Diploma Thesis

Medullary Thyroid Cancer:
The current state of tumour marker and treatment strategies

SUBMITTED BY:

SIDDIQUI ZAINAB GHAZAL

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UNIV.-ASS. MAG. DR.NASSIM GHAFFARI TABRIZI-WIZSY

AO.UNIV.-PROF. DR.PHIL. ROSWITHA PFRAGNER

Graz, 22.04.2015

DECLERATION

I, Siddiqui Zaianb Ghazal, declare that the diploma is on my own account, based upon work actually carried out by me, and that all sources of material have been clearly indicated.

Furthermore I confirm that no part of the work incorporated in the diploma is a quotation from published or unpublished sources, except where this has been clearly acknowledged as such, and that any specific direction or advice received is also properly acknowledged.

Graz, am 22.04.2015 Siddiqui Zainab Ghazal eh

Acknowledgement

Though only my name appears on the cover of this dissertation, many people have contributed to its realization and made this thesis possible.

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Contents

Abbreviations

µg	Microgram
^{131}I-MIBG	Metaiodobenzylguanidine
^{18}F-DOPA	Flourin-18-L-dihydroxyphenylalanine
^{18}F-FDG	Fluorodeoxyglucose
5HT	Serotonin
ACE	Angiotensin-converting-enzyme
ACTH	Adrenocorticotrophic hormone
AEs	Adverse events
APUD	Amine precursor uptake and decarboylation
Array-CGH	Array comparative genomic Hybridization
ASCL1	Achaete-scute complex homolog 1
ATA	American thyroid association
AUC	Area under the curve
CEA	Carcinoembryonic antigen
CEACAM	CEA-related cell adhesion molecule
CgA	Chromogranin A
CgB	Chromogranin B
CGRP	Calcitonin gene related peptide
CI	Confidence interval
C-MET	Hepatocyte growth factor receptor
CT	Calcitonin
CT	Computer tomography
CUX1	Homo sapiens cut-like homeobox 1
d	Day
DCR	Disease control rate
DLT	Dose limiting toxicity
EBRT	External beam radiation therapy
ECG	Electrocardiogram

EGFR	Endothelial growth factor receptor
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FDA	Food and Drug Administration
FMTC	Familial medullary thyroid cancer
FPR	False positive rate
FTC	Follicular thyroid carcinoma
GDNF	Glial-cell-line-derived neurotrophic factor
GEP-NET	Gastroenteropancreatic NET
GFL	Glial cell line-derived neurotrophic factor family of ligands
GFRα	Glial cell line-derived neurotrophic factor family α coreceptor
GRP	Gastrin releasing peptide
HGF	Hepatocyte growth factor
HIF1α	Hypoxia-induced factor 1 alpha
LK2H10	Chromogranin A and related peptides
LR	Likelihood ratio
M2BP	Mac 2 binding protein
MAPK	mitogen-activated protein kinase
MDR	Multidrug resistance
MEN	Multiple endocrine neoplasia
MET	Mesenchymal-epithelial transition
M-FISH	Multiplex fluorescence in situ hybridization
mg	Milligram
ml	Millilitre
ms	Millisecond
MTC	Medullary thyroid carcinoma
mTOR	Mammalian target of rapamycin
NCCN	National Comprehensive Cancer Network
NGF	Nerve growth factor

nmol	Nanomole
NSCLC	Non-small cell lung cancer
NSE	Neuron-specific enolase
ORR	Objective response rate
PAX8	Paired box 8
PFS	Progression free survival
pg	Picogram
Pgr	Progesterone receptor
PHE5	Chromagranin clone PHE5
PHPT	Primary hyperparathyroidism
PI3K	Phosphatidylinositol 3-kinase
PNEN	Pancreatic neuroendocrine neoplasms
PPARG	peroxisome proliferator activated receptor gamma
PTC	Papillary thyroid carcinoma
RECIST	Response evaluation criteria in solid tumours
RET	Rearranged during transfection
RTK	Receptor tyrosine kinase
ROC	Receiver operator characteristic
SCID	Severe combined immunodeficiency
Sg	Secretogranin
SRiF	Somatostatin
T3	Triiodothyronine
T4	Tetraiodothyronine/Thyroxine
TA	Telomerase activity
TBG	Thyronin binding globulin
TdP	Torsade de pointes
TKIs	Tyrosine kinase inhibitors
TSH	Thyroid stimulating hormone
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

Zusammenfassung

Medulläres Schilddrüsenkarzinom (MTC) ist ein neuroendocriner Tumor, der aus Calcitonin-produzierenden parafollikulären C-Zellen entsteht und 5% bis 10% aller Schilddrüsenkarzinome ausmacht. Die jährliche Inzidenz ist bei Männern 0,18/100.000 und bei den Frauen 0.23/100.000 Einwohner. MTC kann sporadisch oder familiär (25% der Fälle) vorkommen. Familiäre Formen sind autosomal dominant entweder als Teil des *multiple endocrine neoplasia syndrome type 2A* (MEN 2A) oder *type 2B* (MEN 2B), oder ohne assoziierte Endocrinopathien als Familiäre MTC (FMTC) vererbt. In lokalisiertem Stadium ist die chirurgische Entfernung aller neoplastischen Gewebe in Hals mit totaler Thyroidektomie, zentraler und bilateraler Halsdissektion, die einzige potentiell kurative Therapie. Allerdings haben zum Zeitpunkt der Diagnose 25% der Patienten bereits Fernmetastasen.

MTC Forschung ist seit 2.5 Dekaden ein wichtiger Schwerpunkt am Institut für Pathophysiologie und Immunologie: Neun MTC-Zelllinien sind etabliert und charakterisiert. Diese Zelllinien sind wichtige Werkzeuge, um die Biologie der MTC zu studieren und Therapeutika gegen MTC zu testen.

Ziel dieser Diplomarbeit ist es, die in der Literatur beschriebenen MTC Tumor-Marker und die neuesten therapeutischen Strategien und Medikamente für die Behandlung vorzustellen. Ein weiteres Ziel ist es, die vorhandenen Informationen über MTC Zelllinien zusammenzutragen, um einen Überblick über ihre Charakteristika zu schaffen.

Abstract

Medullary thyroid carcinoma (MTC) is a neuroendocrine tumour which arises from calcitonin-producing parafollicular C-cells and accounts for 5% to 10% of all thyroid cancers, with an estimated age-adjusted annual incidence of 0.18 for men and 0.23 for women per 100.000 inhabitants. MTC occurs as either a sporadic form or in a familial context (25% of cases). Familial forms are inherited in an autosomal-dominant way, either as part of multiple endocrine neoplasia syndrome type 2A (MEN 2A) or 2B (MEN 2B), or without any associated endocrinopathies as familial MTC (FMTC). Surgical removal of all neoplastic tissue in the neck by total thyroidectomy, central and bilateral neck dissection is the only potentially curative treatment in localized disease. However, at the time of initial diagnosis, at least one quarter of patients has distant metastases.

MTC research is a major focus of Institute for Pathophysiology and Immunology during the last 2.5 decades: 9 MTC-derived cell lines have been established and characterized. These approved cell lines are constantly used to study the MTC biology and to test therapeutic agents.

The aim of this work is to give an overview about the MTC tumour marker and the new therapeutic strategies and drugs for the treatment of MTC. Furthermore, the aim is to assemble the data provided about MTC cell lines and to give an overview of their characteristics.

