid
pT3	Tumor > 40 mm Limited to the thyroid or any tumor with minimal extrathyroidal extension (to sternothyroid muscle or perithyroid soft tissue)	pT3a	Tumor >40 mm Limited to the thyroid
pT4a	Tumor extends beyond the thyroid capsule and invades: subcutaneous soft tissue, larynx, trachea, oesophagus, recurrent laryngeal nerve	pT3b	Tumor of any sizes with gross extrathyroidal extension invading strap muscles (sternohyoid, sternothyroid or omohyoid)
pT4b	Tumor invades prevertebral fascia, mediastinal vessels, or encases carotid artery	pT4a	Tumor invades beyond thyroid capsule and invades any of: subcutaneous soft tissue, larynx, trachea, oesophagus or recurrent laryngeal nerve
pNX	Cannot assess regional lymph nodes	pT4b	Tumor invades prevertebral fascia, mediastinal vessels, or encases carotid artery
pN0	No regional lymph node metastasis	pNX	Cannot assess regional lymph nodes
pN1a	Regional lymph node metastasis in Level VI (pretracheal, paratracheal and prelaryngeal (Delphian) lymph nodes	pN0	No regional lymph node involved
pN1b	Metastasis in other unilateral, bilateral or contralateral cervical (Level I, II, III, IV or V), or retropharyngeal or superior mediastinal lymph nodes	pN1a	Regional lymph node metastasis in Level VI (pretracheal, paratracheal and prelaryngeal (Delphian) lymph nodes
M0	No distant metastasis	pN1b	Metastasis in other unilateral, bilateral or contralateral cervical (Level I, II, III, IV or V), or retropharyngeal or superior mediastinal lymph nodes
M1	Distant metastasis	M0	No distant metastasis
		M1	Distant metastasis

Tumor stage	T	N	M
I	T1a, T1b	N0	M0
II	T2-3	N0	M0
III	T1-3	N1a	M0
IVa	T1-3	N1b	M0
	T4a	Any N	M0
IVb	T4b	Any N	M0
IVc	Any T	Any N	M1

MTC metastasizes to lymph nodes in the neck and mediastinum, and systemically to the liver, lung and bone tissue. In addition to age at time of diagnosis, the tumor stage has been the most important prognostic factor regarding cure and survival (1, 10, 85, 124, 125). However, micro-MTC, tumor diameter ≤ 10mm, does not always mean lower tumor stage, and smaller

tumors have significant rates of poor prognostic features with negative impact on patients survival (126).

Extrathyroidal tumor extension is a negative prognostic factor for survival in patients with MTC (10, 85, 127). Furthermore, in patients with metastatic lymph nodes in the lateral, especially in the contralateral neck, and with high number of metastatic lymph nodes, cure is hard to achieve and there is higher risk to develop distant metastasis (128-130).

Perinodal tumor extension and desmoplastic stromal reaction (desmoplasia) (DSR) is not yet part of routine examination and is not included in the TNM classification. However, an association between perinodal tumor extension in MTC and numbers of metastatic lymph nodes has been found. Machens et al. (124) have established that perinodal extension was determined by the numbers of metastatic lymph nodes, and that it developed independently from extrathyroidal tumor extension. DSR is the presence of a newly formed fibrotic (collagenous) stroma surrounding the invasive epithelial tumor cells not found in the non-neoplastic thyroid parenchyma (131, 132). Associations between DSR and metastatic lymph nodes have been found (133), as some MTCs do not develop a DSR and consequently do not have metastatic lymph nodes (132, 134).

3.5 Somatic mutations and tumor biology

Somatic cancer driver mutations (CDMs) in sporadic MTC have been widely investigated, and more than 3000 samples tested for somatic mutations in MTC are available according to Catalogue of Somatic Mutations In Cancer (COSMIC) (135). In sporadic MTC, there is no germline mutation, but approximately 50% of sporadic MTCs have somatic *RET* mutations (3, 19, 93, 136, 137). Furthermore, the somatic *RET* codon p.M918T mutation in sporadic MTC has a more aggressive clinical course and a less favorable prognosis, as first described by Zedenius et al. in 1994-95 (33, 34). This is in accordance with other studies (35, 36, 38). The *RET* p.M918T mutation is seen less frequently in micro-MTC (138). The *RET* mutation may also show mutational heterogeneity, as *RET* mutations may be found in subpopulations of tumor cells rather than in the entire tumor, indicating that mutations arose later in the course of the disease (138, 139). Somatic *RET* mutations in other exons are also associated with less favorable prognosis

(36, 140). Moura et al. (38) found that sporadic MTC patients with *RET* mutations in exons 15 and 16 were associated with having the most severe prognosis. In addition, they found that patients with other *RET* mutations had the most indolent course and those with no *RET* mutations had an intermediate risk.

In follicular derived thyroid carcinoma, somatic mutations in cancer driver genes in the MAPK/ERK- and PI3K/AKT/mTOR signaling pathways have been known for many years, as *BRAF* in PTC and anaplastic thyroid carcinoma (141, 142), *RAS* mutations in FTC (143) and *PIK3CA*, activating the PIK3CA/AKT pathway, in PTC, FTC or even more in anaplastic thyroid carcinoma (144, 145). Mutation in *AKT1* has been found in metastatic low differentiated thyroid carcinoma (146). In recent studies the role of other CDMs in sporadic MTC have been evaluated. Mutations in *BRAF*, *PIK3CA* and *AKT1* have regularly not been found (40-42), although *BRAF* mutation was found in sporadic MTC in a study by Goutas N et al. (43) in 30 out of 44 (68.2%) MTC samples tested. *RAS* mutations, *HRAS*, *KRAS* or rarely *NRAS* have been found in sporadic MTCs to a varying degree, in 18%-80%, and in the sporadic MTCs without somatic *RET* mutations (3, 37, 39, 41, 88).

It seems that somatic *RET*- and *RAS* mutations are mutually exclusive, as *RET* mutations are found in more aggressive MTC, whereas *RAS* mutations are found in less aggressive MTC (36, 37, 39, 44). The *RAS* signaling pathway is also activated by the *RET* tyrosine kinase receptor (Figure 1), indicating that activation of this pathway, either by *RET* or *RAS* mutation, is important in both hereditary and sporadic MTC tumors (88). *RAS* mutations are present in approximately 10 - 45% of sporadic MTC (37, 39, 41, 44). Still, there is a group of *RET*- and *RAS* mutation-negative MTC tumors where the main genetic changes are not yet known.

3.6 Biochemistry

The parafollicular c-cells secrete the hormone calcitonin involved in the calcium hemostasis (111, 112). Calcitonin is a 32-amino acid monomeric peptide derived from procalcitonin, a precursor peptide derived from preprocalcitonin (3, 147). The half-life of calcitonin in serum is short, 3-30 hours (148). Calcitonin is located in the dense cored secretory granules and secreted from the secretory vesicles (149). Due to diurnal variations in calcitonin

excretion from the cells, the calcitonin value fluctuates without disease progression (150-152). Serum calcitonin, basal or stimulated by pentagastrin or calcium, is the best tumor marker in diagnostics and follow-up of MTC (7, 8, 130, 153). The level of calcitonin in wash out aspirate from FNB can also be analyzed. It is a useful tool for diagnosing MTC, with even higher sensitivity than cytological examination (116, 117, 154).

The calcitonin assays have changed over time with varying sensitivity and specificity. The reference values of serum calcitonin have also varied over time and are related to age, sex, cigarette smoking and food intake (155, 156). In children, the reference values are higher (157). The most recent generation of immunometric assays are highly sensitive and specific for monomeric calcitonin, and cross-reactivity with procalcitonin or other calcitonin-related peptides is less likely (3, 158). This is of importance since sepsis or other general inflammation may cause prolonged elevation of procalcitonin in the tissue (159). A “hook effect” may give false low calcitonin values, normal or slightly elevated, in patients with advanced MTC. Very high serum calcitonin may saturate the binding capacity of the antibodies and cause detection of falsely low serum calcitonin (3, 158, 160). False negative calcitonin in patients with MTC may also be related to tumor cells de-differentiation (158).

Stimulation of calcitonin secretion performed by administration of calcium or pentagastrin may increase sensitivity of diagnosis and evaluation of MTC. However, the tests were more indicative earlier when the calcitonin assays had lower sensitivity (161). Today, the immunometric assays with high sensitivity and specificity make provocation tests less necessary as they offer limited new extra information (3, 150). Stimulation by either calcium or pentagastrin has side effects of varying degrees, such as warmth feeling, nausea, altered gustatory sensation, dizziness, urgency to micturate and abdominal cramping (162).

Serum calcitonin may increase in patients with other disease than MTC, as chronic renal failure, HPT, small cell lung cancer, prostate cancer, mastocytosis and various enteric and pulmonary neuroendocrine tumors (3, 163-169). False positive calcitonin elevation has also been found in other thyroid conditions as autoimmune thyroiditis and thyroid tumors (3, 170, 171), as well as in patients using proton pump inhibitors (169, 170).

As the sensitivity of serum calcitonin in diagnosing MTC is better than ultrasound and FNB with cytological examination, assessments concerning calcitonin screening at the time of diagnostic evaluation of thyroid nodules have been made (7, 172-174). The benefits of diagnosing MTC at lower tumor stage are more successful treatment, it influences surgical strategy (7, 8), and is cost-effective (173). The disadvantages of calcitonin screening are the possibility of false positive elevated calcitonin levels, which can lead to unnecessary thyroidectomies. There are no prospective randomized trials studying the efficacy of serum calcitonin screening in evaluation of patients with nodular goiter, and no strict recommendations exist (3). However, some guidelines recommend calcitonin measurement in patients with thyroid nodules undergoing surgical treatment (150, 175).

The doubling times of calcitonin in follow-up have prognostic impact. It is a practical tool in the evaluation of MTC disease progression. A raised calcitonin doubling time is a negative prognostic factor for recurrence and death (176).

Other secretory biomarkers as CEA, chromogranin A (CgA) and pro gastrin-releasing peptide (proGRP) increase at MTC and might be useful tumor markers, although CgA first increase in aggressive MTC (176-180). CEA is an integral part of the cell membrane of MTC tumor cells, from where it can be released and serve as a tumor marker (149, 181). A postoperative reduction of calcitonin serum levels more than 97% seems to be associated with a less aggressive clinical course, while CEA serum level has lower predictive value (181). The doubling time of CEA serum level has a higher predictive value than that of calcitonin. Hence, measuring both tumor markers is essential for proper risk stratification (176). Furthermore, the neuroendocrine secretory granules contain CgA and the circulating levels of CgA is more related to secretory activity than to the tumor load (158). Increased serum CgA is observed in no more than 50% of MTC patients, mainly those with more advanced and aggressive disease. Hence, CgA is not an optimal tumor marker in MTC (158). The gastrin releasing peptide (GRP) gene encodes proGRP molecules, and is present in MTC tissue (182). In contrast to serum calcitonin, which has to be analyzed immediately or transported frozen to the laboratory, proGRP is stable in blood where it can be measured (183, 184). The ProGRP serum level may also be useful, in

addition to calcitonin, in diagnosing and follow-up of MTC, and can be analyzed in FNB wash out (179, 180).

3.7 Radiology and nuclear medicine

Ultrasound of the neck is the most important preoperative imaging procedure in patients with thyroid carcinoma. Today, high-resolution ultrasound is highly sensitive and therefore valuable in diagnosing the disease, as well as in the disease staging. It is the most important examination for detecting metastatic lymph nodes in the lateral neck compartments (185-187). Kocharyan et al. (187) have reported a positive predictive value (PPV) of 85.4%. However, ultrasound cannot rule out presence of micro-metastasis in the lymph nodes. Further, patients with larger tumors of irregular shape with spiculated margins and a subcapsular location at ultrasound, are more likely to have metastatic lymph nodes in the lateral neck compartments (15).

At extensive neck disease, CT and MRI of the neck are indicated (3). These examinations are more informative than ultrasound concerning relations between tumor tissue and anatomical structures in the neck. To detect lung- and mediastinal metastasis, CT is the most sensitive imaging, three-phase contrast-enhanced multi-detector liver CT and MRI have the highest sensitive for detection of liver metastasis, axial MRI and bone scintigraphy are complementary and most sensitive when it comes to detecting bone metastasis (3, 188). In a study by Giraudet et al. (188), 2-[¹⁸F-fluoro-2-deoxy-D-glucose (FDG) PET/CT (FDG-PET/CT) was found to be less sensitive in diagnosing metastatic disease. However, other studies have shown that FDG- PET/CT and ¹⁸F-dihydroxyphenyl-alanine (F-DOPA)-PET/CT is superior or equal to conventional imaging in detecting metastatic MTC (189, 190).

In patients with suspected MTC recurrence, FDG-PET/CT was by Szakall et al. (191) found to be more sensitive in localizing metastatic lymph node involvement in the neck and mediastinum than MRI and conventional CT. FDG-PET/CT examination identifies patients with progressive disease with more accuracy, whereas F-DOPA-PET/CT detects MTC tumor load with a higher sensitivity (3, 192-194). Bogsrud et al. (195) found that the result of FDG-PET had high prognostic value in patients with suspected residual or recurrent MTC with worse patient's outcome at PET- positive examinations. In addition, calcitonin serum level doubling time was

also shorter for PET-positive compared to PET-negative patients (195). A recent review by Giraudet et al. (196) concluded that PET/CT using FDOPA is the most sensitive radiopharmaceutical in localizing persistent or recurrent MTC. The sensitivity depends on calcitonin levels, with an approximately cut-off at 43 pmol/L (150 pg/mL). In patients with MTC and short calcitonin- or CEA doubling time, FDG PET/CT might be indicated.

3.8 Preoperative diagnostics

The guidelines from the European Thyroid Association (ETA) (175, 197) and the ATA (3), as well as the Norwegian Guidelines (150), are important tools in managing thyroid nodules and MTC. A flow chart of routine diagnostics is presented in Figure 4.

In patients with nodular thyroid disease, anamnestic growth pattern, pain, voice change and family history should be ruled out, clinical examination of the tumor and lymph nodes performed and the vocal cord function examined. Furthermore, biochemical examinations of thyroid stimulated hormone (TSH), thyroid hormones, calcium, parathyroid hormone (PTH), and calcitonin (preferred) should be performed.

Neck ultrasound evaluating the thyroid gland and lymph nodes in the central and lateral neck, including FNB from tumor and pathological lymph nodes if present, are mandatory. However, at tumor diameter < 1 cm with benign appearance in patients without risk of thyroid malignancy, FNB should be avoided unless clinical suspicious metastatic lymph nodes are present at ultrasound examination (3, 14, 150, 175, 197, 198). For better sensitivity, the presence of a cytopathologist or cytotechnician to evaluate the sample at the FNB, is preferable (199). To avoid false-negative MTC by cytology, calcitonin measurement in aspiration needle washout add sensitivity to the FNB diagnostic (117). ProGRP in wash out is a good alternative to calcitonin. At elevated calcitonin and negative cytological examination, cytological cell-block with immunohistochemistry improves the diagnostics (119). Core-needle biopsy, if possible, is an alternative. Excisional biopsy should be avoided.

In patients diagnosed with MTC, it is important to rule out hereditary MTC by *RET* mutation analysis. PCC has to be excluded before thyroid surgery by measurement of urine

catecholamine's or serum metanefrine and normetanefrine. If present, PCC has to be surgically treated prior to thyroid surgery.