1 Background

1.1 Thyroid Gland

The Thyroid gland is a large endocrine organ. It is formed in the early foetal life and recognized as early as 24 days of development. The primitive thyroid comes down to its eventual location in the lower anterior neck by elongation of the thyroglossal duct, which atrophies around the seventh week of life. The mature thyroid has two lobes (each of them with the greatest dimension of about 4cm) which are connected by an isthmus. The thyroid gland weighs about 25 to 35 grams. The origin of the follicles (functional unit of the thyroid) is cord of cells, which exist in the gland at the early stages of development. The follicles are filled with colloid, e.g. secreted thyroglobulin, from which the two hormones, T3 (triiodothyronine) and T4 (tetraiodothyronine or thyroxine) are released (1).

T4 and T3 are found both free and bounded to TBG (Thyronine-binding globulin) in the blood. Peripheral cells are able to take up just free hormone, which then binds to nuclear receptors and causes synthesis of specific protein. T3 is more active than T4, but mostly T4 is secreted and then deiodinated in peripheral tissues to T3 (1).

Almost all organs are affected (body´s overall metabolic activities, both anabolic and catabolic are increased) by thyroid hormone. Basal metabolic rate and metabolism of lipids, carbohydrates and proteins are stimulated; body heat, gluconeogenesis and glycogenolysis are increased and synthesis of many other hormones, enzymes and structural proteins is promoted by thyroid hormone. TSH (thyroid stimulating hormone) controls the function and structure of thyroid and the secretion of T3 and T4 causes the suppression of pituitary TSH. Dietary supply of iodine is very important and crucial for the thyroid hormone production (1).

The thyroid gland contains parafollicular or C cells as well, located close to the follicles. The C cells originate from neural crest and their main product is calcitonin, but chromogranin and synaptophysin are also products of these cells (1).

1.2 Tumours of Thyroid Gland

Thyroid tumours are either benign (noncancerous) or malignant (cancerous) growths.

1.2.1 **Benign Tumours**

1.2.1.1 **Follicular Adenoma of the thyroid**

Follicular Adenoma (the most common tumour of the thyroid, solitary circumscribed, and completely surrounded by thin fibrous capsule) is a benign neoplasm having follicular differentiation (the cells are arranged in follicles just like the normal thyroid) and clonal origin. The female to male ratio is 7:1. Patients affected from follicular adenoma are euthyroid persons and have a “cold” nodule. It occurs frequently in areas with iodine deficiency, but can also occur as part of Cowden syndrome and in irradiated glands. Cystic changes, Haemorrhage and fibrosis are commonly seen with this tumour. There are several histologic subtypes of this adenoma (embryonal, foetal, simple, colloid, oncocytic cell, and atypical adenoma) and it is important to distinguish them from thyroid cancers (particularly follicular carcinomas) (1).

The macroscopic and microscopic features of FTC are shown in figure 1 (see below). Careful evaluation of the capsule for capsular or vascular invasion is very important to make this distinction. Malignant tumours can develop in association with, or within benign nodules (1).

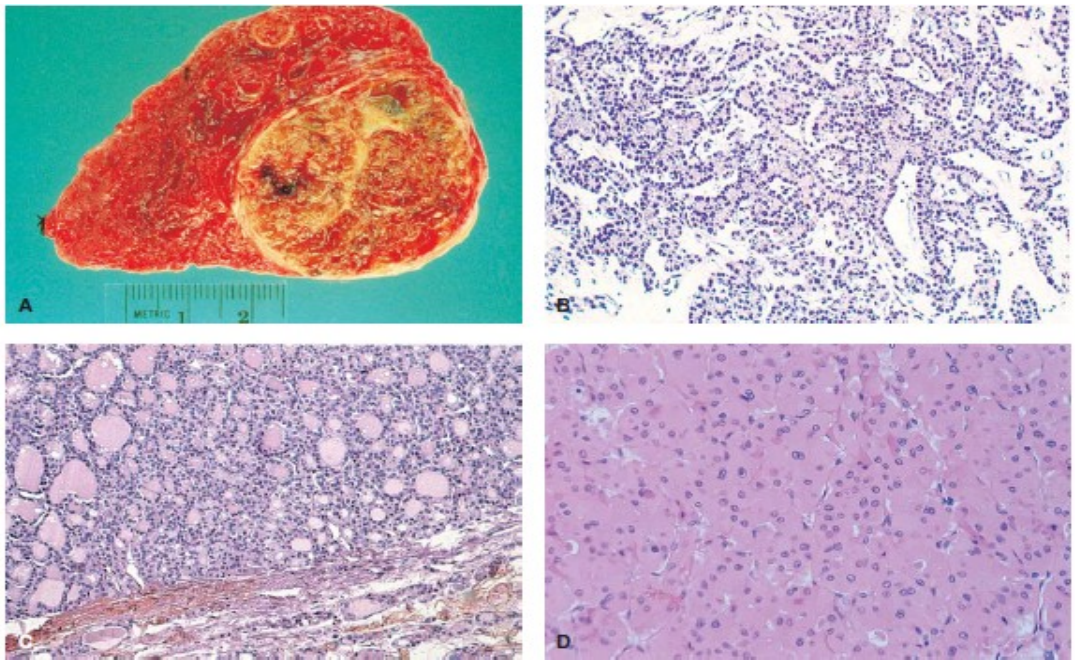

Figure1: Follicular adenoma A: colloid adenoma **B:** Embryonal adenoma C**:** Foetal adenoma **D:** Oncocytic cell adenoma **(1)**.

1.2.1.2 **Papillary Hyperplastic Nodules**

Children and young women are affected by papillary hyperplastic nodules. These Nodules are well circumscribed and well encapsulated and contain papillae having variable sizes. The stalks of these papillae may contain small follicles. Papillae are lined by cuboidal cells with follicular nuclei. The centres of nodules are cystic and most often contain colloid like material. The papillary hyperplastic Nodules are often misdiagnosed as papillary cancer (1).

1.2.2 **Thyroid Cancer**

Thyroid cancer is the most common endocrine malignancy, with an increasing incidence over the last few decades throughout the western world (2). Thyroid carcinoma develops either from follicular or parafollicular cells and is diagnosed by fine-needle biopsy. It has three subtypes namely papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), and medullary thyroid cancer (MTC)

1.2.2.1 **Papillary thyroid carcinoma**

PTC is the most common thyroid cancer and makes up to 90% of sporadic thyroid carcinoma cases. Most often the female patients are affected and the female to male ratio is 3:1, but any age, even children can suffer from it and elderly men have a worse prognosis. Although several associations have been identified, the aetiology of PTC is still unknown (1).

The cut surface and histological aspects of PTC are demonstrated in figure 2.

Increased incidence of later PTC is observed in children (the younger children take up more radioactive iodine) and adults, whose neck was exposed to external radiation. For example a greater incidence of PTC in Japan (in survivors of atomic bomb explosions) and around Chernobyl can be mentioned (1).

Epidemiologic studies have shown that first degree relatives of patients who suffer from PTC have a 4- to 10-fold higher risk for papillary thyroid carcinoma. Papillary thyroid carcinoma occurs in association with familial adenomatous polyposis

syndrome as well. Somatic mutation of the *RET* on chromosome 10, BRAF mutations and RAS mutations are common in PTC. Papillary cancer can arise anywhere in the gland and are firm, solid and white-yellowish. They have irregular borders and these lesions can be multiple and sometimes encapsulated (1).