Figure 4. Flow chart of diagnostics in patients with MTC. (*150 pmol/L (500pg/mL))

Determination of serum CEA should be performed together with serum calcitonin before thyroid surgery is undertaken. In disease staging, CT or MRI of the neck is indicated if locally advanced neck disease is suspected, as well as laryngo-tracheo-broncho-oesophagoscopy. At calcitonin >150 pmol/L (500 pg/mL), and if metastatic MTC is expected, evaluation of metastatic disease is indicated with CT, MRI and/or PET/CT (3, 8).

3.9 Surgical treatment

Surgery is the only means of potential cure for MTC if performed to a sufficient extent, (3, 4, 8, 13). Total thyroidectomy and dissection of lymph node compartments in the central neck, level VI is standard treatment when there is no evidence of metastatic lymph nodes or systemic disease (3, 14).

MTC is occasionally diagnosed by lobectomy because of follicular neoplasia Bethesda group IV or uncertain preoperative diagnosis. If the patient is biochemically cured after lobectomy, the revised ATA guidelines 2015 (3) recommend no completion thyroidectomy unless the patients has a *RET* germline mutation, a significant elevated basal or stimulated serum calcitonin level postoperatively, or residual MTC by imaging examinations. Since there is no data to base this recommendation on, individual decisions should be made in order to follow a patient with sporadic MTC with regular follow-ups without complementary thyroid surgery (3).

Figure 5: UICC / Robbins compartment levels in the neck.

Stack BC, Jr., Ferris RL, Goldenberg D, Haymart M, Shaha A, Sheth S, et al. American Thyroid Association consensus review and statement regarding the anatomy, terminology, and rationale for lateral neck dissection in differentiated thyroid cancer. Thyroid. 2012;22(5):501-8

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In patients with metastatic lymph nodes in the lateral neck, therapeutic dissection in the lateral neck compartments, levels II to V is mandatory (3, 14). The levels, or compartments, in the central and lateral part of the neck are illustrated in Figure 5 (200). The metastatic lymph node patterns are more aggressive and widespread in patients with MTC than in PTC patients, and meticulous dissection of the lymph node compartments is necessary (201-203).

Although imaging indicates that there is no metastatic lymph nodes visible in the lateral neck, there is yet no consensus regarding prophylactic dissection in the lateral neck compartments. Major studies have analyzed diagnostic predictors for the necessity of prophylactic lateral neck dissection considering tumor features at ultrasound (15), calcitonin level (8, 9, 204) or metastatic lymph node load in the central neck compartments (14, 16). This will be further discussed later on in the thesis.

The surgical procedure in patients with hereditary MTC is equal to the treatment in patients with sporadic MTC. However, due to the bilateral disease in hereditary MTC, lobectomy is not sufficient in patients with *RET* gene mutation and hereditary MTC (3). Prophylactic thyroidectomy is recommended to MEN2 screening patients with *RET* gene mutation, and the timing and extent of thyroid surgery should be based on preoperative basal serum calcitonin levels, and as outlined by the ATA guidelines (3, 27, 98) (Table 1).

Figure 6: Prophylactic thyroidectomy and bilateral central neck dissection in a patient with MEN2A, ATA risk class high. The arrows point to the recurrent laryngeal nerve and the parathyroid gland.
Informed written consent is available

Prophylactic central neck dissection of the lymph node compartments in *RET* gene carriers is recommended in patients with a calcitonin above 11.7 pmol/L (40 pg/mL), as lymph node metastasis rarely occurs when calcitonin is below 11.7 pmol/L (40 pg/mL) (3, 9, 21, 29, 100). Thyroid surgery in a patient with MEN2A is presented in Figure 6.

MTC develops very early in life in patients with MEN2B (23). Thyroidectomy is recommended as early as possible and during the first year of life (3, 31, 32). Thyroid surgery has to be performed before 4 years of age to achieve biochemical cure (32)

Possible per- and postoperative complications at thyroid surgery are injuries to the recurrent laryngeal nerve (RLN), the external branch of the superior laryngeal nerve and the vagus, phrenic, accessory and hypoglossal nerve, as well as the sympathetic plexus. At thyroidectomy, injury to the RLN is the most severe. The RLN palsy might be bilateral, unilateral, temporary or permanent. At unilateral palsy, the patient gets a hoarse voice and dyspnea, and at bilateral palsy permanent or temporary tracheostomy might be necessary. Permanent damage to the RLN occurs approximately between 0.3 and 3%, and transient palsy in up to 12% (205-209). Intraoperative nerve monitoring (IONM) is used by most endocrine surgeons today. A study by Bergenfelz et al. (210) reported reduced risk of permanent vocal cord palsy in the patients where IONM was used, and there was no bilateral RLN injury in these patients. Furthermore Brauckhoff et al. (211) found that continuous nerve monitoring may prevent RLN palsy by timely recognition of imminent nerve lesions.

Postoperative hypoparathyroidism, transient or permanent, following dissection in the central neck compartments was reported to be 17.6% and 4.4%, respectively, in a study by Polistena et al. (207). In another study, Toniato et al. (205) reported permanent hypoparathyroidism in 3.6% of the patients having total thyroidectomy without central neck dissection and in 13.2% with the dissection. Bergenfelz et al. (209) found that 9.9% and 4.4% of the patients had hypocalcemia and treatment with vitamin D analogues after 1-6 weeks and 6 months of follow-up, respectively. In this study as well, the risk increased with the dissection of lymph node compartments. If the parathyroid cannot be preserved in situ, parathyroid auto transplantation in the sternocleidomastoid muscle is recommended (206, 207).

Postoperative bleeding is rare but might be life-threatening due to tracheal compression. In this case, the patient requires immediate re-operation. The re-operation itself harbor additional risk of RLN palsy and hypoparathyroidism (206, 209).

Following lateral neck dissection, nerve injury to the accessory nerve gives the most severe morbidity, resulting in reduced mobility of the shoulder and pain (206, 207, 212). At neck dissection in the lateral left side of the neck, injury to the thoracic duct at the junction of the subclavian and internal jugular vein may occur (207, 213). The fistula might heal by conservative medical treatment like nutritional and pharmacological treatment (213), such as total parenteral nutrition and Somatostatin. However, in the absent of recovery, surgical approach is necessary. After lateral modified (651 patients) and radical (17 patients) neck dissection, Polistena et al. (207) reported intra- and postoperative hemorrhage in 2% and 0.29%, respectively, respiratory distress in 0.29%, lesions of the facial nerve in 0.44%, the vagus nerve in 0.14%, the phrenic nerve in 0.14% the hypoglossal nerve in 0.29%, permanent lesion of the cervical plexus in 0.29%, salivary fistula in 0.14% and chylous fistula in 1.04%. Lesions of the accessory nerve were transient in 1.34% and permanent in 0.29% of the patients.

3.10 Follow-up and recurrent disease treatment

Biochemical cure after primary surgery is crucial to the patient's prognosis. In some, often more recent studies and in the Norwegian guidelines (2017), biochemical cure is defined as no detectable calcitonin (11, 150, 214). However, in other studies biochemical cure has been defined as basal calcitonin below upper normal limit (9, 172, 215). Postoperative biochemical cure has been reported by Jung et al. (215) as the best predictor for recurrence- free survival, as 94,7% of the patients maintained biochemical cure status at follow-up. Machens et al. (9) showed that preoperative basal calcitonin levels greater than 146 pmol/L (500 pg/mL), best predicted the failure to achieve biochemical cure. In patients with biochemical disease and no structural disease visible at imaging, observation without reoperation in the neck is adequate procedure (3, 216). In the same study by Machens et al. (9), metastasis in the lymph nodes started to appear at calcitonin level 11.7 pmol/L (40 pg/mL) before primary surgery, and at 3 pmol/L (10 pg/mL) in the re-operating setting. In patients with metastatic lymph nodes, distant metastasis began at

calcitonin level of 117 pmol/L (400 pg/mL) before primary surgery and at 44 pmol/L (150 pg/mL) in the re-operative setting. 50% of the patients had distant metastasis at calcitonin level of 1463 pmol/L (5000 pg/mL), and nearly 100% at a level above 5852 pmol/L (20,000 pg/mL). The number of organs with distant metastasis increase with increased serum calcitonin (9, 217).

Regular follow-up includes radiologically examination, at least ultrasound of the neck, and biochemical analysis as calcitonin, CEA, PTH, calcium, TSH and free thyroxin. The follow-up frequency varies depending on the patient's postoperative biochemical and clinical status. The newly published Norwegian Guidelines in 2017 (150) recommend to measure calcitonin and CEA every 6 months for the first two years after primary surgery, and then annually. Patients with a slightly raised postoperative calcitonin level and with no visible structural disease, should be followed with active surveillance, as the biochemical signs of disease might be stationary for many years (3, 150). According to the Norwegian guidelines (150), stimulation test in follow-up is rarely indicated, but should be considered following lobectomy 3-6 months after surgery. However, the interpretation of the stimulation test might be difficult (150). Calcitonin may also increase at stimulation in benign nodular thyroid disease, thyroiditis and non- MTC thyroid carcinoma (170).

In patients with biochemical cure, ultrasound of the neck is recommended annually for the first 5 years, then less often (150). Additional imaging studies are indicated at increasing levels of calcitonin and CEA. Metastatic disease might be ruled out by a multimodal approach including combinations of CT, MRI, bone scintigraphy, FDG-PET/CT and F-DOPA-PET/CT. By the ATA guidelines, additional imaging is recommended at postoperative calcitonin above 44 pmol/L (150 pg/mL) and according to calcitonin doubling time (3).

Calcitonin- and CEA doubling time are indicators for disease recurrence and survival and a tool in assessing tumor progression (217). A meta-analysis by Meijer et al. (176), showed that the highest predictive power was found for the doubling time classification 0-1 year versus >1 year. CEA doubling time had a higher predictive value than calcitonin doubling time. A calculator provided by ATA determines the doubling times of serial serum calcitonin and CEA

measurements (3); www.thyroid.org/thyroid-physicians-professionals/calculators/thyroid-cancer-carcinoma

The follow-up of hereditary MTC follows the same principles. In addition, in the MEN2 patients the possibility of developing PCC, and also HPT in MEN2A patients, necessitates measurements of metanephrine, normetanephrine, calcium and PTH regularly.

In assessing patients with recurrent MTC disease in need for recurrent treatment, multidisciplinary approach and decision making are recommended. In patients with persistent or recurrent disease in the neck, re-operative neck surgery with systematic compartment dissection may be beneficial for in long-term survival and possible biochemical cure (3, 201, 218, 219). In patients with previously extensive surgery, or in palliative setting with threat of local compression or invasion of vital organs in the neck, as well as systemic symptoms of metastatic tumor burden like pain, flushing and diarrhea, operative procedures such as resection of only grossly metastatic lymph nodes are the treatments of choice (3, 5, 219). In patients with distant metastasis, extensive loco-regional surgery in the neck is not indicated (3).

The risk of complications in recurrent neck surgery is higher, but the higher risk of injuring the RLN and parathyroid in the central neck can be reduced by the lateral surgical approach (201). In the neck compartments previously surgically treated, percutaneous injection of ethanol in recurrent metastatic lymph nodes, guided by ultrasound, has showed to be a very good alternative to surgery in metastatic lymph nodes in PTC (220, 221). No study so far has demonstrated similar effect in MTC metastatic lymph nodes in the neck, but percutaneous ethanol injection in metastatic liver lesions from MTC has been discussed (222).

Metastatic MTC is incurable, and the management goals are to provide loco-regional disease control, palliate symptoms due to hormonal excess like diarrhea, palliate symptoms due to metastases like pain or bone fracture and control metastases that threaten life, such as bronchial obstruction or spinal cord compression (223). This can be achieved by palliative surgery, external beam radiation therapy (EBRT), radiofrequency ablation, chemoembolization or systemic therapy with tyrosine kinase inhibitor (TKI) (3, 223-229).

Chemotherapy and EBRT have not been effective treatment in MTC (219). No obvious benefit in OS or local or regional relapse-free rates has been found at EBRT (230, 231), but there are no prospective studies exploring this pathway. In general, adjuvant EBRT has been reserved for selected patients with high risk of loco-regional recurrence where benefit is observed (3, 231-233).

Treatment with TKI reduces the gain of function in the downstream signal transducer pathways in the tumor cell, with partial tumor response and extended progression-free survival (PFS) (Figure 1). However, the effect is transient and resistance to treatment will occur (87, 229, 234-236). The effect of Vandetanib and Cabozantinib in the treatment of patients with advanced, unresectable, locally advanced or metastatic MTC have been investigated in two phase 3 trials (237, 238), and a significantly increased PFS and biochemical response were found. However, there was no improved OS. The ATA guidelines (3) recommend to offer patients with significant tumor burden, and symptomatic or progressive metastatic disease according to RECIST (Response Evaluation Criteria in Solid Tumors), systemic treatment with TKIs targeting both *RET* and *VEGFR* (vascular endothelial growth factor) receptor tyrosin kinases. Both Vandetanib or Cabozantinib can be used as single-agent first-line systemic therapy in patients with advanced, progressive MTC.

4. Aims of the thesis

4.1 General aims

There has been a lack of knowledge concerning trends in diagnostics and surgical treatment in both hereditary (MEN2) and sporadic medullary thyroid carcinoma (MTC) in Norway in recent times. The present studies are the first nationwide population-based studies concerning MTC in Norway during the last decades. Furthermore, population-based studies strengthen the results credibility with the possibility of generalization.

Consequently, one aim of the thesis was to evaluate the clinical course, calcitonin as predictor in thyroid surgery and prognostic factors for outcome in patients with MTC, both hereditary and sporadic, in Norway during the last decades. These clinical aspects are the subjects of research which is continuously evaluated and discussed internationally. Another aim was to evaluate and discuss molecular biological aspects.

4.2 Specific aims

- I.** To evaluate the clinical course of MTC, as well as its predictive and prognostic factors, in all known *RET* positive MEN2A patients diagnosed in Norway from 1974 to 2015.
- II.** To compare time related trends in diagnostics and surgical treatment, including prognostic factors for biochemical cure and disease-free survival in all the patients with MTC, sporadic and hereditary, in Norway from 1994 to 2016.
- III.** To evaluate the relation between preoperative basal serum calcitonin level and tumor stage, and the ability of the calcitonin level to predict the extent of surgery needed in the lateral neck compartments in patients with MTC.
- IV.** To evaluate long time follow-up of MEN2B patients in Norway, including somatic cancer driver mutations in *HRAS*, *KRAS*, *NRAS*, *BRAF*, *PIK3CA* and *AKT1* in their tumor tissue.

5. Material and methods

5.1 Patients and data collection

The multicenter studies in **paper I-IV** are retrospective, population-based and nationwide, and cover four different, but overlapping cohorts. The studies are part of the Norwegian MTC project and are based on a systematic search within all four Norwegian departments of medical genetics, as well as clinical and histopathological registrations in the regional centers dealing with MTC. In addition, the Cancer Registry of Norway provided information on patient names and treatment centers of all patients registered with MTC in the Cancer Registry of Norway during 1994-2016. All living patients were invited to participate in the studies. Two patients declined to participate in **paper I**. In **paper II**, nine patients declined to participate, and six patients declined in **paper III**. While these patients were not part of the specific data presentation, they were included in the epidemiological calculations of incidence and prevalence. In **paper IV**, analysis of somatic cancer driver mutations were performed prospectively.

Data collection was performed by reviewing patient's files. In **paper I**, the data collection was done by the PhD candidate herself for the patients treated at Oslo University Hospital (OUH) and Haukeland University Hospital (HUH). For the patients treated in the remaining two regional hospitals, the Norwegian MTC-project collaborators at the institutions did the data collection. In **paper II-IV**, the PhD candidate herself collected all the data at the four regional hospitals.

The different cohorts in **papers I-IV** are summarized in Table 3 and described below. Even further details concerning definitions, as well as inclusion- and exclusion criteria, are described in the Material and method sections in the papers.