PTC has many morphologic types; some have good prognoses such as microcarcinoma, follicular variant of papillary cancer, encapsulated tumours and papillary tumours of the usual type. Diffuse sclerosis type, tall cell type and columnar variants have most often worse prognoses. PTCs often show psammoma formations (Figure 2) and the level of calcium is normal in serum (1).

PTC invades lymphatics and spreads to regional cervical lymph nodes and in 25% of PTC cases direct extension into the soft tissues is observed. Haematogenous metastases occur seldom, most often to the lungs (1).

The prognosis of PTC is excellent and life expectancy of the patients who suffer from PTC differs little from that of the normal population. The outcome is worse in men over 50 years, but in children the prognosis is very good even if they have lung metastases (1).

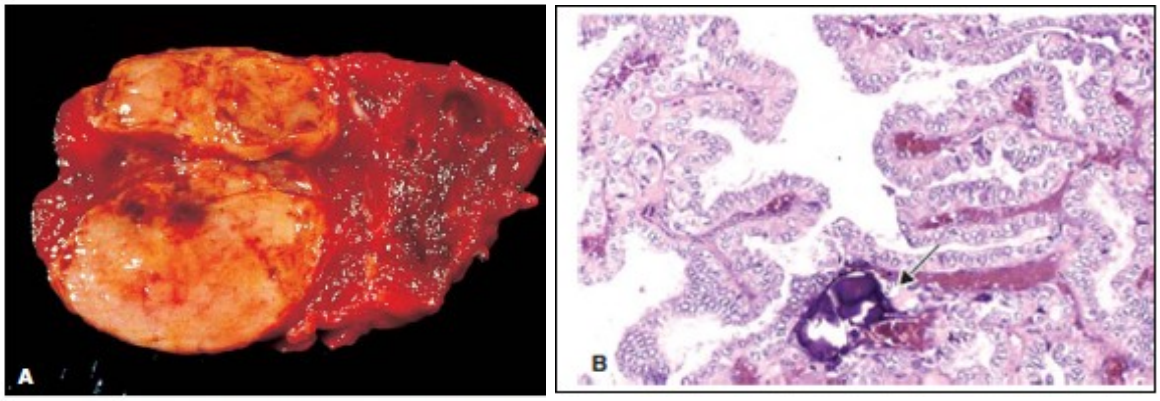

Figure 2: Papillary thyroid carcinoma: A: Macroscopic feature of PTC. **B:** Histological feature of PTC (the arrow indicates psammoma body) **(1)**.

1.2.2.2 **Follicular thyroid carcinoma**

FTC (a purely follicular carcinoma with no papillary or other elements) is rarely fatal and is responsible for 15-20% of the thyroid tumours. The patients are most often older than 40 years (it is very rare in children) and the female to male ratio is 3:1. In regions where iodine is added to salt, FTC is uncommon and irradiation of neck increases the frequency of these tumours (1).

Mutations in RAS oncogene and rearrangement of the PAX8/PPARG (Paired box 8/peroxisome proliferator activated receptor gamma) are reported in 20%-45% of FTC. Mutations of P^{53} and PTEN tumour suppressor genes and imbalances in chromosomes 3p, 7q, 11 and 10q are also reported in FTC. FTC has two types: minimally invasive and widely invasive (1).

Minimally invasive FTCs have very good prognosis (cure rate of at least 95%). On the other hand the widely invasive type has worse outcome (cure rate of 50%) (1).

The treatment of FTC is unilateral lobectomy and the metastases are treated with radioiodine (1).

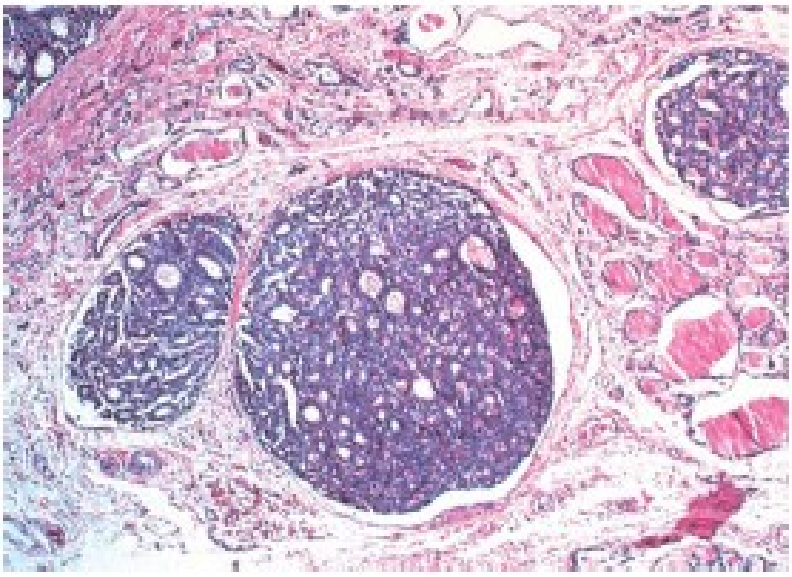

Figure 3: FTC with vein invasion **(1)**.

1.2.2.3 **Medullary thyroid carcinoma (MTC)**

MTC is the subject of this diploma. The information about this cancer is below.

2 Introduction

2.1 **Medullary thyroid carcinoma**

MTC originates from the C-cells of the thyroid and occurs either in sporadic or familial form. Patients who suffer from the FMTC often have MEN type 2A and 2B. Patients with multiple endocrine neoplasia type2B develop tumours in infancy and the development of tumours in patients with MEN type 2A occurs in adolescents. Sporadic MTCs occur later in life and its female to male ratio is 1.5: 1. The FMTC is inherited in an autosomal dominant way and has an equal sex distribution (female to male ratio=1:1). In 25% to 70% of sporadic MTC mutation happens in *RET*, most of the time at codon 918 (3-5) .

The macroscopic and histologic features of MTC are shown in figures 4 and 5 (below).

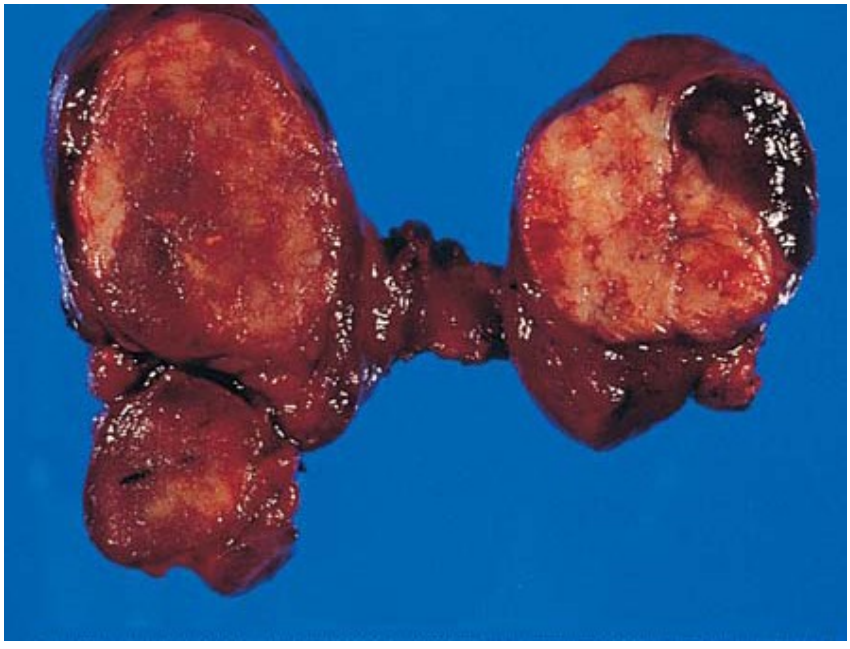

Figure 4: Macroscopic feature of MTC **(1)**.

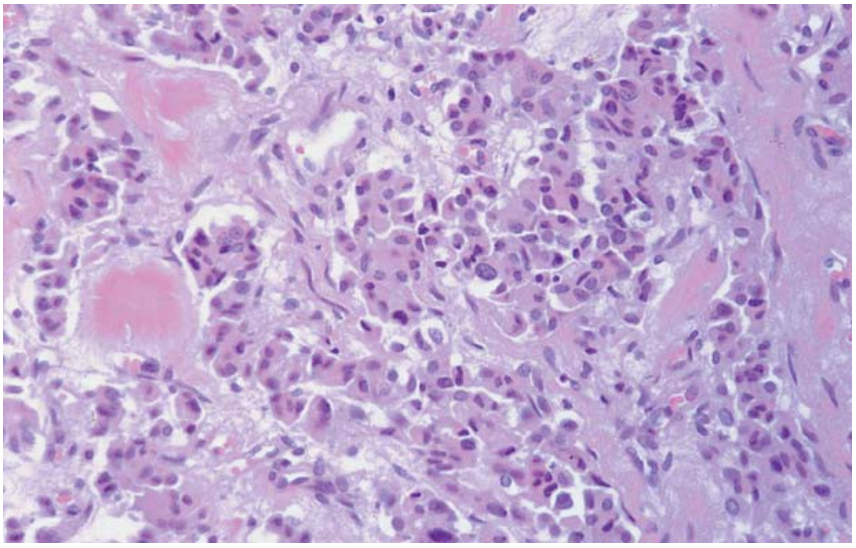

Figure 5: Microscopic feature of MTC **(1)**.

2.2 **Multiple endocrine neoplasia**

The term ‘‘multiple endocrine neoplasia’’ or MEN was used by Steiner in 1960s. He reported about 3 various endocrine disorders (6) . In the first disorder MEN1, Werner Syndrome, patients with familial pituitary, parathyroid, and pancreatic islet cell tumours were characterized. The second disorder MEN2, Sipple syndrome, illustrated patients with familial pheochromocytomas, medullary thyroid cancer and hyperparathyroidism. Papillary thyroid carcinomas and non-familial parathyroid tumours were described in the third group of endocrine disorders (MEN3) (6).

Today multiple endocrine neoplasia (MEN) shows three autosomal dominant (AD) disorders: MEN1, MEN2A, and MEN2B (6).

MEN1 is a disorder which is inherited in an autosomal dominant way. It is caused by germ line mutation of the MEN1 gene, a tumour suppressor gene, which is located on chromosome 11q13 and prohibits cell proliferation. The protein menin is encoded by 11q13. It is a nuclear protein (7) which plays a role in DNA replication and repair, transcription, and chromatin modification (8). The two-hit hypothesis of Knudson (9) says that prior to the tumour cell development; both alleles of such a gene have to be inactivated. In people harbouring MEN 1 gene germ line mutations, all cells of the body have one mutated MEN 1-allele, which may be indicated as first hit according to the two-hit hypotheses. In case if the normal second allele is mutated through an

additional somatic mutation (second hit), tumour may develop. Patients who are affected from MEN 1 are likely to have pituitary adenoma, parathyroid hyperplasia or adenoma, insulinoma and gastrinoma. But, over 20 various endocrine tumours such as thymic, bronchial, gastric and duodenal carcinoids and adrenocortical lesions and non-endocrine tumours such as facial angiofibromas and collagenomas have been reported in patients with MEN1 (10).

MEN1 syndrome manifests usually in late adolescence or early adulthood. A pituitary adenoma, associated with MEN, in a five year old child has been reported (10,11).

Multiple endocrine neoplasia type 2 is an autosomal dominant (AD) syndrome which is caused by germ line activating mutations of *RET*. About 95% of patients suffering from MEN2 have a *RET* mutation, which can be identified (12).

The *RET* proto-oncogene is located on chromosome 10q11.2, has 21 exons and encodes a tyrosine kinase. This tyrosine kinase is primarily expressed in neuroendocrine and neural cells (13).

The Prevalence of MEN2 is about 2.5 in 100,000. MTC, Pheochromocytomas and PHPT are seen in MEN2 patients. MEN2 is classified into 3 subtypes: MEN 2 A, FMTC, and MEN2B, which correlate with high risk for MTC (14).

2.3 **MTC in MEN 2**

As mentioned earlier, MTC develops from the parafollicular cells of the thyroid gland. These parafollicular or C cells produce calcitonin, serotonin, ACTH and somatostatin. This cancer represents more than 5% of all thyroid carcinomas. Patients with FMTC have tumours that are often bilateral and multicentric, which is not the case in sporadic form. C-cell hyperplasia causing elevated serum calcitonin precedes the development of FMTC. A large number of MTCs occur in the superior on third of the thyroid gland. High levels of calcitonin (>1,000pg/ml) and CEA are sensitive indicators of MTC. (15).

Patients who suffer from MTC often have neck pain, a palpable neck mass, or diarrhoea because of the high levels of calcitonin. If the patients have dysphagia and hoarseness then MTC is advanced. The aggressiveness of MTC depends on the *RET*

mutation. In the beginning, metastasis occurs in cervical or mediastinal lymph nodes, and later metastases are seen in the lung liver, and bone (15).

Over 90% of MEN 2A patients and almost all MEN 2B patients develop MTC, and it is usually the first clinical manifestation of MEN2 (10).

In 2009, ATA guidelines and recommendations for the screening and treatment (age of prophylactic thyroidectomy) of patients with hereditary MTC (according to the specific *RET* mutation) are published (Table 1) (12).

Table I. Aggressiveness of MTC according to *RET* mutation and the recommended age of prophylatctic thyroidectomy **(12)**.

ATA level	Codons	Aggressiveness of MTC	Age of prophylactic thyroidectomy
A	768,790,791,804,891	Lowest	After 5 years[a]
B	609,611,618,620,630	Low	Before 5 years[a]
C	634	High	Before 5 years[a]
D	883,918	Highest	Within first year of life

2.4 The C cells (parafollicular cells of the thyroid)

The C cells were recognized by Baber EC in 1876 for the first time, he identified them in the thyroid of the dog in a parafollicular location. They were named parenchymatous cells (16,17).

Foster *et al.* (16,17) related the cells to calcitonin production in the canine thyroid. Bussolati and Pearse (17,18) showed the calcitonin content of the C cells in the porcine thyroid. They studied these cells with immunofluorescent. So the name C cell applied by Pearse, in 1966, (17,19) is most applicable, because it identifies these cells with the calcitonin production (17).