In the MEN2A cohort study, **paper I**, the study includes data from 65 out of 67 patients with confirmed MEN2A-causing *RET* mutations registered in Norway. Since 1974, 60 patients underwent thyroid surgery and five patients were not yet surgically treated. The median postoperative follow-up was 9.9 years (range 0-39.5 years). The patients were divided into index and screening patients. The *RET* consortium, diagnostic tools and surgical approach have changed during the time period. To account for these time-dependent disparities and minimize

time bias, the patients and analyzes were divided into pre*RET*-era and *RET*-era groups, including those who had thyroid surgery before January 1st 1994 and after, respectively.

Table 3: The different cohorts in papers I-IV. Inclusion and exclusion

	Time period of inclusion	Total number of patients	Patients included in the epidemiology ¹	Patients excluded	Patients included in the data presentation ¹	Surgically treated patients	Censoring date of observation
Paper I	1974-2015	67	67	2	65	60	March 1 st 2015
Paper II	1994-2016	237	237	9	228	201	March 1 st 2017
Paper III	2003-2016	160	N/A	66	94 ²	94	March 1 st 2017
Paper IV	1988-2017	6	6	0	6	6	July 1 st 2018

¹Patients who declined to participate were not part of the specific data presentation, but they were included in the epidemiological calculations of incidence and prevalence ²Only patients with available preoperative calcitonin before surgical intervention and surgically treated patients were included. N/A: Not applicable.

The cohort study in **paper II** covers all patients with sporadic and hereditary MTC during 1994-2016. Of 237 patients found, 228 patients were included. Surgery was performed in 201 patients. To evaluate time trends in diagnostics and treatment of MTC, the patients were divided into two time periods, 1994 to June 2005 and July 2005 to 2016.

Due to more sensitive calcitonin analysis from 2003, the study in **paper III** was limited to patients with MTC treated during 2003-2016. Preoperative analysis of calcitonin before surgical intervention was available in 94 patients. These patients were included in the study. The patients were grouped according to preoperative calcitonin level. To minimize bias related to stage migration during follow-up, the data were compared in prognostic groups according to lymph node- and distant metastatic status after completed primary surgery and during follow-up. Patients without distant metastasis (M0) and no metastatic lymph nodes (pN0), metastatic lymph nodes in the central- (pN1a) or lateral neck compartments (pN1b) at primary surgery and with no

disease-progression to next tumor level during follow-up (NPNL) were grouped as (N0-NPNL), (N1a-NPNL) and (N1b-NPNL), respectively, a total of 58 patients.

The MEN2B cohort study, **paper IV**, included all registered MEN2B patients born in Norway and treated during 1988-2016. A total of six MEN2B patients were found.

5.2 Treatment definitions and follow-up

The treatment of MTC in Norway is regionalized. However, for some patients in the study period, diagnostic surgery has been performed at local hospitals prior to the patients being referred to one of the four regional hospitals. Definitions and follow-up procedures in **papers I-IV** are identical. Primary surgery of the thyroid and lymph nodes were performed within one or more procedures. Completed primary surgery was defined as one or more surgical procedures within the first year. Dissection of lymph node compartments and number of surgical procedures were evaluated at completed primary surgery, and, if applicable, cumulatively after repeat surgeries. The total number of surgical procedures (**paper I**) included revision neck surgeries along with mediastinal surgery and surgeries for other metastatic disease. In **paper II**, the ratio between metastatic and total number of dissected lymph nodes was calculated. The term dissected lymph nodes is referred to as resected lymph nodes in the present thesis, which is more precise. However, the content and results are the same.

Regular cancer specific follow-up included at least serum calcitonin analysis and ultrasound of the neck. Radiological- and nuclear medical examinations as CT, MRI or PET/CT were performed at indication. Follow-up was generally performed early postoperatively, every 3-6 months in the first postoperative year and then once a year. However, the frequencies have varied depending on the patient's biochemical and clinical status.

Biochemical cure was defined as basal calcitonin below limit of detection after total thyroidectomy or basal calcitonin within normal range when lobectomy alone was performed. Biochemical cure was evaluated after completed primary surgery. The lowest measured calcitonin value after completed primary surgery and before known disease progression and recurrent treatment was used to evaluate biochemical cure. At latest follow-up, biochemical

disease was defined as measurable basal calcitonin without visible tumor at imaging examinations, or basal calcitonin above normal range after lobectomy without structural disease. Persistent clinical disease was defined as radiologically proven distant metastasis at diagnosis and/or remnant disease in the mediastinum and neck. Cause of death were divided into MTC specific death and death due to other cause than MTC, known or unknown cause.

5.3 Ethics

The Regional Committee for Medical and Health Research Ethics (REC) of Western Norway approved the MTC NOR project with the included studies, case number 2013/1499. All patients included in the studies, or their parents if children, gave written, informed consent to have their data include in the studies. When the children reached 16 years during the study period, they even gave an additional written informed consent, according to the terms of REC. The consent also includes permission to present data in the very rare inherited MTC with few patients harboring the syndrome, for instant as a case report with one or with very few patients. Furthermore, the consent also includes presentation of anonymized images without person-recognition. The studies did not require extra examinations or follow-up appointments for the patients. Finally, REC granted permission to include deceased patients.

The acquisition of information on patient's names and treatment centers provided by the Cancer Registry of Norway was approved by REC.

The data collection from the four regional hospitals was made possible due to signed agreements between the institutions.

5.4 Biochemical analysis

As outlined in the Introduction chapter, the calcitonin assays have changed over time with varying sensitivity and specificity, and the limit of detection have changed. The reference values of serum calcitonin have also varied over time and are related to age, sex, food intake and cigarette smoking (155-157, 239). In children, the reference values are higher than in adults (157, 239). The most recent generation of immunometric assays are highly sensitive and specific for monomeric calcitonin. During the study periods, calcitonin was analyzed by routine

immunoassay in two laboratories; OUH and HUH. From 2003 calcitonin was analyzed by the more sensitive immunochemiluminometric assays (ICMAs); Immulite® Siemens and later on Roche COBAS® Module E. The time- and assay related variation in limits of detection are described in the Material and methods section in **papers I-III**, respectively.

The reporting of calcitonin values in either metric or SI units differs between countries. The study results were reported in pg/mL in **paper I** and pmol/L in **papers III and IV**. Since calcitonin values are reported in SI unites in Norway, the calcitonin values are reported in pmol/L with pg/mL in parenthesis in the present thesis, also when referring to studies reporting calcitonin values in pg/mL. The conversion factor between pg/mL and pmol/L is 0.2926 (pg/mL x 0.2926 = pmol/L). The reference values at OUH were < 2.1 and < 3.1 pmol/L (7.2 and 11 pg/mL), and at HUH < 1.6 and < 2.2 pmol/L (5.5 and 7.5 pg/mL) in females and males, respectively. In **paper I**, 3.1 pmol/L (11 pg/mL), the OUH upper reference value for men, was used as the cut off for normal range for both females and males. In the later papers, and according to the more commonly used cut-off in other publications, 3.0 pmol/L (10 pg/mL) was chosen as cut-off value for upper normal limit, for both sexes (**papers II-IV**). According to the ATA recommendations, prophylactic lymphadenectomy in the central neck compartments is recommended for patients with a preoperative calcitonin levels > 11.7 pmol/L (40 pg/mL) (3, 21). As such, in **paper I**, 11.7 pmol/L (40 pg/mL) was used as the cut-off for possible lymph node metastases.

Analysis of stimulated calcitonin and CEA were performed to a limited degree and were not reported.

5.5 Genetic analysis

RET mutation analysis was performed in blood cells collected in EDTA tubes and performed according to international standards, using reference sequence NM_020975.3 and NM_020975.4. Mutation analysis were performed by sequencing of exons 10, 11, 13-16. Exon 8 has not routinely been analyzed before autumn 2016. The method has been described by Sjursen et al. (240).

In **paper I** and based on recent scientific knowledge, genetic analyses were repeated in stored blood from three surgically treated patients, with no change in the clinical consequences. Risk stratification was based on the mutated codon and ATA risk classification. For the tandem mutation p.Q781R/p.S904C, the risk classification was not determined, but the phenotype was FMTC (MEN2A). In the study, the mutation was included in the ATA risk class A (ATA moderate).

5.6 Histopathological analysis

Each of the four regional hospitals performed separate histopathological examinations of thyroid specimens implemented in line with the applicable WHO/IARC tumor classifications (121). MTC tumor stage in **papers I-III** was classified according to the American Joint Committee of Cancer (AJCC) TNM classification, 7th edition (UICC 2010), the current classification during the data collection (121, 122). In **paper IV**, the MTC tumor stage was classified according to the 8th edition (UICC 2017) (123). The differences between the 7th and 8th edition are shown in Table 2.

At primary surgery, pTNM was defined as the total pTNM after primary surgery. In **paper I**, patients with dissection of lymph node compartments at primary surgery and metastatic lymph nodes were classified as pN1, and pN0 when there was no metastatic lymph nodes. Patients without lymph node compartment dissection at primary surgery were classified as pNx. The presence of CCH was investigated in all patients with no histological evidence of MTC. In **paper II and III**, when indicating the MTC tumor stage, unknown preoperative metastatic status (Mx) and no lymph node compartment dissection (Nx) were interpreted as no metastasis (M0) and no metastatic lymph nodes (pN0) in the patients who achieved biochemical cure after primary surgery.

5.7 DNA extraction and somatic mutation analysis

The somatic mutation analysis in **paper IV** were performed in 2018. The analysis of mutations in *HRAS*, *KRAS*, *NRAS*, *BRAF*, *PIK3CA* and *AKT1* were performed on formalin-fixed paraffin-embedded tissue (FFPE) from the primary tumor in the thyroid gland (**paper IV**). All the analyses were performed at HUH, Bergen. The DNA was extracted from the FFPE as earlier

described (241), and further using the NucleoSpin DNA FFPE kit (Machery-Nagel GmbH & Co.KG, Düren, Germany) on an automated platform (Genomic StarLet, Hamilton, Reno, Nevada). The method is described in detail in the Material and methods section in **paper IV**.

Detection of mutations in *BRAF* (codon 600), *KRAS* (codons 12, 13, 59 and 61), *NRAS* (codons 12, 13, 59 and 61), *PIK3CA* (codons 542, 545, and 1047) and *AKT1* (codon 17) were performed using an allele-selective multiplex polymerase chain reaction (PCR) kit (CRC-RT48, EntroGen, Inc. Woodland Hills, CA) screening against 50 different somatic mutations by real-time PCR. The PCR analysis were performed essentially as indicated by the provider of the kit. The codons 12, 13 and 61 of the *HRAS* gene were analyzed by PCR and Sanger's direct sequencing. PCR amplification was performed using the Qiagen Master Mix (Qiagen) with primers CTGAGGAGCGATGACGGAAT (sense) and TATCTCCACTCGGACCGCG (antisense) for *HRAS* codons 12 and 13 and primers GGATTCCTACCGGAAGCAGG (sense) and ATGTCCACTTGGGGCACTCC (antisense) for *HRAS* codon 61. The PCR amplification and sequencing analysis in the somatic mutation analysis are described in detail in the Material and methods section in **paper IV**.

5.8 Statistics

Data were analyzed using SPSS Statistics for Windows. Differences were explored by univariate analysis. For continuous variables not following the normal distribution, non-parametric tests for independent samples as Mann-Whitney U test and Kruskal Wallis test were used. Group differences and associations between categorical variables, the numbers of subjects, were analyzed using a Pearson Chi-square 2-sided test. Predictive and prognostic factors for developing MTC, **paper I**, and postoperative biochemical cure in **paper I and II** were explored in univariate analysis, and in multivariate analysis using logistic regression. In **paper II**, DSS were analyzed by Kaplan-Meier survival analysis, with the log-rank test applied to explore differences in univariate analysis. For multivariate survival analysis, the proportional hazard regression analysis (Cox) was applied, with the Omnibus test as lratio (likelihood ratio) test for differences. In **paper III**, factors predicting biochemical cure, preoperative calcitonin level and disease in the lateral neck compartments were explored in multivariate analysis using logistic regression. Statistical significance was set at < 0.05 .

6. Summary of results

6.1 Paper I

A Nationwide Study of Multiple Endocrine Neoplasia Type 2A in Norway: Predictive and Prognostic Factors for the Clinical Course of Medullary Thyroid Carcinoma.

The relation between hereditary MTC and *RET* gene mutation has only been known since 1993. Predictive and prognostic factors have been evaluated in many major studies to find indicators predicting the optimal time for and extent of prophylactic thyroid surgery. The guidelines change continuously due to increased knowledge. The aim of this study is to contribute to the discussion through an evaluation of the clinical course of MTC as well as the predictive and prognostic factors in the MEN2A patients diagnosed in Norway from 1974 to 2015.

The incidence of MEN2A was 1:66,438 live births per year and the prevalence 1:84,672 person years (Errata chapter, point 1). Of the 65 patients included in the present study, 60 underwent thyroid surgery. MTC was observed in 100% of the index patients and in 45% of the screening patients. At primary surgery, all the 13 patients with metastatic lymph nodes had preoperative basal serum calcitonin levels ≥ 20 pmol/L (68 pg/ml), and all the 17 patients without central lymph node compartment dissection and preoperative basal calcitonin < 11.7 pmol/L (40 pg/mL) were biochemically cured. By multivariate analysis, when correcting for time related bias as the *RET*-era was considered separately, preoperative basal calcitonin was found to be significant predictive factor for MTC superior to age at thyroid surgery. Furthermore, the only significant prognostic factor for postoperative biochemical cure was nodal status at primary surgery.

Preoperative calcitonin alone can serve as a guide for best timing of prophylactic surgery in the central neck compartment. Prophylactic thyroidectomy has to be performed in patients with MEN2A at elevated calcitonin but before metastatic lymph nodes occur.

6.2 Paper II

Trends in Diagnostics, Surgical Treatment and Prognostic Factors for Outcome in Medullary Thyroid Carcinoma in Norway. A Nationwide Population-based Study.

This population-based nationwide study addresses lack of knowledge concerning trends in diagnostics, surgical treatment and outcome of MTC in Norway in recent times. The cohort includes data for 228 out of the 237 patients (96%) with MTC in Norway, sporadic and hereditary, during 1994-2016. Surgical treatment was performed in 201 patients. The study compares trends over two study periods.

MTC accounted for 4.2% of thyroid carcinomas during 1994-2016. During the two study periods, the incidence increased from 0.18 to 0.25:100,000 per year. Preoperative diagnostics improved with significantly increased use of calcitonin, ultrasound and FNB. Furthermore, patients were diagnosed at an earlier tumor stage, and significantly more patients were cured in the second than in the first study period. The 5- and 10-year DSS were 88 and 82%, respectively, and the 5-year DSS was improved from 84% in the first study period to 90% in the second. By multivariate analysis of the patients with metastatic lymph nodes, significant independent prognostic factors for cure were low ratio between metastatic and total number of resected lymph nodes and no extrathyroidal extension. Significant independent prognostic factors for DSS were no distant metastasis, younger age and low ratio between metastatic and total number of resected lymph nodes.

Disease control in patients with MTC has improved in Norway. Patients with MTC in Norway receive diagnostics and treatment at an international level. Furthermore, in patients with metastatic lymph nodes in the lateral compartments of the neck, therapeutic neck dissection performed with meticulous dissection has prognostic impact on cure and survival.

6.3 Paper III

The Role of Calcitonin in Predicting Extent of Surgery in Medullary Thyroid Carcinoma. A Nationwide Population-based Study.