The C cells originate from neural crest, as it was said by Pearse (17,19) in 1966. These cells share a common origin with the adrenal medullary chromaffin cell, intestinal enterochromaffin cell, pituitary corticotrophs and melanotrophs, and islet cells. This series of cells was described as APUD (amine precursor uptake and decarboxylation) cells by Pearse (17,20).

APUD cells originate from the neural crest in the mammals, including man as reported by Smith (17,21).

In the embryonic neural tube, these cells are present as clusters of neuroectodermal cells and do not participate in the infolding of the neural plate. They play no role in the closure of the dorsal ectoderm as well. The thyroid gland is then an organ containing the C cells (17,21,22).

The primary function of the C cells is the production of calcitonin (17).

2.5 **Molecular biology and key pathways in the MTC**

MTC can be caused either sporadically (due to the somatic mutations) or familial (because of a germline mutation). Sporadic medullary thyroid cancer makes up to 80% of all MTC cases (23,24). The mutations are frequently observed in *RET*, but can also involve MET and VEGFR. The mutations promote tumour development and growth (24).

2.5.1 **Rearranged during transfection**

RET is a proto-oncogene, which is located on chromosome 10. *RET* activates multiple signalling pathways that promote cell cycling, motility, and survival (24-26). *RET* mutations are observed in more than 50% of sporadic cases of MTC and in almost all inherited cases (24,27). Somatic *RET* mutations correlate with advanced stages of MTC at diagnosis. Theses mutations were associated with a worse prognosis, in one study (23,24). In 80% of MTC patients, M918T was the most common *RET* mutation (24).

Moura *et al.* studied *RET* mutation in 51 patients with sporadic MTC and 64.7% had somatic mutations. No statistically significant variations in clinical or pathologic findings were found between *RET* -positive individuals and *RET* -negative ones. But, in subgroup analysis and examination, sporadic cases of medullary thyroid carcinoma with exon 15 and 16 mutations had a higher number of lymph node metastases, multifocal tumours and persistent MTC. Detectable calcitonin at last evaluation, and stage IV disease had also been observed in the mentioned cases (28).

A number of TKIs can inhibit the *RET* receptor, in addition to targeting other receptors, such as VEGFR. These tyrosine kinase inhibitors are cabozantinib, vandetanib, sunitinib, and axitinib. The degree of *RET* inhibition relative to inhibition of other receptors differs a lot in the mentioned TKIs and they have different potency against *RET* (24,29)

An *in vitro* study compared these 4 agents and delivered that cabozantinib is the most potent inhibitor in MEN 2A MTC and vandetanib in MEN 2B MTC, which shows that mutation-specific therapy may deliver advantage in MTC therapy (24,30).

The TKIs are mentioned in detail in the results of this work.

Figures 6 and 7 show structure of *RET*; *RET* signalling pathways, and the strategies for targeting its activation.

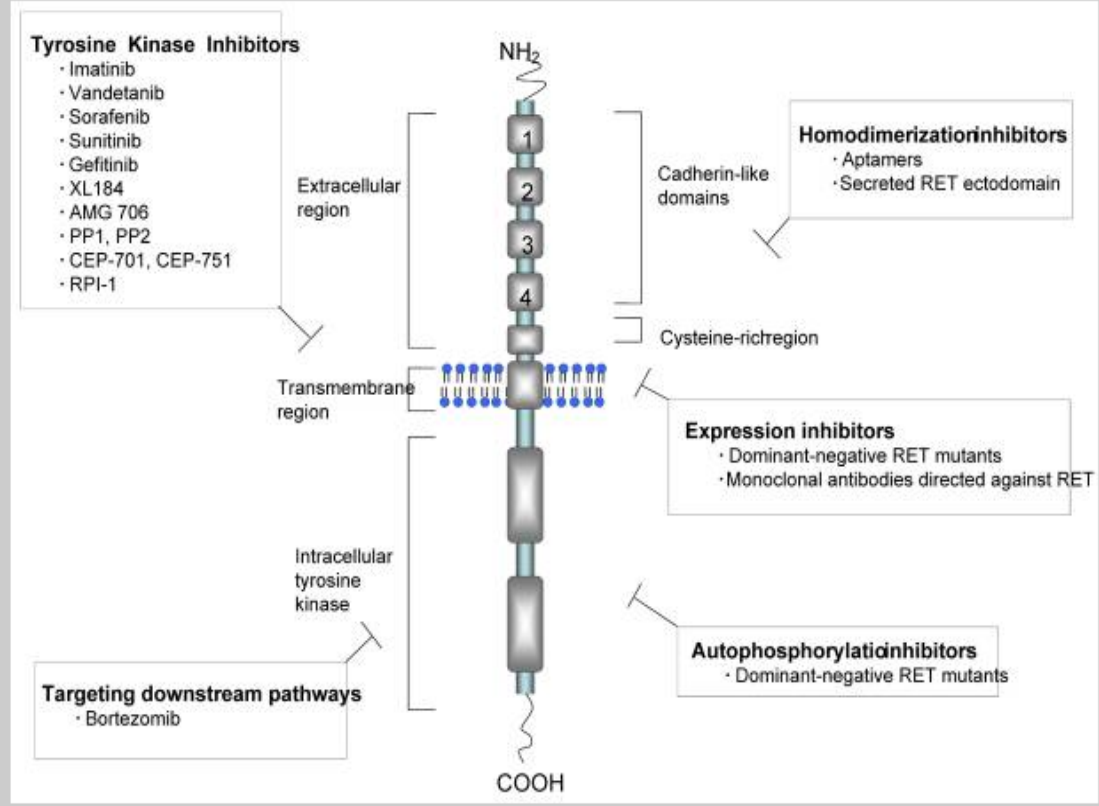

Figure 6: Structure of *RET* proto-oncogene and the strategies to inhibit its activation **(25).**

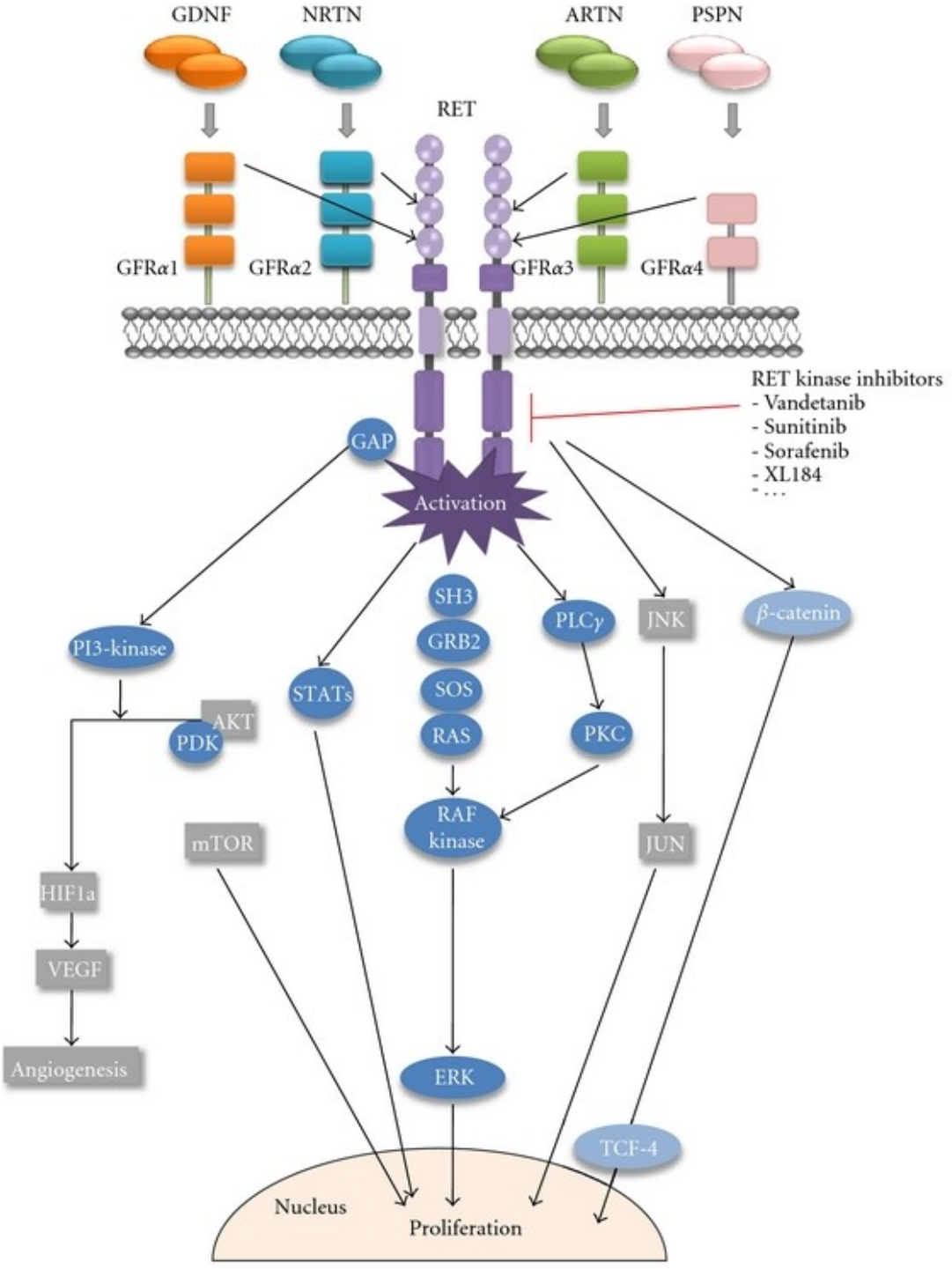

Figure 7: Signalling pathways of *RET* proto-oncogene and the targeting mechanism of TKIs **(26)**.

2.5.2 **Vascular endothelial growth factor receptor**

A number of other mutations correlate with the development of medullary thyroid cancer as well such as mutations of VEGFR. VEGF and its receptor (VGFR) affect angiogenesis and are very important signalling pathways in many cancers (28).

Elevated expression of VEGF-A, VEGFR1, and VEGFR2 has been shown in medullary thyroid cancer (50% of primary MTCs and 75% of distant metastases) (28,31,32). Expression of VEGF and its receptors in MTC is demonstrated in figure 8 (see below).

Angiogenesis (to ensure a supply of nutrients) is important for tumour growth and invasion, and as previously pointed to, VEGF/VEGFR (VEGF receptors 1, 2, or 3) have a key role in many cancers (24,33). In malignancy, the VEGF/VEGFR pathways are usually constantly activated. This activation is caused either by mutation of the receptor, or by tumour-dependent upregulation of VEGF expression (24,31,32).

But, antiangiogenic treatment cannot induce durable remissions in many malignancies, if it is applied as a single therapy. And that it can lead to increased tumour invasion and metastasis (24,34). Because alternative pathways, such as MET (see below) are activated, in response to tissue hypoxia and inhibition of VEGF/VEGFR. In a preclinical mouse model of pancreatic carcinoma, reduction of tumour size was caused by anti-VEGF treatment, when it was applied as a single therapy, but it conducted increased local invasiveness and liver metastases. On the other hand, the inhibition of VEGFR and MET (with cabozantinib) caused reduction in both tumour size and invasiveness, and prevented the development of metastases as well (24,35).

The expression of VEGF and its receptors are illustrated in Figure 8 (see below)