Predictors for prophylactic lateral neck dissection in patients with MTC have been evaluated and discussed in many major studies. There exist no uniform guidelines. In this study, the relation between preoperative basal serum calcitonin levels and tumor stage is evaluated, as well as the ability of calcitonin level to predict the necessity of prophylactic surgery in the lateral neck compartments in patients with MTC. Due to more sensitive calcitonin analysis since 2003, the study was limited to patients with MTC treated after 2003, thus 94 surgically treated patients were included. The patients were grouped according to calcitonin levels and prognostic groups, as described in the Material and method chapter above.

At calcitonin levels ≤ 500 , 501-1000 and > 1000 pmol/L (≤ 1709 , 1712-3417 and > 3417 pg/mL), metastatic lymph nodes in the lateral compartment of the neck were found in 16%, 50% and 71% of the patients, respectively. In the prognostic groups, 19% of N0-NPDL patients had calcitonin > 500 pmol/L and 17% of N1b-NPDL patients had calcitonin ≤ 500 pmol/L.

By multivariate analysis, preoperative basal calcitonin could not predict the need for prophylactic surgery in the lateral neck compartments, but was significantly related to tumor diameter. However, significant factors related to such metastatic lymph nodes were extrathyroidal extension, and biochemical cure was less likely achieved in these patients.

6.4 Paper IV

Mutation Screening of HRAS, KRAS, NRAS, PIK3CA and AKT1 in Medullary Thyroid Carcinoma in Multiple Endocrine Neoplasia Type 2B

MTC in MEN2B develops in early childhood and have poor prognosis, mostly due to late diagnosis and hence a more advanced tumor stage. In Norway six patients with MEN2B, all with *de novo* p.M918T mutation, were diagnosed and treated from 1988 to 2016. All the patients presented with the MEN2B phenotype. The median age at diagnosis was 12.5 years (range 8-34). The patients had tumor stage IV at time of diagnosis. At censoring date July 1st 2018, they were alive at median age of 26 years (range 19-49). Despite the advanced tumor stage at time of diagnosis, the MTC tumor behavior has not been aggressive, but has rather shown a slow disease progression.

In sporadic MTC, somatic cancer driver mutations (CDMs) are well investigated, and *RET*-mutations and other CDMs have, with a few exceptions, been found to be mutually exclusive. In sporadic MTC, *RET* p.M918T mutation is associated with a more aggressive disease. However, little is known about the presence of CDMs in MEN2B related MTC harboring germline *RET* pM918T mutation. Somatic CDMs in the Norwegian MEN2B patients were evaluated and are reported in this paper. CDMs were analyzed in FFPE tumor tissue from all the patients. Somatic mutations in *HRAS*, *KRAS*, *NRAS*, *BRAF*, *PIK3CA* and *AKT1* were not found in any patient.

Further research into multifactorial aspects in genetics, epigenetics and tumor microenvironment properties is needed to explore prognostic differences in patients with advanced MTC experiencing slow disease progression. In sporadic as well as hereditary MTC, research on multifactorial prognostic factors related to molecular biology is needed.

7. Methodological considerations

7.1 Study design, data collection and bias.

Prognostic factors in diagnostics as well as surgical decision making are the main issues in daily clinical work, when dealing with patients with MTC. Decisions regarding medical therapy should be evidence based. Retrospective evaluations of diagnostics, treatment and outcome add information in order to improve internal and national routines in diagnostics and treatments, improve understanding of the disease-trends as well as create new questions for further research. Confirmation or disconfirmation of already established scientific truths are crucial. Population-based studies are critical in epidemiological studies, and valuable in scientific research. Furthermore, and based on the knowledge of the already treated patients, prospective studies in diagnostics and treatment, as well as molecular-medicine, can be designed.

The Norwegian MTC project (MTC-NOR) seeks to explore these issues. The studies are multicentric, nationwide and population-based, adding strength to the studies.

Epidemiological observational cohort studies are suited in describing distribution of a disease, as well as trends in diagnostics, treatment and outcome over time (242). The study population must represent the target population. Nationwide studies, covering the entire patient population in a country over time are suitable, and the results can be projected to a larger target population. Including all patients with a certain disease in a time interval will reduce bias.

Nationwide, retrospective studies of a certain disease have the advantage of the ability to include all affected patients, with reduced selection bias. However, in retrospective studies, there might be missing values leading to a reduced number of cases included in the specific analysis, especially in multivariate analysis. The possibility of time and information bias with reduced statistical power might occur. **In paper II**, the lack of information and missing variables in the first study period may have limited the study, including the possibility of stage migration. Furthermore, in **paper III**, as there was no preoperative calcitonin analysis in 28% (39/139) of the surgically treated patients, these patients were excluded from the study. Hence, the possibility of reduced power and diagnostic selection bias exist, as patients with MTC randomly discovered

might have less severe disease. However, the variation in correlations between preoperative calcitonin levels and extent of disease in the present and included study population, remain valid. In the multivariate analysis in **papers I-III**, reduced number of patients with complete datasets constitute a limitation, as low numbers in some variables might cause statistically type II error. On the other hand, in prospective randomized studies, there will be complete datasets of variables. However, it will be hard to include all patients with a certain disease in the country over time. Hence, there might be institutional selection bias as the referral hospitals might treat more severe cases than other hospitals. Further, it will take time to reach the follow-up end point.

In retrospective cohort studies, it is important to be aware of time dependent bias due to time dependent differences in technical examinations and analysis methods, treatment differences and changing methods of evaluation in follow-up. Loss of follow-up might also create bias and threaten the validity. It is important with precise definition of the groups analyzed, as well as comparing similar groups in the analysis. For example, in **paper I** in the pre*RET*-era, patients were diagnosed later and cured less often than patients in the *RET*-era. Furthermore, dissection of lymph node compartments was less frequently performed in the pre*RET*-era. Current calcitonin assays are much more sensitive than those used previously. By analyzing the data for predictive and prognostic factors for the pre*RET*-era and the *RET*-era groups separately, these time-dependent disparities were taken into account, and controlled.

In Norway, four regional centers are involved in genetic counseling, and took part in identifying patient names and data collection in **paper I**, minimizing selection bias. The *RET* gene mutation analysis is also centralized. Hence, we can assume that all registered MEN2A patients constitute the majority of MEN2A patients in Norway in the *RET*-era. However, it can not be ruled out that MEN2A patients who were diagnosed in the pre*RET*-era may have been missed. In **paper II-IV**, the regionalization of MTC-treatment and the clinical data collection combined with data from the Cancer Registry of Norway, made these population-based studies possible and complete with minimal to none selection bias. Accuracy and reduced interpretation bias of the variables are for the most part due to data collection by one person with in-depth understanding of endocrine surgery.

It is important to compare the study results with other similar studies, and repetitive resembling results strengthen the significance of the achieved results. The studies in the thesis (**paper I-IV**) cover research on a broad spectrum of diagnostics and surgical treatment of MTC. As will be further outlined in the Discussion chapter below, the study results are both coincident and non-coincident with the studies referred to in the Introduction chapter.

7.2 Ethical considerations

These studies followed the ethical regulations governing clinical research in human beings according to the Act of Health Research in Norway. Study approval from the Regional Ethical Committee and informed written content from the patients were achieved. Un-identification of data and anonymity was maintained. By adhering to these regulations, the patients' privacy and data protection were properly secured.

In consent-based studies, the sample size might be reduced leading to a reduced power. However, in the studies constituting this thesis, only two, nine and six patients declined to participate in the studies in **paper I, II and III**, respectively. All registered MEN2B-patients treated in Norway participated in the study in **paper IV**. The study power was considered not reduced. Furthermore, the patients did neither need extra follow-up beyond the regular follow-ups, nor undergo additional examinations. Therefore, the studies represented no extra burden for the patients.

Signed inter-institutional agreements, ensure the institutions ownership to study data and strengthen the nationwide study cooperation.

7.3 Diagnostic environment, surgical treatment and follow-up

During the last decades, diagnostic centers for patients with nodular thyroid disease have been established in Norway as well as abroad, permitting ultrasound of the neck, FNB and biochemical- and clinical evaluation by a multidisciplinary team, resulting in more uniform diagnostics and treatment. In addition, a novel surgical specialty comprising breast- and endocrine surgery has been established in this country according to international standards. Prior to this, there was no formal education in thyroid surgery in Norway. Assumingly, the

establishment of diagnostic centers and formal education and training in thyroid surgery might increase attention to heighten and uniform the diagnostics and surgical- and oncological treatment.

Surgical procedures have changed over time and the extent of especially dissection of lymph node compartments in the neck might have differed during time and between institutions. The follow-up has also been improved over time. With more sensitive calcitonin assays, high resolution ultrasound, more skilled examiners and increased use of other imaging as CT, MRI and PET/CT, higher frequencies of disease recurrence will be discovered, followed by treatment of recurrent disease. Consequently, when studying these phenomena over a length of time, bias might occur. So with all this joint progress, the patients might achieve a more successful treatment with increased possibility of cure.

7.4 Biochemical analysis

As calcitonin analysis have become more precise and sensitive in recent times, the limit of detection has thus improved due to lower measurable calcitonin concentrations. As the definition of biochemical cure was “not detectable calcitonin”, the changing methods of analysis might have influenced the biochemical cure results over time (**paper I-III**).

Furthermore, as the most recent generations of immunometric assays are more sensitive, cross-reaction with procalcitonin or other calcitonin-related peptides is less likely to occur (3, 158).

Calcitonin reference values are influenced by age, gender, body mass index, food intake and cigarette smoking (155-157, 239). In children, especially neonates, reference values as high as 11.7 pmol/L (40 pg/mL) are reported in the literature (157). A recent study by Eckelt et al. (239) including 6090 serum samples of 2639 subjects, comprising newborn and children as old as 5 years of age, showed an accelerated decline in calcitonin levels with age, with higher values in boys than in girls. The study presents calcitonin reference ranges in children from 3 months to 18 years of age, and the highest normal calcitonin value was 21.3 pmol/L (72.7 pg/mL) in a neonate of 3 months. However, in the studies in **papers I-IV**, all the patients with MTC were six years or

older at thyroid surgery. The influence of cigarette smoking was evaluated in a study by d'Herbomez et al. (155) who by analyzing 287 blood samples, showed that cigarette smokers had higher calcitonin levels than non-smokers. Furthermore, the influence of food intake was examined by Zaved et al. (156) who demonstrated that food intake raised serum calcitonin levels. The papers constituting the current thesis have not taken into account the probable influence of tobacco smoking and food consumption on calcitonin blood levels.

Due to the retrospective nature of the studies, no standardized follow-up schedule, including calcitonin analysis, existed for patients who completed primary surgery. However, in the studies, this was compensated for by using the lowest measured calcitonin value after primary surgery and before known disease progression and any recurrent treatment, when possible postoperative biochemical cure was evaluated. In **paper II**, the median time until the first postoperative calcitonin analysis after primary surgery in the biochemical cured patients was 3 months (range 0.03-181) and in the not cured patients 2.1 months (range 0.03-98) ($p = 0.094$). In a very recent study by Machens et al. (243), time to calcitonin normalization after surgery for node-negative and node-positive MTC was evaluated. It shows that calcitonin levels normalize within one week, and within two weeks in those with MTC with metastatic lymph nodes and preoperative calcitonin levels of 146-292 pmol/L (500.1-1000 pg/mL). In patients with MTC and metastatic lymph nodes, and preoperative calcitonin exceeding 292 pmol/L (1000 pg/mL) as well as more than 10 metastatic lymph nodes, time to calcitonin normalization is prolonged.

7.5 Genetics analysis

RET mutation was first described in 1993, and the analysis was gradually implemented in 1994. In **paper II**, 78% of the patients with MTC was not offered *RET* mutation analysis throughout the entire study period. However, that improved from 66% in the first study period to 85% in the second. Assumingly, the MTC-NOR project may raise the level of attention related to offering *RET*-mutation analysis to 100% of the patients with MTC.

The method of the *RET*-mutation analysis has been perfected over the years, and as recommended the exons 10, 11 and 13-16 have been included in the routine analysis. In addition, from the autumn of 2016 sequencing of exon 8 was also included in the routine analysis. In

Norway today, all exons are analyzed by whole genome sequencing using next generation sequencing technology.

7.6 Histopathological analysis

The four regional hospitals handling MTC also performed histopathological examinations of the thyroid specimens obtained from biopsies and operations in their respective institutions. Variations in interpretation of the MTC specimens might have occurred. However, the histological diagnosis of MTC was in accordance with WHO/IARC tumor classifications (121) allowing uniform assessment. These classifications have changed during the study period, and was taken into account when reviewing the patient's files, including the original histopathological reports and classified according to the 7th revision of the TNM classification (UICC 2010) in **papers I-III** (122) and the 8th revision in **paper IV** (123). Using the same TNM version when comparing the study patients reduces bias due to more uniform groups. However, there might be missing information especially in older records leading to missing variables.

While MTC might be difficult to diagnose, IHC evaluation increases the accuracy in diagnosis. However, interpretation of IHC stained slides can be partly subjective and therefore require an experienced pathologist (244). There are neuroendocrine carcinomas of the thyroid that do not produce calcitonin. They are high-grade, very rare and generally have a poor prognosis, as recently described by Chorny JA et al. (245).

Histopathological reports today contain more detailed information than they did earlier, in most part due to a more extensive examination of the specimens including all non-metastatic lymph nodes. This may influence the ratio between metastatic and total number of resected lymph nodes between the two study periods in **paper II**. However, the number of resected lymph nodes did not differ significantly in the two periods. Furthermore, the ratio is a composed parameter and demanding to interpret, as the ratio rise with increasing number of metastatic lymph nodes and decreasing number of resected lymph nodes. However, the more lymph nodes resected, the lesser risk of leaving metastatic lymph nodes behind, is still applicable. The resected lymph nodes are called dissected in **paper II**.

7.7 Somatic mutation analysis

Polymerase chain reaction (PCR) is a highly sensitive technique, especially in detecting nucleotides, even in a very limited material when DNA amounts down to a few nanograms are used (244). When PCR is performed on DNA extracted from FFPE, awareness of variations in tumor content relative to non-neoplastic cells, is necessary. False negative results might occur if normal cells are dominant in the specimens (244). This occur more frequently in FFPE tissue than in fresh/frozen samples (241). The standardized methods in the automatic platforms, or next generation sequencing, enables the analysis of somatic mutation using a small amount of tumor-specific DNA. The methods are robust with high level of sensitivity reported in previous studies, > 94%, which is higher than that of Sanger sequencing (244, 246, 247). There is minimal risk of obtaining false positive and false negative results.

7.8 Statistics

Statistical validity depends on using the right statistical analysis and effect measures (248). The sample size must be sufficient to give power to the study and reduce the possibility of statistical type I and type II error. In a relatively rare disease as MTC, and especially in the MEN2A- and MEN2B syndromes, obtaining large enough sample size with high power in a nationwide study in Norway can seem unachievable due to the small number of inhabitants. To add more patients, international multicenter studies ought to be applied. That may, however, give raise to ethical and organizational issues.

As described by Benestad and Laake al (248) and Aalen et al. (249), the significance level is the probability of rejecting a null hypothesis that is actually true, and should be low. It is most often set to 5%, ($p < 0.05$). Rejecting a null-hypothesis that is true is a type I error. The probability of rejecting a null hypothesis that is false, is the statistical power of the study and the outcome that is desired. The power needs to be high, usually minimum 80%. Accepting a null-hypothesis that is false is a type II error. The probability of type II error is one minus power. This means the probability of type I error increase if the level of significance is higher than 0,05, and the probability of type II error increase at decreasing power and decreasing sample size. In **paper I-III**, missing variables resulted in reduced datasets with reduced variables in some of the multivariate analysis, and type II error might have occurred.

By univariate analysis a certain risk or probability is calculated between two variables (cause and effect), without considering if there are other variables (confounders) that might influence either the cause or effect variable. Hence it is important to be aware of possible confounders (248, 249). In the statistical analysis in **papers I-III**, as described in the Material and methods chapter, possible confounders were controlled for in the adjusted, multivariate analyses. Hence, the true relations between the possible causes and the effect were explored, when evaluating predictors and prognostic factors in **papers I-III**. Generally, in epidemiological studies, multivariate analysis has to be added to the univariate analysis for better and more truthful scientific understanding. However, if there are missing variables and the total sample is low, the type II error might occur. With many possible confounders and missing values in many variables, there will be few patients with a full set of variables and the multivariate analysis may therefore not be applicable. Hence, it may be necessary to make a selection of the most relevant confounders in the multivariate analysis.

8. Discussion

8.1 Summary and impact

During the last decade, disease control in patients with MTC has improved in Norway, because of earlier detection of the disease and better disease outcome. Preoperative calcitonin levels is a good marker for timing of prophylactic thyroidectomy in *RET* gene carriers, but not for predicting the need for prophylactic surgery in the lateral neck compartments in patients with MTC. Tumor stage is still the main prognostic factor governing cure and survival, but surgical accuracy has a significant independent prognostic impact.

The multicenter studies included in the thesis are the first nationwide and population-based studies dealing with MTC in Norway in recent times. The study results concur with other major studies from other countries and international guidelines. The quality of health care for MTC-patients in Norway is high and on level with comparable countries. However, in other countries with larger populations, nationwide population-based studies are difficult to perform. In this aspect, Norway has an advantage, because of the Population Registry and the Cancer Registry of Norway.

As the studies comprising the thesis have evaluated different aspects in preoperative diagnostics, including ultrasound and calcitonin, as well as different surgical aspects, the studies have impact on the clinical management of MTC as well as the associated research. The recommended timing and extent of thyroid surgery in patients with hereditary MTC have changed during the relative short time the connection between *RET*-gene mutation and MEN2A has been known. Previously, the recommendations were generally based on genetic risk classification and the patient's age. However, in recent time, the timing of the surgery is more based on preoperative levels of serum calcitonin (Table 1) (3, 250, 251). The study in **paper I** from 2016 has contributed to this trend, and is referred to in the recent review of Raue et al. (250).

By studying diagnostic- and treatment trends regarding MTC-patients the last decades, the study in **paper II** found that disease control as well as survival has improved, including improved

survival. Furthermore, subjects as calcitonin screening, the extent of both therapeutic- and prophylactic dissection in the lateral neck compartments and other prognostic factors for outcome have been discussed during the last years and are still discussed. The studies in **paper II and III** are supplementary in this research and future discussion.

The studies included in this thesis will have further impact in future research. They provide a basis for further research concerning MTC in Norway. In **paper IV**, molecular biological aspects as somatic CDMs in MEN2B related MTC are evaluated and discussed. Consequently, further studies in MTC and molecular biology can be designed, and deeper insight into molecular biology will reveal several disease mechanisms and hopefully development of further targeted systemic therapies.

The project has been nationwide and multidisciplinary and has included the four regional hospitals in Norway. Consequently, many collaborators from these hospitals and a variety of subject fields working with MTC have been involved. The impact has been, and will be further on, an increased knowledge about MTC in Norway as well as increased interests in thyroid research. Beyond this, knowledge about trends of diagnostics and surgical treatment in MTC in Norway during the last decades (**paper II**) is definitive of national as well as international interest, especially for comparable countries according to population size and socioeconomic level.

As shown in the Summary of results chapter above, the research questions raised in the four studies have been answered. The coherence in, as well as the impact of the research, will later be discussed in more detail, when discussing the study results.

8.2 Epidemiology

The calculated incidence of patients with MEN2A of 1:66,438 live births per year in Norway during 1965-2015 (**paper I**), which is slightly higher than the incidence reported by Machens et al. (58), involving German patients. They found that the minimum overall incidence of *RET* gene mutation (both MEN2A and MEN2B) over a 50-year period (1951-2000) was 1:100,000-1:200,000 live births per year. However, in the Norwegian study period, which lasted

until 2015, the slightly raised incidence might be an expression of the effect of ongoing genetic screening, thus discovering more MEN2A screening patients. The prevalence (1:84,672) of MEN2A patients in Norway was lower than the incidence. Usually, prevalence is well above incidence as the observed prevalence is a function of several previous years of incidence. However, dealing with an inherited disease, it is generally considered more relevant to calculate incidence per live births than for all person years. With incidence defined as the number of cases per live births, the incidence in **paper I** is the proportion of newborns that carry the *RET* gene mutation. This definition will lead to approximately equal incidence and prevalence in stable populations. Only small changes in time trends or mortality could yield an incidence in **paper I** that exceeds the observed prevalence. In addition, the calculated incidence applies to ages up to 50 years, whereas the prevalence applies to the entire lifetime. With an average annual birth rate of 58,465 in Norway during 1965-2015 (252) together with the incidence of 1:66,438, 0.88 MEN2A individuals carrying *RET* gene mutations would be estimated to be born each year. Given the average life expectancy of 80 years in Norway and the low mortality rate for MEN2A patients, the prevalence can be calculated as 1:73,786. The possibility exists that a few MEN2A patients may have been missed in **paper I**, especially older index patients born before 1965, with low MTC penetrance and MTC not yet diagnosed at the censoring date of the study. However, the number of new families with MEN 2A declined asymptotically in the decades after 1994, suggesting few undiscovered families.

The incidence of MEN2B of 1:482,967 (**paper IV**) was slightly higher than the incidence of 1:700,000, reported by Machens et al. (58), assuming that the majority of the MEN2B patients born in Norway since 1968 are included in the study, and that the selection bias is minimal. However, single MEN2B patients born after 1968 might still be underrepresented. In association with the studies in **paper II and III** concerning all patients diagnosed with MTC since 1994, the Cancer Registry of Norway provided information about all patient names registered with MTC during 1994-2016. Hence, missed MEN2B patients diagnosed after 1994 is not likely. However, since the six patients were born on average every fifth year, and no MEN2B patients have been diagnosed during the last 20 years, two to four MEN2B patients should be anticipated not yet diagnosed.

The population-based study in **paper II** covered 96% of all Norwegian patients treated for MTC from 1994 to 2016. During this period the Cancer Registry of Norway recorded 5610 patients with primary thyroid carcinoma (253), among these, MTC accounted for 4.2% (237 patients) of thyroid malignancies in Norway. The frequency is in the middle of 1-10% as shown in older and more recent studies from other countries (1, 3), and it is slightly higher compared to the frequency of 3.8 % for the time period 1970-1985 in Norway reported by Akslen et al. in 1990 (2). The increased incidence, from 0.18 to 0.25:100,000 person years in the first and second study period referred to in **paper II** is higher than reported in 1990, 0.15:100,000 person years (2). Gatta and the *EUROCORE* group (72) reported in 2006 a world standardized incidence of 0.13 and a calculated incidence between 0.11 and 0.21 per 100,000 person years. A recent publication by Mathiesen et al. (254) included 474 MTC patients diagnosed in Denmark between 1960 and 2014 and recorded a mean age-standardized incidence of all MTC of 0.19 per 100,000 per year, which is the same frequency compared to the Norwegian study discussed in **paper II**. When stratifying the study population into sporadic and hereditary MTC, an incidence of 0.13 and 0.06 per 100,000 per year, respectively, was found. Furthermore, the annual percent change (APC) in incidence for all MTC, sporadic MTC and hereditary MTC was 1.0, 2.8 and -3.1, respectively, and there was no significant changes (254). This reflects a well-functioning genetic screening program in Denmark.

Many studies, both abroad and in Norway, have shown an increased incidence of thyroid carcinomas over the last decades. This also applies to the MTC. In the US, the APC in MTC was reported to be 0.7% and 1.87% during the period from 1974 to 2013 and 1993 to 2012 (73-75). Whether the rise in incidence is due to a more frequent and widespread use of diagnostics, such as ultrasound with FNB and other radiological imaging methods, or if the raise in incidence is a real, are discussed (73, 76-80). The association with obesity and a high body mass index have been observed for all major histological types of thyroid carcinoma except MTC, and were stronger for thyroid cancer mortality than thyroid cancer incidence (73, 82, 83).

8.3 Trends in preoperative diagnostics, treatment and outcome

Preoperative diagnostics in Norway have significantly improved over the last decades. MTC was detected earlier with a higher proportion of tumor stage I disease, and more patients achieved biochemical cure in the second study period compared to the first (**paper II**).

Lobectomy is commonly not recommended. However, occasionally MTC is diagnosed by lobectomy with normalized postoperative calcitonin, and surveillance is preferred above further surgical treatment. In **paper II**, lobectomy was performed more frequently in the second study period and the patients were monitored consistent with the ATA guidelines (3). Few studies regarding safety of lobectomy alone in MTC have been performed. However, a recent retrospective study by Randle et al. (255) compared patients with less than total thyroidectomy with patients with total thyroidectomy, when MTC was restricted to the central neck. They found less multifocal disease and less extrathyroidal extension in the less than total thyroidectomy group. By adjusted, multivariate analysis, the extent of initial resection did not significantly change DSS in patients with MTC, confined to the central neck.

In all patients with dissection of lymph node compartments in **paper II**, the ratio between metastatic and resected lymph nodes was significantly reduced in the second compared to the first study period. However, in the patients with metastatic lymph nodes the ratio was not significantly different between the two periods. This suggest that the extent of the dissected lymph node compartments did not increase, but the number of patients without metastatic lymph nodes (pN0) were significantly higher in the second study period.

To compare time related trends in the study in **paper II** with studies from other countries, a search in PubMed for resent studies concerning time-related trends in diagnostics, treatment and outcome of MTC during the last decades was performed, and the results are categorized in Table 4 (11, 12, 172, 214, 215).

Table 4: Review of the literature regarding trends in diagnostic, presentation, primary treatment and outcome in patients with MTC during the last decades; in recent studies from US, Germany, Italy, Israel, South Korea and Norway¹.

Series	Country	Period	Gender	Age	Tumor diameter	Tumor stage ³	Cured after thyroid surgery	DSS 5 years
	Study type	Included patients in the period	F vs M	Year	mm	SEER (L-R-D)		
	Included patients			Mean (SD) ⁴ or Mean (95%CI) ⁵ or median (range) ⁶	Mean (SD) ⁴ or Mean (95%CI) ⁵ or median (range) ⁶	TNM (I-IV)	Distant disease	
	(n)	(n)	(%) <i>p</i> -value	≤50 vs >50 (%) <i>p</i> -value	≤10 vs >10 (%) <i>p</i> -value	(%) <i>p</i> -value	(%) <i>p</i> -value	% <i>p</i> -value
Randle RW et al (2017) (12)	US	1983-1992 (353)	61 vs 39	50 (19) ⁴	28 (31) ⁴	50 vs 37 vs 12		86
	PB	1993-2002 (793)	59 vs 41	51 (18) ⁴	28 (22) ⁴	50 vs 36 vs 15	N/A	83
	SEER	2003-2012 (1794)	58 vs 42	54 (17) ⁴	26 (30.5) ⁴	52 vs 34 vs 14		89
			<i>p</i> =0.68	<i>p</i> <0.001	<i>p</i> =0.24	<i>p</i> =0.57		<i>p</i> <0.001
Machens A et al (2016) (172)	Germany	995-2000 (38)	50 vs 50	54 (49;58) ⁵	26 (20;33) ⁵		24	53 ⁷
	Single center	2001-2005 (78)	55 vs 45	54 (50;57) ⁵	20 (16;24) ⁵		19	57 ⁷
		2006-2010 (103)	55 vs 45	57 (54;59) ⁵	15 (13;18) ⁵		8	64 ⁷
		2011-2015 (103)	53 vs 47	59 (56;62) ⁵	20 (16;24) ⁵		5	73 ⁷
			<i>p</i> >0.99	<i>p</i> =0.05	<i>p</i> =0.018		<i>p</i> <0.001 ⁸	<i>p</i> =0.0016
Torresan F et al (2018) (11)	Italy	1980-2000 (87)	56 vs 44	57 vs 43	22 vs 78	I-II vs III-IV		Died of MTC
	Single center	2001-2015 (168)	56 vs 74	48 vs 52	40 vs 60	49 vs 51		30
			<i>p</i> =0.95	<i>p</i> =0.16	<i>p</i> =0.003	70 vs 30		8
						<i>p</i> =0.0016	<i>p</i> <0.0001	<i>p</i> <0.001

Hirsch D et al (2018) (214)	Israel	1981-1995 (43)	60 vs 40	39 (17) ⁴	26 (19) ⁴	35 vs 65 ⁹	27 ¹⁰	81
	Multi-Center	1996-2005 (54)	56 vs 44	51 (18) ⁴	27 (18) ⁴	41 vs 58 ⁹	43 ¹⁰	83
		2006-2016 (85)	52 vs 48	54 (18) ⁴	24 (18) ⁴	33 vs 67 ⁹	48 ¹⁰	92
			NS	<i>p<0.001</i>	NS	NS	NS	NS
Jung KY et al (2016) (215)	Korea	1982-2000 (71)	61 vs 39	41 (13) ⁴	25 (13) ⁴		11 ¹¹	40 ¹²
	Multi-Center	2001-2005 (55)	65 vs 35	46 (13) ⁴	24 (16) ⁴		6	49 ¹²
		2006-2010 (132)	64 vs 36	50 (14) ⁴	16 (13) ⁴		7	74 ¹²
		2011-2012 (73)	63 vs 37	51 (15) ⁴	17 (16) ⁴		8	76 ¹²
			<i>p=0.952</i>	<i>p<0.001</i>	<i>p<0.001</i>		<i>p=0.726</i>	<i>p<0.001</i>
Opsahl et al (2018)¹ (Paper II)	Norway	1994-06/2005 (73)	48 vs 52	52 (7-82) ⁶	20 (2-75) ⁶	15-13-11-61		35 ¹⁴
	PB							
	NW	07/2005-2016 (128)	63 vs 37	57 (6-84) ⁶	20 (1-125) ⁶	35-19-10-36		58 ¹⁴
			<i>p=0.045</i>	<i>p=0.051</i>	<i>p=0.661</i>	<i>p=0.004</i>	<i>p=0.002</i>	<i>p=0.172</i>

All numbers, % and SD are reported without decimals and adjusted to the nearest number, consequently the total will be 99% or 101% at some occasions. PB: Population based. SEER: Surveillance, Epidemiology, and End Results Program. NW: Nationwide. DSS: Disease specific survival. N/A: Not applicable. NS: No significance.

¹ Included in the thesis. ² Total thyroidectomies with any lymph node dissection. ³ Stage - definition: SEER: Local / Regional / Distant - TNM (Table 2). ⁴ Mean (SD). ⁵ Mean (95%CI) ⁶ Median (range). ⁷ Not available information in 8, 6, 6 and 8 patients. ⁸ Significant after Bonferroni correction for multiple testing. ⁹ Available information regarding tumor stage in 29/43, 50/54 and 79/85 patients respectively. ¹⁰ No available information in 6/43, 3/54 and 10/85 patients. ¹¹ No available information in 9, 6, 4 and 0 patients in the respective four groups. ¹² Information about biochemical cure was only available in patients diagnosed after 1994, i.e. no available information in 23, 4, 11, 2 patients, and *p<0.001*, compared to 1982-2000. ¹³ Information available in 180 patients. ¹⁴ No information in 4 and 3 patients respectively.