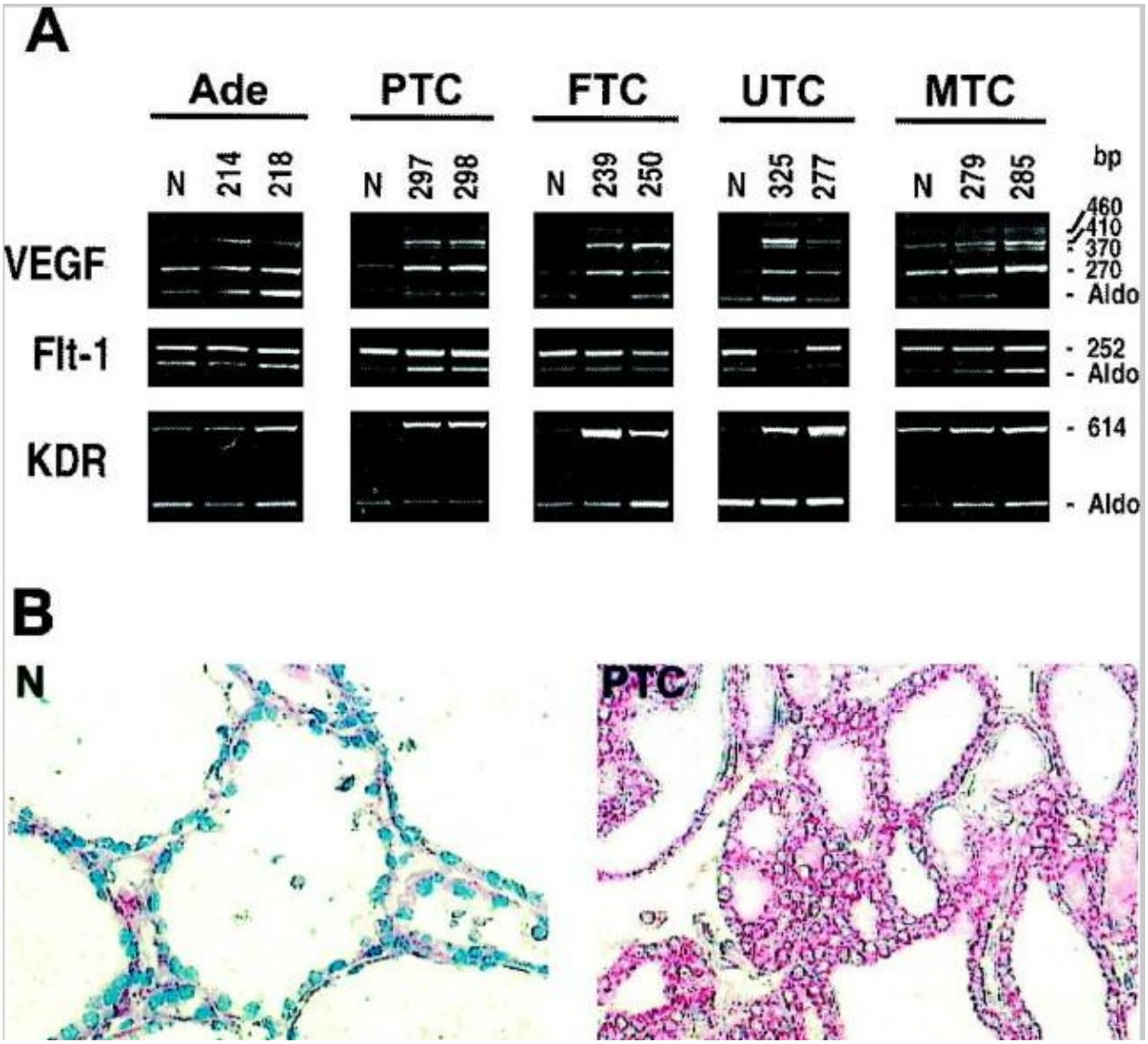

Figure 8: Expression of vascular endothelial growth factor and its receptors in various thyroid tumours. **A:** Total RNA was derived from normal thyroid tissues and various thyroid tumours. Semiquantitative RT-PCR evaluation was done with the use of 5 µg of total RNA and intron-spanning primers specific for VEGF, Flt-1, and KDR. Particular oligonucleotides were used to amplify various VEGF isoforms. 4 bands were visualized that correlated to 189aa (460 bp), 165aa (410 bp), 145aa (370 bp), and 121aa (270 bp). Aldolase signal (Aldo) shows the relative intensities of the bands. The numbers at the top of the figure show various patients. **B:** Immunoperoxidase staining of vascular endothelial growth factor in normal and neoplastic thyroid tissues. Magnification, ×200. N, normal thyroid; Ade, follicular adenoma; PTC, papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; UTC, undifferentiated thyroid carcinoma; MTC, medullary thyroid carcinoma **(32)**.

2.5.3 **Mesenchymal epithelial transition**

Mesenchymal epithelial transition is a proto-oncogene, which codes for the hepatocyte growth factor receptor (HGFR). In normal tissue, the activated receptor stimulates cell division and motility, especially in endothelial cells. It helps in angiogenesis and wound healing as well (24,36).

Mutation, overexpression and amplification of MET is observed frequently in tumours (24,37).

Activated c-MET, hepatocyte growth factor receptor, promotes cell replication and reduces apoptosis causing tumour cell survival and conferring malignant potential, and aids detachment and migration of the tumour cells, which results in invasiveness and formation of metastasis (24,38).

If MET is activated, in response to VEGF inhibition, these effects of MET act as “escape pathways”, causing tumour progression. Increased expression of MET together with hepatocyte growth factor has been shown in a subset of medullary thyroid cancer (24,39)

RET signalling activity can directly cause increased expression of c-MET (24,40).

Figure 9 demonstrates Signalling pathways of MET, *RET* and other RTKs (Receptor tyrosine kinases).

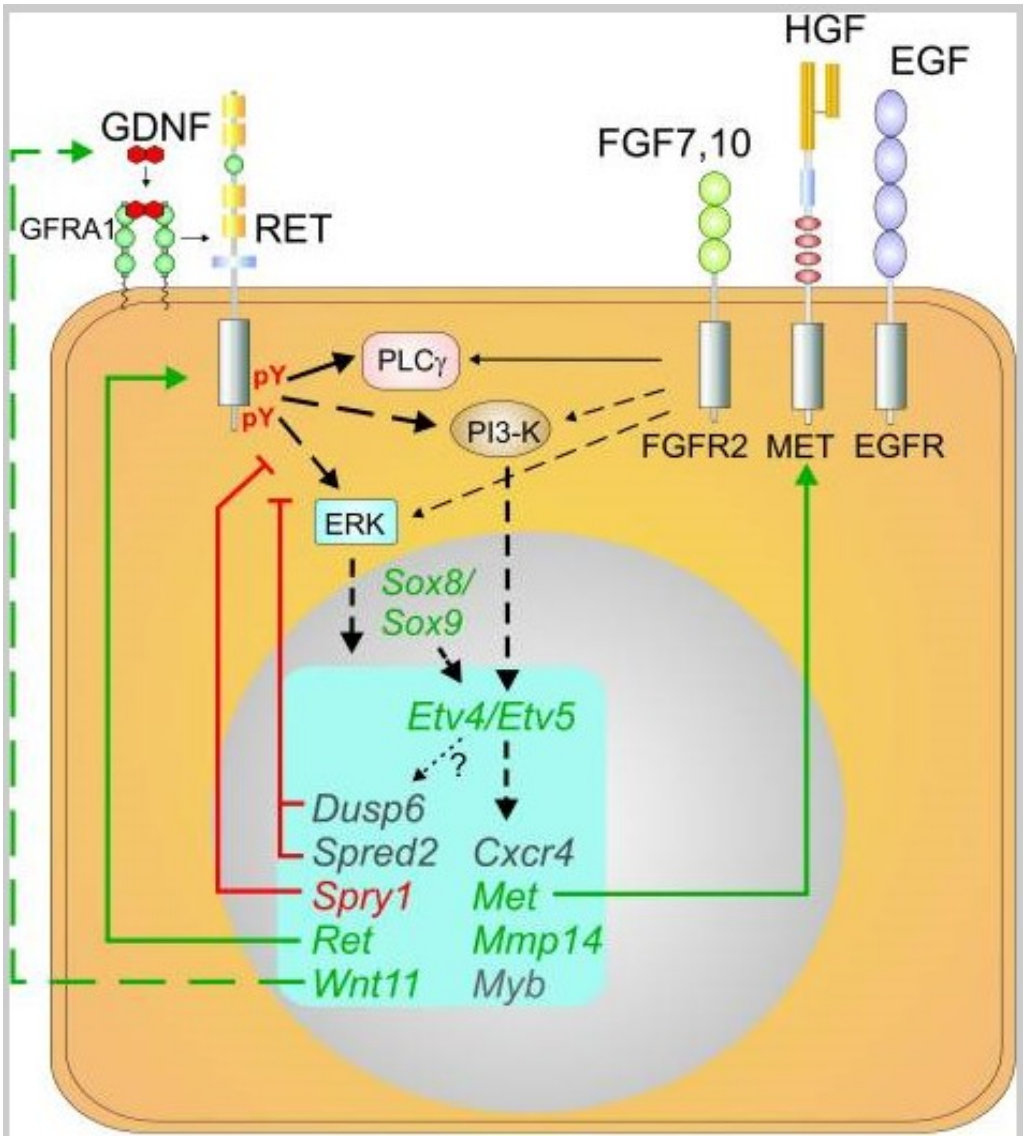

Figure 9: Signaling pathways of mesenchymal epithelial transition (MET), rearranged during transfection (*RET*) and and other receptor tyrosine kinase (RTK) **(40)**

2.5.4 **Other alterations**

The epidermal growth factor receptor correlates with regulation of cell growth, proliferation, and apoptosis and its overexpression has been demonstrated in some MTCs. Tissue microassay studies indicated that 20% of MTCs were strongly reactive for epidermal growth factor receptor and that MTCs harbouring the most aggressive *RET* mutations had reduced epidermal growth factor receptor expression (28,41).

It has also been reported that fibroblast growth factor receptor 4 is overexpressed in medullary thyroid carcinoma (28,41).

H-RAS mutations (in 56% of *RET* negative sporadic MTC) and activation of the mammalian target of rapamycin (mTOR) intracellular signalling pathway (in hereditary MTC) are examples of alternative genetic pathways that play a role in MTC development (28,42).