In the Norwegian study (**paper II**), the disease control was improved over time. The patients with MTC were diagnosed at an earlier tumor stage ($p = 0.004$) and more patients became biochemically cured ($p = 0.002$) in the second compared to the first study period. The patients were older at diagnosis with borderline significance ($p = 0.051$), and there were more female patients with MTC in the second than in the first study period ($p = 0.045$).

Furthermore, the 5-year DSS was improved from 84% in the first study period to 90% in the second but the difference was not statistically significant ($p = 0.172$), perhaps due to a shorter follow-up in the second study period.

The trends in the Norwegian study coincide with other reported studies in Table 3. Disease control improved over time in four of the studies (11, 12, 172, 215), there was reduced tumor diameter and / or tumor stage at time of diagnosis in three of the studies (11, 172, 215) and finally, the patients were older at time of diagnosis in four (12, 172, 214, 215). Furthermore, in the previous mentioned epidemiological study by Lim et al. (74) from the US, the annual incidence-based mortality rate in MTC also dropped during 1994-2013 with an APC like -0.7%. The trends in diagnostics- and treatment outcome in Norway are completely in line with international trends, showing a worldwide improvement in diagnostic environments with earlier detection of the disease and thus a better outcome.

8.3.1 Diagnostic centers – Calcitonin screening

During the last decades, new diagnostic centers for patients with nodular thyroid disease offer ultrasound of the neck, FNB and biochemical- and clinical evaluation by a multidisciplinary team, yielding a better diagnostic accuracy (256). This again results in early diagnosis at a lower tumor stage as well as a raise in incidence. Further, the widespread use of ultrasound might be a major causal factor to initiate elucidation of small thyroid nodules. In **paper II**, ultrasound before surgery was a significant prognostic factor in favor of biochemical cure by univariate analysis, but not by multivariate analysis in the selected group of patients with metastatic lymph nodes.

As mentioned in **paper II**, calcitonin analysis was performed more frequently in the second study period (73%) compared to the first (55%). Ideally, calcitonin in 100% of patients

with MTC should have been achieved. Interestingly, preoperative calcitonin analysis was by univariate analysis significantly more frequently performed in the not biochemical cured patients compared to the cured ($p = 0.004$). Hence, patients with MTC randomly discovered might have less severe disease. Performed calcitonin analysis was not a significant prognostic factor in favor of biochemical cure by multivariate analysis. As outlined in the Introduction chapter, the sensitivity of calcitonin analysis today is better than ultrasound and FNB with cytological examination, and assessments concerning calcitonin screening at the time of diagnostic evaluation of thyroid nodules have been made (7, 172-174). The benefit of diagnosing MTC at lower tumor stage is more successful treatment. Knowledge of the calcitonin levels before surgery also influence surgical strategy (7, 8). As further outlined in the Introduction chapter, the disadvantages of calcitonin screening are the possibility of false positive elevated calcitonin levels, which can lead to unnecessary thyroidectomies. Furthermore, as multiple analyses have to be performed to diagnose patients with MTC, it raises the question of cost-effectiveness. However, a study by Cheung et al. (173) concluded that routine serum calcitonin in patients undergoing evaluation for thyroid nodules is assessed to be cost effective in the US. The national guidelines in Norway do not recommend calcitonin screening in general, but calcitonin analysis should be considered for all patients with thyroid nodules undergoing thyroid surgery (150). There are no prospective randomized trials evaluating the efficacy of serum calcitonin screening to standard evaluation of patients with nodular goiters. The ATA guidelines contain no strict recommendation (3). However, the European Thyroid Association Cancer Research Network and European Panel of Experts meeting 2009 (175) suggest that preoperative measurement of calcitonin should be mandatory in all patients with thyroid nodules for whom surgery has been indicated. The recent study by Frank-Raue et al. (257) recommend calcitonin screening in nodular goiter, as it helps to identify the rare MTC at an early stage.

There is a possibility of false positive elevated calcitonin levels, and recent studies have evaluated the basal serum calcitonin cut-off thresholds for diagnosing MTC. Allelein et al. (258) found that the most precise basal calcitonin thresholds for the identification of MTC were ≥ 10.2 pmol/L (35 pg/mL) in females (sensitivity: 87.3%, specificity: 87.5%, PPV: 98%, NPV: 50%) and ≥ 13.5 pmol/L (46 pg/mL) in males (sensitivity: 93.6%, specificity 95.0%, PPV 97%, NPV 90%). Frank-Raue et al. (257) found the best cut-off values to differentiate micro-MTC from

CCH and other unspecific calcitonin elevations as 8.8 pmol/L (30 pg/mL) threshold in females and 17.6 pmol/L (60 pg/mL) in males. Based on this study, the Thyroid Section of the German Society of Endocrinology recommends thyroidectomy in women at serum calcitonin values > 8.8 pmol/L (30 pg/mL) (grey zone 6-8.8 pmol/L (20-30 pg/mL)) and in men at serum calcitonin values > 17.6 pmol/L (60 pg/mL) (grey zone 8.8-17.6 pmol/L (30-60 pg/mL)). Calcitonin of lower values should be re-evaluated at 3-6 months intervals, and with rising calcitonin levels, MTC is likely. However, it is necessary to bear in mind that the cut-off values might vary according to the different assays used. In **papers II and III** it was the population of patients with diagnosed MTC that was evaluated and not a population of patients with a variety of thyroid nodules. Recommendations concerning calcitonin screening based on these studies can not be made. However, in **paper I** concerning MEN2A, the median preoperative calcitonin levels in the patients with MTC was 52 pmol/L (range 1.1-2048 pmol/L), (177 pg/mL (range 3.7-6999)), with the lowest measured calcitonin value of 1.1 pmol/L (3.7 pg/mL). Metastatic lymph nodes were not found in patients with preoperative calcitonin < 20 pmol/L (68 pg/mL). However, in **paper III**, concerning both hereditary and sporadic MTC, 13 out of 94 patients (14%) with MTC had preoperative calcitonin ≤ 20 pmol/L (68 pg/mL) with median calcitonin value 4.7 pmol/L (range 1.1-20) (16 pg/mL (range 3.7-68)), meaning that seven of these patients had calcitonin levels ≤ 4.7 pmol/L (16 pg/mL). Metastatic lymph nodes were found in three out of the 13 patients (23%) with calcitonin values ≤ 20 pmol/L (68 pg/mL). In the era of calcitonin screening, and in the patients with slightly elevated calcitonin and no disease found at ultrasound of the neck, thyroidectomy performed at the recommended cut-offs outlined by Allelein et al. (258) and Frank-Raue et al. (257) are relevant guidelines. However, as shown in **paper I and III**, there is wide grey-zones, and most importantly, if healing is to be achieved, the thyroidectomy must be performed before metastatic lymph nodes occur.

8.3.2 Genetics

Compared to reports from abroad, Norwegian MEN2A patients (**paper I**) had a predominance of lower risk in the ATA A and B groups (ATA moderate), and fewer patients in the higher risk ATA C group (ATA high) (23, 92, 95, 100). When analyzed alone, the *RET*-era group had an even higher proportion of ATA A cases relative to when all patients were considered. For the pre*RET*-era group, MTC in low risk MEN2A (FMTC) patients was more

likely to be misclassified as sporadic MTC compared to the *RET*-era patients. For these reasons, making comparisons between results for the patients in this study with those from older reports may not be right. This has also been illustrated in a recent thematic review by Machens and Dralle (251) based on major studies by Eng et al. (1996) (95), Frank-Raue et al. (2010) (259) and Machens et al. (2013) (260). More penetrant *RET* mutations as, ATA high and highest, were overrepresented in early MEN2 studies as the classification was based on phenotype. In the molecular *RET*-era, the classification is more based on *RET* mutation analysis, since these are offered to almost every patient with MTC nowadays. Consequently, the distribution between the different ATA risk groups are more correct, and misclassification of the ATA low risk MEN2A as sporadic MTC is less likely. Comparing with a recent Danish study by Mathiesen et al. (261) there was a disproportionately high number of codon 611 mutation in 13 families. In Norway, only two families were found with codon 611 mutation and in one the index patient was living in Denmark, and the patient was not included in the **paper I** study.

One family referred to in **paper I** carried a single p.Q781R mutation. According to the 2015 ATA Guidelines (3), a double *RET* mutation in the same allele involving *RET* codon 804 (p.V804M) and the *RET* mutations p.Y806C, p.S904C, p.E805K, or p.Q781R were reported as being atypical MEN2B. Similar germline double mutations in *RET* have also been identified in families with a FMTC variant of MEN2A (3). Meanwhile, a study by Maschek et al. (262) reported a patient with a single p.Q781R mutation in *RET*, while Nakao et al. (263) reported both a single p.Q781R and double mutation with p.Q781R/p.V804M in the same family. Based on these findings, stored blood samples from the Norwegian patients who carried the p.Q781R *RET* mutation were re-analyzed (**paper I**). Each of the three patients carried a double *RET* mutation in the same allele that involved p.Q781R and p.S904C, but not p.V804M. However, the clinical consequences were not influenced. This double mutation has not previously been reported, and thus should be further evaluated. At primary surgery all three patients had MTC with a phenotype of MEN2A, and thus this *RET* mutation was classified as ATA A (ATA moderate) (3, 263).

Previous studies have reported that double *RET* mutations might change the clinical phenotype of the corresponding single *RET* mutation (263, 264). Patients with a single p.V804M

or p.Q781R mutations presented with the MEN2A or FMTC (now included in MEN2A) phenotype (262, 265). Patients with p.V804M mutation combined with mutations in p.Y806C, p.S904C, p.E805K or p.Q781R, presented with MEN2B phenotype (105-107, 263) with development of MEN2B at 20 to 30 years of age, but with more aggressive MTC (3). Furthermore, in patients with double mutation in p.V804M combined with p.V778I or p.R844L the phenotype was classified as FMTC (now MEN2A) (266, 267). However, in the family with p.V804M/p.V778I mutation, the patients had corneal nerve thickening which might be present in MEN2B patients. These reports illustrate that in some patients with single or double mutation, classification according to phenotype might differ and is difficult. In addition, patients with p.A883F mutation in MEN2B have a more indolent form of MTC than patients with codon p.M918T mutation (268, 269). Based on the current studies, patients with p.A883F mutations have been moved from the ATA highest-risk to the ATA high-risk, which actually is MEN2A (3).

It is recommended to offer *RET* mutation analysis to all patients with MTC (3, 150). The number of patients with MTC having *RET* mutation analysis referred to in **paper II** improved significantly in the second study period compared to the first (85% vs. 66%). In a recent nationwide study from Denmark 2019 by Mathiesen et al. (270), the proportion of *RET* gene testing in the overall MTC cohort during 1997-2013 was 87%, and in the adjusted cohort after excluding patients diagnosed with hereditary MTC by screening, the percentage was 83%.

8.4 Timing and extent of thyroid surgery – Predictors

To obtain biochemical cure, surgery should be performed at an early stage of the disease and to a sufficient extent (3, 4, 8, 13).

8.4.1 Prophylactic thyroid surgery in MEN2A patients – Guided by calcitonin levels

Major studies have been carried out to identify indicators predicting the optimal timing and extent of prophylactic thyroid surgery (27, 28). The treatment recommendations of American Thyroid Association (ATA, 2009/2015) (3, 21) are presented in Table 1.

Predictors for discovering MTC development were evaluated in **paper I**. Adjusted, multivariate analysis were performed. When analyzing either the *RET*-era patients alone or the patients in the pre-*RET* and the *RET*-era altogether, preoperative basal serum calcitonin was a predictive factor for MTC superior to age at time of thyroid surgery. Bringing in genotype in the analysis, preoperative calcitonin was not statistically significant but had the highest significance in the analysis. The loss of statistical significance when analyzing the three parameters together might be due to the small number of patients, type II error. Nevertheless, the results rendered in **paper I** support that preoperative basal serum calcitonin level is the main guiding factor for best timing of thyroid surgery, which is consistent with findings in a French cohort, reported by Rohmer et al. (98). In addition, the prospective study by Elisei et al. (27) was the first study that discussed the relevance of serum calcitonin in the management of *RET* gene carriers. Among the surgically treated patients with detectable basal serum calcitonin, intrathyroidal tumors were found when the calcitonin levels were below 17.5 pmol/L (60 pg/mL), whereas either metastatic lymph nodes or larger tumors were observed when the calcitonin levels exceeded 17.5 pmol/L (60 pg/mL). No correlation between serum calcitonin level, age, and *RET* mutation type was observed. This study concluded that personalized timing of thyroid surgery could be based on serum calcitonin levels (27), which correlates with the results and conclusion in **paper I**.

In **paper I**, all patients with metastatic lymph nodes at the time of surgery had preoperative basal serum calcitonin levels ≥ 20 pmol/L (68 pg/mL), and all patients who have not had lymph node resection in the central neck compartments and with preoperative basal calcitonin levels below 11.7 pmol/L (40 pg/mL) were biochemically cured, assuming that these patients were without metastatic lymph nodes. These results correlate with the study by Schreinemakers et al. (100), where none of the patients with metastatic lymph nodes at primary surgery had a basal serum calcitonin level below 11.7 pmol/L (40 pg/mL). Furthermore, the same result was found by Machens et al. (9), showing a cumulative risk of metastatic lymph nodes at primary surgery at basal calcitonin levels above 11.7 pmol/L (40 pg/mL). Metastatic lymph nodes have not been reported at calcitonin levels ≤ 8.8 pmol/L (30 pg/mL) (98, 250). In the 2015 ATA guidelines (3) the classifications and recommended age at thyroid surgery were adjusted (Table 1). Because of the high sensitivity of monomeric calcitonin measurements, the time of thyroid surgery should be based on non-stimulated serum calcitonin levels alone. Furthermore, the ATA

Guidelines (3) also recommend neck dissection in the central compartments in patients with a calcitonin levels above 11.7 pmol/L (40 pg/mL) since lymph node metastasis rarely occurs when serum calcitonin is below this level (3, 9, 100). The results in **paper I** coincide with these recommendations.

As a summary of **paper I**, basal serum calcitonin is the main guidance for best timing of thyroid surgery. Thyroidectomy should be performed when basal serum calcitonin levels are elevated but before metastatic lymph nodes occur, that is ≤ 20 pmol/L (68 pg/mL) in **paper I**, and ≤ 8.8 -11.7 pmol/L (30-40 pg/mL) recommended by other authors (3, 98). Recent reviews by Machens and Dralle (251) and by Raue and Frank-Raue (250) support the same recommendations. Optimal timing for thyroid surgery is provided by early identification of *RET* gene carriers in childhood with risk assessment and analysis of serum calcitonin. Further, the patients are monitored until thyroidectomy is performed. That is when the serum calcitonin level is elevated, but before the occurrence of metastasis. Thus, biochemical cure can be achieved (250, 251, 271). First screening of *RET* gene mutation should be performed at less than one year of age for patients in highest-risk group, one to five years for high-risk group and five years for moderate-risk group (98, 250).

In patients with high-risk mutations in *RET* gene codon 634, penetrance variate. MTC might occur very early in life and the physician must be alert. The ATA guidelines (3) recommend thyroid surgery at or before 5 years of age based on serum calcitonin levels. In **paper I**, 20 (7 index-and 13 screening patients) out of 60 surgical treated MEN2A patients (33%) had codon 634 mutations. MTC was found in 18 (90%) of these patients. However, by multivariate analysis, preoperative calcitonin was significant predictive factor for MTC prior to age at thyroid surgery ($p = 0.009$). Furthermore, significant predictors for MTC were age prior to genotype ($p = 0.024$) and preoperative basal calcitonin prior to genotype with borderline significance ($p = 0.057$). In a study by Bussires et al. (2018) (272), including 19 MEN2A patients with thyroidectomy before 18 years of age, eight patients had a high risk mutation (in codon 634). All the patients had MTC and median age at time of surgery was 4.6 years (range 2.4-10.8). However, the preoperative calcitonin level was elevated in all. Furthermore, in a study by Machens et al. (2018) concerning disease progression of genotype-specific hereditary MTC,

including 184 patients with MTC and high-risk codon 634 mutation, the mean age at thyroidectomy was 19.0 years (95%CI [15.8;22.2]) (range 1-69) in patients without and 31.1 years (95%CI [27.8;34.4]) (range 9-63) in those with metastatic lymph nodes. In a recent study by Voss et al. (273), patients with high-risk and moderate-risk *RET* mutations were found to have similar OS and development of distant metastasis after diagnosis, and therefore a similar aggressive clinical course. The authors concluded that high-risk connotes increased disease aggressiveness, and therefore the *RET* mutation classification should be by disease onset (early vs late) rather than by risk (high vs moderate). However, these facts are related, as the MTC onset in patients with high-risk codon 634 mutation is generally earlier than MTC onset in patients with moderate-risk (272, 274). In **paper I**, genotype was not found to be a prognostic factor for postoperative biochemical cure. However, low number in each variable as well as a higher number of variables analyzed, might have caused statistical type II error.

8.4.2 Phenotype and thyroid surgery in patients with MEN2B

MTC in MEN2B develops already during the first year of life (23, 275), and the cure rate dramatically drops in patients treated later than 4 year of age (32, 275). Thus, recognition of the non-endocrine MEN2B phenotype in very young children, and before MTC becomes incurable is the most important factor for disease control in MEN2B patients. Hence, surgery should be performed as early as possible. The classic nevinomas and habitus are hardly visible before 4 year of age but, chronic obstipation caused by IGNM, failure to thrive and tearless crying represent MEN2B symptoms consistently expressed early, and during the first year of life (32, 110). The former ATA 2009 guidelines (21) recommend that differential diagnosis in patients with IGNM should include MEN2B, and ganglioneuromatosis histology (Figure 7) indicate germline *RET* gene testing.

Based on a very recent multicentric study by Castinetti et al. (275), including 345 MEN2B patients, it was concluded that thyroidectomy performed before one year of age is associated with a high probability of cure. However, the reality is that the majority of children with MEN2B will be diagnosed after this recommended age. Thus, it was confirmed that every health-care provider should be aware of the extra-endocrine features as well as the recommendations for

thyroidectomy before the age of one year. They found that all patients with available data ($n = 287$) had at least one extra-endocrine feature.

Figure 7:
Intestinal ganglio-
neuromatosis
(IGNM).

Histologic slide,
imaging the
intestinal neurinoma
(arrow)

*Informed
written consent
is available*

As reported in **paper IV**, all the patients presented with the classical phenotype. Nевrinomas were not observed before 4 year of age, but obstipation or megacolon were observed early in life in two of the study patients. The study by Castinetti et al. (275) found that MTC-specific survival curves did not show any significant difference between patients who had thyroidectomy before and after the age of one year ($p = 0.2$), but the difference was significant in disease remission between the two study groups ($p < 0.0001$). Recent studies by Brauckhoff et al. (32) and Raue et al. (250) have demonstrated that the ability to diagnose MEN2B at an earlier age and an earlier tumor stage have improved during the last years. (32, 250).

8.4.3 Prophylactic lateral neck dissection – The role of calcitonin levels

In patients with MTC, high-resolution ultrasound is important in disease staging, enabling the detection of even very small metastatic lymph nodes, although not micro-metastasis. Calcitonin is a good tumor marker in the management of MTC. The increase in calcitonin values seems to be proportional to tumor load, making the hormone a good marker for pre-therapeutic staging and surgical strategy planning (7-9).

The relation between preoperative basal serum calcitonin levels and tumor stage, and the ability of calcitonin level to predict the extent of surgery needed in the lateral neck compartments in patients with MTC, were evaluated in **Paper III**. Preoperative levels of calcitonin were significantly related to the extent of disease comprising primary tumor size, tumor stage, the numbers of metastatic lymph nodes in the neck and metastatic systemic disease. Metastatic lymph nodes in the lateral neck compartments had negative prognostic impact. However, based on this study a clear cut-off in calcitonin value could not be predicted and forecast the extent of surgery needed in the lateral neck compartments, as patients with calcitonin ≤ 500 pmol/L (1709 pg/mL) had metastasis in the lateral neck, whereas other patients with calcitonin > 1000 pmol/L (3417 pg/mL) did not. Hence, in patients with MTC and without distant metastasis, other factors might influence the calcitonin level in addition to the extent of disease in the neck.

Major studies have been carried out in order to identify calcitonin cut-off that could predict the need for prophylactic dissection of lymph node compartments in the lateral part of the neck (8, 204). In patients with normal preoperative neck ultrasound, elective prophylactic surgery in the central and ipsilateral lateral neck compartments at serum calcitonin > 5.9 pmol/L (20 pg/mL) and in the contralateral lateral neck compartments at calcitonin levels > 59 pmol/L (200 pg/mL) have been suggested (8). In a study by Chandese et al. (204), ipsilateral lateral metastatic lymph nodes were found at a preoperative calcitonin level of 11 pmol/L (38 pg/mL). Hence, the ATA guidelines of 2015 (3) recommend consideration of prophylactic surgery in the lateral neck compartments based on serum calcitonin levels in patients without distant metastasis, but the Task Force did not achieve consensus regarding this recommendation. Guidelines from the United Kingdom (14) recommend ipsilateral prophylactic lateral neck dissection based on the presence of metastatic lymph node lode in the central neck compartments. Machens et al. (16) found that the presence of up to three metastatic lymph nodes in the central neck compartments, or more than four metastatic lymph nodes, predicted risk of metastatic lymph nodes in the ipsilateral lateral neck compartments in 77% and 98%, respectively. In the study in **Paper III**, the median number of metastatic lymph nodes in the N1a-NPDL group was one (range 1-6).

Paper III established that extrathyroidal tumor extension was significantly predictive for metastatic lymph nodes in the lateral neck. In a study by Oh et al. (15) ultrasound features in the

primary tumor at preoperative neck ultrasound were evaluated. They showed that patients with larger tumors of irregular shape with spiculated margins and a subcapsular location were more likely to have metastatic lymph nodes in the lateral neck compartments. These features can represent extrathyroidal extension. These results are confirmed in **paper III**.

Preoperative ultrasound of the neck in evaluation of thyroid nodules was performed in all the study patients in **paper III**. High-resolution ultrasonography in experienced hands is the most valuable method for detecting metastatic lymph nodes in the lateral neck compartments, in both papillary and medullary thyroid carcinomas (185-187). However, a negative examination does not rule out a possible presence of micro-metastasis in the lymph nodes. Kocharyan et al. found that preoperative ultrasonography had a PPV of 85.4% in detecting metastatic lymph nodes in the lateral neck compartments (187).

Although metastatic lymph nodes might be found in the lateral neck compartments despite a low preoperative calcitonin value, preoperative calcitonin level does not offer strict guidance for when to operate. As reported in **paper III**, 28% of the patients without metastatic lymph nodes in these compartments, had preoperative basal calcitonin levels above 500 pmol/L (1709 pg/mL). Further, as outlined in the Introduction chapter, dissection in the lateral compartments includes risk of surgical complications with nerve injuries as the most severe among these (206, 207). The dilemma then is the possible presence of undetected micro-metastasis versus the risk of morbidity due to surgery. Hence, in patients without any sign of distant metastasis, prophylactic lateral neck dissection should be considered as a second operation, if biochemical cure is not achieved after primary surgery. Polistena et al. (276) concluded that postponed surgery of the lateral compartments is a valid option in order to limit unnecessary complications without affecting adequate lymph node clearance. However, the level of preoperative calcitonin together with assessment of nodal involvement of the central compartment compensate the diagnostic limits of ultrasound (276).

Other and recent studies provide concurrent issues and results as discussed in **paper III**. In a study by Pena et al. (277) the outcome in patients with MTC and no radiological evidence of metastatic lymph nodes in the lateral neck, was evaluated. Patients with no lymph node removal

were compared with patients who had undergone prophylactic lymph node clearance in the lateral neck compartments. There was no significant difference in biochemical cure rate and 5-year OS in the two groups. On the other hand, in a study by Fan et al. (278) it was found that only thyroid capsule invasion was independent significantly predictor for metastatic lymph nodes in both the central and lateral compartments, and they recommend prophylactic lateral neck dissection at thyroid capsule invasion. The study also showed a high incidence of cervical metastatic lymph nodes at CEA levels above 30 µg/L.

Studies have shown that desmoplastic stroma reaction (DSR) as a possible predictor for disease aggressiveness and lymph node metastasis in the lateral neck compartments (132, 133). Scheuba et al. (132) found patients without DSR and tumor stage pT2, lacking metastatic lymph nodes in these compartments. Hence, they proposed to avoid dissection in the lateral compartments in MTC patients without DSR. One can assume that patients with larger tumors without DSR by itself have high calcitonin values, and the calcitonin level is not reflecting the extent of the disease in the lateral neck compartments but the tumor diameter. The relations between no DSR, tumor diameter and calcitonin levels should be further investigated. Aubert et al. (279) found that the abundance of desmoplastic stroma and high levels of miR-21 expression, but not preoperative levels of calcitonin, were strong independent indicators for metastatic lymph nodes in patients with large sporadic MTC, despite no preoperatively proven metastatic lymph nodes. Furthermore, they outlined that DSR can be evaluated on frozen sections with perioperative guidance for the extent of surgery (279). However, multivariate analysis did not show a differentiation between metastatic lymph nodes in the central and lateral parts of the neck. As patients with large MTC routinely will have lymph node resection in the central neck, it is recommended to evaluate DSR in the final histological examination. Further, in patients with no satisfactory drop in calcitonin level after primary surgery together with DSR presence, a prophylactic lateral neck dissection in a second séance should be indicated.

8.5 Prognostic factors for disease-control in MTC

Prognostic factors for biochemical cure and DSS in MTC have been widely studied the last decades, and age and tumor stage at time of diagnosis have been found to be the main significant prognostic factors (1, 10, 11).

8.5.1 Age, histopathological factors and calcitonin

In order to minimize both time-and biological biases when analyzing the prognostic factors that determine biochemical cure in the MEN2A patients (**paper I**), they were grouped according to different time periods and biology. When adjusted for time bias and examined by multivariate analysis with a reduced number of parameters, only metastatic lymph nodes (pN) at primary surgery, representing the extent of malignancy, was a statistically significant independent prognostic factor for not achieving biochemical cure in patients analyzed in the *RET* era groups. In addition, when data from all patients in both the pre-*RET* era and the *RET* era period, and all patients with MTC were analyzed, performed central neck dissection at primary surgery was found to be a negative prognostic factor for receiving biochemical cure. This fact reflects the tumor stage, as MEN2A patients with none or a less severe MTC, more infrequently have undergone selective neck dissection. However, the significance was not persistent when the analyzes were adjusted for time bias. When all the patients in the entire study period were analyzed, it was found that an increasing preoperative serum calcitonin level was a significant prognostic factor for not obtaining biochemical cure. However, this was not the case when the cohort was adjusted for time bias. In other reports, prognostic factors for biochemical cure in MEN2A were genotype, preoperative calcitonin level, and age at thyroid surgery. But, the most important predictor of survival was tumor stage at diagnosis (1, 3, 4, 21, 99, 100). This includes the presence of metastatic lymph nodes, which concurs with the results in **paper I**.

In **paper II**, when analyzing both hereditary and sporadic MTC, the tumor diameter was not found to be prognostic factor for outcome, neither for biochemical cure nor DSS. Kazaura et al. (126) concluded that micro-MTC does not always mean an early tumor stage, and that smaller tumors have significant rates of poor prognostic features with impact on survival. The study in **paper III** showed a significant relation between tumor diameter < 20 mm and preoperative calcitonin < 500 pmol/L (1709 pg/mL). Furthermore, 30% of the patients in the prognostic groups without metastatic lymph nodes in the lateral neck compartments, had tumor diameter > 20 mm. In **paper III** it is concluded that tumor diameter did not predict postoperative biochemical cure. The only independent prognostic factor for biochemical cure found in this study was presence of metastatic lymph nodes laterally in the neck.

Extrathyroidal extension was an independent prognostic factor for biochemical cure in **paper II**. In a study by de Groot et al. (85) and one by Modigliani et al. (10), extrathyroidal extension and disease stage were found to be the only prognostic factors for biochemical cure. A recent, nationwide study from US, Youngwirth et al. (280) included 3415 patients with MTC from 1998 to 2012. The study showed that patients with extensive extrathyroidal tumor extension were experienced a reduced OS, compared to those with no extrathyroidal extension. But, minimal extrathyroidal extension was not significantly associated with reduced OS. Based on these findings it is suggested that the handling of extrathyroidal extension should be included in the thyroid cancer treatment guidelines. In the 8th version of TNM (123), the earlier pT3 is divided in pT3a and pT3b, as pT3b is a tumor of any sizes with gross extrathyroidal extension (Table 2). In a study by Ito et al. (2018) (281), extrathyroidal extension was independently correlated with distant recurrence. Furthermore, extrathyroidal extension, tumor diameter > 4 cm, and M1 status each and one independently affected carcinoma-related mortality.

In **paper III** 36 patients had preoperative calcitonin levels between 21 and 500 pmol/L (72-1709 pg/mL), median 152 pmol/L (519 pg/mL). Of these, 22 (61%) of 36 patients achieved biochemical cure. However, there was no significant difference between the cured and not cured patients with regard to preoperative calcitonin levels. Machens et al. (9) showed that preoperative basal calcitonin levels greater than 146 pmol/L (500 pg/mL) best predicted the failure to achieve biochemical remission. Furthermore, Jung et al. (215) found that postoperative biochemical cure, defined as calcitonin < 3 pmol/L (10 pg/mL), was the best predictor for recurrence-free survival, as 94.7% of the patients in whom biochemical cure was achieved, remained so at follow-up.

In a Danish study published in 2019, Mathiesen et al. (282) evaluated patients with both hereditary and sporadic MTC. They found that patients with hereditary MTC diagnosed by screening, patients without regional metastases and patients with stage I,II and III disease may have survival rates as the population in general. In addition, the presence of metastases predicted reduced DSS, while absence of regional metastases predicted long-term biochemical cure. These results coincide with the results in **papers II and III**.

In a recent study by Jayakody S et al. (283) it was found by multivariate analysis that preoperative calcitonin values together with age, venous invasion and MEN2 status were significant independent predictors for disease-free survival. Patients with sporadic MTC were older and had a poorer prognosis. However, the study did not distinguish between index- and screening MEN2A patients and no MEN2B patients was included. These facts constitute a bias concerning evaluating the difference in disease aggressiveness between hereditary and sporadic MTC. The results point at the value of well-functioning genetic screening programs, detecting the *RET* gene mutation carriers at a younger age followed by surgery at a lower tumor stage.

Advanced MTC combined with low preoperative calcitonin level was found in the study population (**paper III**). Dedifferentiation in advanced MTC with loss of ability to produce calcitonin has been reported (284). However, Frank-Raue et al. (285) found prognostic heterogeneity in calcitonin negative MTC patients, from long-term survival to rapid progressive disease at simultaneously high Ki-67 and somatic *RET* p.M918T cell-mutation. Ki-67 and somatic *RET* p.M918T mutation were not evaluated in the patients with advanced MTC in **paper III**.

According to previous knowledge, MTC in MEN2B correlates with poorer prognosis in MTC, mostly related to late diagnosis at more advanced tumor stage (3, 32). The MEN2B patients studied in **paper IV** were diagnosed with MTC at tumor stage IV at the median age of 12.5 years (range 8-34). At the latest date of study observation, July 1st 2018, the six MEN2B patients were alive with slow progressive disease at median age of 26 years (range 19-49). Median follow-up was 166 months (range 20-354). Also Yoshimoto et al. (286) found less aggressive tumor progression in 23 Japanese MEN2B patients, with 92% 10 years survival rate. Furthermore in the study by Raue F et al. (287) from 2018 evaluating 68 patients with *de novo* MEN2B and 7 patients with familial MEN2B, the cancer-related survival rates at 5, 10 and 20 years were found to be 85, 74 and 58% respectively. Significant more patients had tumor stage I and II, and more patients were cured with a higher survival trend, with borderline significance ($p = 0.058$), after the year of 2000 compared to before (287).

8.5.2 The ratio between metastatic and resected lymph nodes

In the patients with metastatic lymph nodes (**paper II**), the number of metastatic lymph nodes was significantly higher in the patients without biochemical cure compared to the cured patients. The number of resected lymph nodes, however, was not significantly different between the two groups. Furthermore, by multivariate analysis the ratio between metastatic and the total number of resected lymph nodes was found to be a significant prognostic factor for both biochemical cure and DSS. This validates the importance of meticulous dissection of neck compartments to achieve a low ratio in patients with metastatic lymph nodes. The more lymph nodes resected, the less risk of leaving metastatic lymph nodes behind. This is consistent with other studies, emphasizing the importance of systematic and meticulous neck dissection in patients with MTC (201-203). In population-based, but not nationwide study by Qu et al. (288), a higher ratio was found to predict inferior survival. Leggett et al. (289) observed likewise, but not in analyzed subgroup of patients with metastatic lymph nodes. In a very recent population-based study by Meng et al. (290), using the SEER 18 database, included 1466 patients with MTC during from 1998 to 2013, the number of metastatic lymph nodes showed prognostic power in survival analysis with significantly reduced DSS. Age and tumor size were also the most important prognostic factors in MTC DSS, according to this study.

8.5.3 Molecular biology in MTC

The *RET* gene mutation leads to gain of function with increased downstream signaling in both MAPK/ERK and PIK3CA/AKT/mTOR signaling pathways (86, 87, 89). In a study by Murakami et al. it was found an association between the clinical phenotype of MEN2B and higher levels of activation of *PIK3CA* and phosphorylation of its downstream signaling molecules, when comparing to MEN2A (108). Very few MEN2B patients have been included in previous studies analyzing cancer driver genes in MTC (40-42). The study in **paper IV** was undertaken to evaluate the clinical course of MTC in relation to possible coexistence of somatic CDMs, in *HRAS*, *KRAS*, *NRAS*, *BRAF*, *PIK3CA* or *AKT1*, that might affect disease aggressiveness. However, no mutation in *PIK3CA* or in the other cancer driver genes were found. This finding corresponds to earlier studies including very few MEN2B patients (40-42).

In sporadic MTC, *BRAF* mutation was found by Goutas N et al. (43) in 68.2% in the MTC samples tested. *HRAS*, *KRAS* or rarely *NRAS*, have been found to a varying degree, in 18-80%, and in sporadic MTCs lacking the somatic *RET* mutation (3, 37, 39, 41). However, Moura et al. (39) found *HRAS* mutations in one sporadic *RET*-positive patient. It seems that somatic *RET*- and *RAS* mutations are mutually exclusive, with *RET* mutations seen in more aggressive MTC and *RAS* mutations in less aggressive (37, 39, 41, 44). Recent results from exome sequencing indicate that, besides mutations in *RET*, *HRAS* and *KRAS*, no other recurrent driver mutations were present in MTC (44, 87). In the previously mentioned study by Jayakody et al. (283), *HRAS* p.A61R was analyzed by IHC in tumor tissue from 18 patients with MEN2A and 82 patients with sporadic MTC. No *HRAS* mutation was found in the MEN2A patients and 12 of 82 patients with sporadic MTC had *HRAS* positive tumor tissue. This suggests that detection of *HRAS* in tumor tissue by IHC could exclude the need for *RET* germline examinations in these patients. However, in all patients with MTC it is crucial to have excluded at least the possibility of the MEN2 related PCC before thyroid surgery by analyzing serum metanephrine and normetanephrine.

Already in 1994 and 1995 Zedenius et al. (33, 34) described the relation between presence of somatic p.M918T *RET* gene mutation in tumor tissue and poorer outcome, later confirmed by other studies (35, 36). A rapid method for DNA extraction from FNB for *RET* mutation analysis by PCR with the intention of targeted therapy was developed in 1998 (291). Furthermore, in the recent study by Cote et al. (292) the presence of somatic *RET* mutation in plasma was evaluated in 50 patients with *RET* p.M918T mutations detected in tumor tissue. In 16 of these patients the somatic *RET* mutation was detected in circulating cell-free DNA in plasma and by a blood test. The authors found that the liquid biopsy was able to detect *RET* p.M918T mutations in patient plasma with high specificity but low sensitivity. In addition it was found that the detection of *RET* p.M918T in plasma correlated with a worsened OS. More precisely, the detection of the mutation indicated a poorer outcome than did the calcitonin doubling time. This may play a role in monitoring treatment response.

The findings of the study in **paper IV** concur with the literature concerning sporadic MTC and somatic mutations, with mutually exclusive *RET*-mutations and other CDMs (37, 39,

41, 44). However, as patients with sporadic MTC and the somatic p.M918T *RET* mutation might have a poorer outcome than the MEN2B study patients with *de novo* p.M918T mutation and slow progressive disease, further research should be performed. Patients with somatic and hereditary *RET*- p.M918T mutations should be compared, and mainly, even more intensive mutational search would be needed to draw more firm conclusions in MEN2B related MTC.

Cell heterogeneity by copy number alternations (CNA) in MTC have been studied. Ciampi et al. (293) found by multivariate analysis that only *RET* mutation and advanced clinical stage correlated with poorer outcome. Furthermore, 30% of the 65 patients studied harbored *RET* CNA in variable percentage of the cells. Cell heterogeneity as *RET* CNA was considered to be a poor prognostic factor in patients with *RET* mutation. Romei et al. (294) studied *RET* mutation heterogeneity in 65 patients with primary advanced MTC and their metastasis. In patients with sporadic MTC, genetic intra-and inter-tumor heterogeneity was found in only 20% of the patients, which could justify the relatively moderate level of disease aggressiveness in these patients. In three patients with somatic p.M918T mutation in the primary tumor tissue, there was no *RET* mutation in subgroups of the metastatic lymph node tissue. Patients with MEN2B have germline *RET* p.M918T mutation. It should further be explored if the same development of tumor heterogeneity and CNA in metastatic tumor tissue, both in lymph nodes and distant metastasis, also in MEN2B related MTC might reduce tumor aggressiveness.

Two of the six study patients in **paper IV** received TKI treatment. Therapy with TKI reduces the gain of function with partial tumor response and extended PFS, but the effect is transient and resistant to treatment will occur (87, 229, 234-236) (Figure 1). In two phase 3 trials, patients with advanced, unresectable, locally advanced or metastatic MTC have been studied (237, 238). In the study by Wells et al. (237) significant increased PFS and biochemical response in patients treated with Vandetanib were found with increased response to TKI at p.M918T mutation in patients with sporadic MTC. In the study by Elisei et al. (238), significantly increased PFS was also found, but the tumor response and PFS were independent of *RET* mutation status. The prolonged PFS with Cabozantinib treatment was also observed in a subgroup of patients who had prior TKI treatment (229, 238). OS did not improve in either of the studies. However, in a

recent study by Schlumberger et al. (295), increased OS was found in patients treated with Cabozantinib in the subgroup of *RET* p.M918T mutation.

This thesis opens for new research questions. To better understand prognostic differences in patients with MTC, the different MTC tumor behavior, as well as development of new targeted therapies in advanced MTC, further research concerning multifactorial aspects in genetics, epigenetics and tumor microenvironment properties is needed. However, there has been widespread research activity in molecular biology in recent times, uncovering new knowledge and further creating new research questions. A few studies are mentioned below.

In addition to the prognostic implications of germline and somatic gene-mutations in patients with MTC, epigenetic regulations, such as DNA methylation, micro RNA deregulation and post-translational histone modification, alters gene expression independently of the changes in the primary DNA sequence (296). All these modifications implicated in the tumorigenesis of MTC, and transcription of the *RET* proto-oncogene, the main driver of MTC, can also be modulated by epigenetic alterations as further described in the review by Joo et al. (296).

In a study by Cavedon et al. (297), the downregulation of miRNA-224 in MTC, with low miR-224 expression, was associated with higher preoperative calcitonin levels, more severe tumor stage, more patients with persistent disease and disease related death. Furthermore, miR-224 represented an independent prognostic marker in MTC by multivariate analysis, and finally miR-224 was upregulated in patients with *RAS* mutated MTC and in patients with a better prognosis.

Oczko-Wojciechowska et al. (298) found a significant association between the localization of *RET* mutations and the expression of three genes as Neuronatin (*NNAT*), suggested to be a tumor suppressor gene; *CDC14B*, involved in cell cycle control, and Neurotrophin receptor tyrosine kinase 3 (*NTRK3*), a tyrosine receptor kinase that undergoes rearrangement in PTC. This suggests that these genes are significantly deregulated in tumors with MEN2A-like and MEN2B-like mutations. In MTC, both genetic predisposition and epigenetic regulation play a significant role and are important factors to understand the differences in the

phenotypes of MTC subtypes (298). However, when analyzing the whole-gene expression profile of MTC in 86 MTC samples, with regard to the type of *RET* gene mutation and the cancer genetic background, the study results indicate a rather homogeneous MTC gene expression profile in hereditary and sporadic MTC (298).

Furthermore, a profound modification of tumor microenvironment has been associated with the *RET* MEN2 associated oncoproteins, and is discussed in the thematic review of Castellone and Melillo from 2018 (299). The oncoproteins influence the surrounding stroma, activating cancer associated fibroblasts which represents the main cellular entity involved in desmoplasia, promoting cancer-associated inflammation and suppressing anti-cancer immune response. These mechanisms might be exploited to develop new anti-cancer treatment and new prognostic markers.

The *TERT* gene (Telomerase Reverse Transcriptase) is normally suppressed. However, in cancer cells *TERT* is over-expressed, and telomerase activation is observed in many human cancers (300). The maintenance of telomeres, achieved by activating the ribonucleoprotein telomerase through the telomerase reverse transcriptase component encoded by the *TERT* gene, increase cancer cell survival and proliferation (300, 301). Mutations in the *TERT* gene promotor region have to a greater extent been associated with more aggressive follicular derived thyroid carcinoma than less aggressive types (302, 303). Regularly, no such association with MTC has been found (303, 304). Furthermore, Wang et al (300) presented genetic and epigenetic background as well as protein expression profiles in relation to telomerase activation in MTC, in a Swedish study from 2016. They found that increased copy numbers in some telomerase positive MTC correlated with late tumor stages at the time of diagnosis in patients with MTC. They also reported that increased *TERT* promoter methylation in MTC tumor tissue correlated with telomerase activity and association with poor survival in MTC patients. By studying the protein expression profile, the study group found different profiles between telomerase positive and negative MTC tumors, but no clear relationship with the *TERT* expression could be defined based on this study. However, the mRNA expression of *XRCC5* was increased in MTCs compared to normal thyroid tissue.

9. Conclusions and implications

Nationwide population-based retrospective cohort studies are important in epidemiological research. The selection bias is minimal, and the results can be generalized.

In Norway, MTC disease control has improved, with earlier detection and better outcome. Timing and extent of prophylactic thyroidectomy in patients with MEN2A can be based on preoperative basal serum calcitonin levels, but not the necessity of prophylactic lymphadenectomy in the lateral neck compartments in patients with MTC. Tumor stage is still the main prognostic factor for cure and survival. However, surgical accuracy has significant independent prognostic impact on outcome.

Paper I: Preoperative basal serum calcitonin level alone can serve as an indicator for optimal timing and the extent of thyroid surgery for MEN2A patients.

Paper II: The preoperative diagnostics in MTC have improved over time in Norway, with increased therapeutic control. A low ratio between metastatic and resected lymph nodes predicts better outcome in patients with metastatic lymph nodes.

Paper III: Preoperative basal serum calcitonin levels cannot predict the need for prophylactic lateral neck dissection in patients with MTC. Further prospective randomized studies are warranted.

Paper IV: Despite the advanced tumor stage at the time of diagnosis, the MTC tumor behavior has not been aggressive, but has rather shown slow disease progression in the Norwegian MEN2B study patients. Somatic cancer driver mutations in *HRAS*, *KRAS*, *NRAS*, *BRAF*, *PIK3CA* or *AKT1* were not found. More research on the genetic, epigenetic and tumor microenvironment properties of these lesions is needed.

10. Future perspectives

Research into genetics, epigenetics and tumor microenvironments properties is the future of research in MTC. Evaluating genetic as well as somatic mutational analysis, in combination with other interfering factors in the microenvironment, are warranted. Factors in the microenvironment such as extracellular matrix, stromal cells and immune cells, play a critical role in MTC (299). Relations between cancer associated fibroblasts in desmoplasia, as well as the relation between oncoproteins found in MTC and their influence on extracellular stroma, cancer associated fibroblasts and cancer associated immune-inflammatory component should be further evaluated.

Furthermore, implications of epigenetic regulations, such as DNA methylation, micro RNA deregulation and post-translational histone modification, on MTC tumor genesis and transcription of the *RET* gene warrant to be studied further. Still more, copy number alternations and heterogeneity of mutations in primary versus metastatic tumor tissue should be explored. Possible additional relations between Telomerase Reverse Transcriptase gene (*TERT*) expression, telomerase activity, *TERT* promotor methylation and protein expression profile in MTC should also further be evaluated.

Patients with advanced MTC cannot be cured by surgical treatment alone. TKI is today the only systemic treatment and are indicated at metastatic progressive MTC. The treatment effect is increased PFS with transient effect, the side effects are profound (237, 238, 299). Combinations of targeted therapies with other agents, including immunotherapy has shown to enhance drug efficacy, overcoming resistance and reducing side effects (299, 305). Treatment with TKI together with immunotherapy in MTC should be explored even more.

Stand-alone retrospective and prospective clinical research or in combination with molecular biological research is needed. Additional factors predicting thyroid lobectomy as sufficient surgical treatment in selected patients with sporadic MTC should be explored. Finally, a prospective case-control study evaluating prophylactic lateral neck dissection in patients with MTC should be designed.

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12. Errata list

1. **Paper I:** The prevalence of MEN2A at censoring date was 1:84,672, and not 1:79,462. There was a mistyping during calculation page 4, first column, line 33. Furthermore, 61 patients, and not 62, were living in Norway at censoring date, page 4, first column, line 30. This is according to the data described in the result paragraph, section of clinical and follow-up data for index- and screening patients, page 6, first column. However, the content in the discussion remains the same.
2. **Paper I:** On page 11, first column, line 25, reference 36 should be moved to page 11 second column line 13, and replace “37”.
3. **Paper I:** Conversion factor for pmol/L to pg/mL is 0.2926 and not 0.296. There is a typing error on page 3, second column, line 30. However, the correct conversion factor is used for the calcitonin values and the analysis.
4. **Paper II:** On page 8, first column, line 3, the incidence in Norway 1970-1985 was reported by Akslen in 1990 and not 1985.

13. Disclaimer

The study has used data from the Cancer Registry of Norway. The interpretation and reporting of these data are the sole responsibility of the authors, and no endorsement by the Cancer Registry of Norway is intended nor should be inferred.