Ras-Raf-MEK-ERK pathway and its interaction with the phosphatidylinositol 3-kinase (PI3K)-AKT-mTOR pathway has also been researched in medullary thyroid carcinoma. Ras promotes cell functions like proliferation, differentiation, apoptosis and senescence. Phosphatidylinositol 3-kinase is one of the most important effector pathways of Ras. Especially, *RET*-negative MTC has a high prevalence of Ras mutations and 68% of *RET*-negative medullary thyroid carcinomas had Ras mutations (28,41).

2.6 History, physical examination, and laboratory testing of MTC

Most patients suffering from sporadic MTC have a palpable mass (43-46). Due to the fact that C cells are often located in the posterior part of the thyroid gland, these tumours are most often found in the posterior thyroid and usually cause compression of the trachea or the oesophagus, local invasion and nerve involvement. Dyspnoea, and cough, dysphagia, and hoarseness are the typical symptoms (43,46). Neuroendocrine symptoms such as diarrhoea, flushing, and weight loss may also be caused because of the elevated calcitonin secretion to the serum (44,46). Because germline mutation in the *RET* is observed in about 25% of MTC, the presence of other affected family members and possible syndrome correlated tumours must be considered and noticed very carefully. MEN2A, MEN2B, and familial MTC are the hereditary forms of MTC. Pheochromocytoma and hyperparathyroidism are also included in MEN2A, and could be identified (45-47). 75% of hereditary MTC are caused by MEN2A and medullary thyroid carcinoma is observed in >95% of MEN2A. MTC in MEN2A is typically multifocal and bilateral. MEN2B also contains pheochromocytoma and neuromas that can be screened on physical examination.

Almost 100% of patients suffering from MEN2B develop aggressive MTC at a very young age (43,45,46,48).

Calcitonin may authenticate the diagnosis of MTC. A small percent of normal patients may have marginally high levels of serum calcitonin, but mostly MTC is diagnosed when the calcitonin level is elevated (>100 pg/ml). Additionally, the degree of calcitonin elevation is associated with tumour volume. Nodal metastases become visible at basal calcitonin levels of 10-40 pg/ml (normal range <10 pg/ml), and calcitonin levels of >150 pg /ml typically correlate with distant metastases (43,46,49,50). CEA is another useful marker of MTC and more than half of the MTC patients have elevated CEA levels. Extensive disease, lymph node involvement, and distant metastases have association with a serum CEA level of >100 ng/ml (normal range <2.5 ng/ml), and it has been shown that even levels >30 ng/ml are correlated with an inability to do curative surgery (43,46,49).

Genetic testing must be offered to all patients with MTC. As it is mentioned before, the *RET* proto-oncogene, which is located on chromosome 10q11.2 has an activating mutation in FMTC and about 25% of the patients with MTC will harbour a germline mutation (46,47). The MTC aggressiveness differs according to the specific *RET* mutations and there are guidelines for the timing of performing prophylactic thyroidectomy for each mutation (45,46,51). The significance of performing surgery in patients with identified mutation before the development of carcinoma cannot be underscored. The prognoses of MTC in patients who have the cancer at the time of surgery is worse than in patients who have not developed carcinoma yet (46,52,53). Pheochromocytoma and hyperparathyroidism with urinary/plasma metanephrines, serum calcium, and parathyroid hormone levels should be evaluated, when multiple neuroendocrine neoplasia type 2 is suspected (45,46,49).The diagnosed pheochromocytoma has to be managed before the MTC (46).

2.7 **Diagnosis of MTC**

As pointed to before, patients with sporadic MTC typically have a solitary thyroid nodule. These nodules are with or without palpable cervical lymphadenopathy. MTC

is diagnosed by fine needle aspiration, which shows neuroendocrine cells and positive immunohistochemical staining for MTC tumour markers such as calcitonin, CEA, and CgA (28,54).

National Comprehensive Cancer Network (NCCN) recommends a basal calcitonin and CEA level, pheochromocytoma screening, serum calcium, genetic counselling, screening for *RET* mutation, neck ultrasound, and CT with contrast of the chest, in the primary evaluation of MTC (28,55).

As indicated previously, basal calcitonin levels have been demonstrated to be associated with tumour size and stage of disease. Calcitonin levels <100 pg/ml correlate with a median tumour size of 3 mm and when its levels are >2,000 pg/ml then additional imaging for metastatic disease must be done (28,54).

As it is mentioned before, information for risk stratification is also provided by CEA levels. Levels >30 ng/ml are mentioned to be predictive for central and lateral cervical lymph node involvement (in70% of patients). Bilateral nodal disease and distant metastasis were correlated with CEA levels of >100 ng/ml (28,54).

Poorly differentiated and progressive disease has loss of calcitonin expression and elevated CEA levels. When MTC is diagnosed at an early age then it can have good prognosis (28,42).

2.8 **Operation of MTC**

Prophylactic removal of the thyroid gland should be offered to patients with identified *RET* mutation before they develop medullary thyroid carcinoma. As pointed to before, the timing of surgery depends on the time of MTC development in different *RET* mutations (46).

The benefits of surgery must be considered and put against possible complications before the prophylactic surgery is offered to the patients. Almost 100% of patients with rearranged during transfection (*RET*) mutation will develop MTC and sooner or later will have lymph node involvement and distant metastases if the prophylactic

surgery is not done (43,45-47). Patients who have undergone prophylactic thyroidectomy have better prognosis and less recurrence than the patients with MTC at the time of surgery (46,56). Only about 1% of the MTC patients experience permanent complications after the operation by experienced surgeons; but, this number is increased if central node dissection is done (46,57,58). The extent of surgery that is essential in the prophylactic thyroidectomy has been discussed controversially. There is no controversy about the total thyroidectomy but it is about the addition of routine central lymph node dissection (45,46,49).

6% of the patients have clinically occult MTC with nodal metastasis and in this case the routine central lymph node dissection should be done. In addition, long term complications of central lymph node dissection are minimized by parathyroid gland autotransplantation (46,59). On the other hand the people who are against routine central neck dissection bring the argument that clinically occult MTC is very rare in young children ,under the age of ten years, and therefore it should only be done in selected patients. The chance of developing occult nodal disease is very low, when preoperative ultrasound is done and serum calcitonin (basal and/or stimulated) and CEA levels are evaluated, in such case the risk of permanent hypoparathyroidism is higher than the benefits of a prophylactic neck dissection (43,46,49).

In clinically evident MTC, a minimum of a total thyroidectomy and bilateral central neck dissection must be done. When lymph nodes in the lateral compartment are involved then lateral neck dissection should be added (45,46,49). About 81% of patients with palpable tumours have Central neck nodal disease (46,60). In clinically evident MTC, addition of a central neck dissection has good effects on the cure rates of the disease than thyroidectomy alone (46,61). In a central neck dissection, all lymph nodes and fibro-fatty tissue from the level VI compartment are completely removed. Careful dissection of the recurrent laryngeal nerve along its entire length and meticulous dissection of the parathyroid glands are important in a level VI lymphadenectomy (43,46).

Routine lateral lymph node dissection is not recommended in the present ATA guidelines (46,49). 14-80% of MTC patients have ipsilateral lateral nodal metastases and contralateral lateral nodal metastases have been mentioned in 19-49% of MTC cases (46,60,62). If ultrasound or physical examination indicate the presence of lateral lymphadenopathy then an ipsilateral lateral lymphadenectomy can be added (45,46,49).

The lymph node compartments in surgical intervention of MTC are presented in Figure 10.

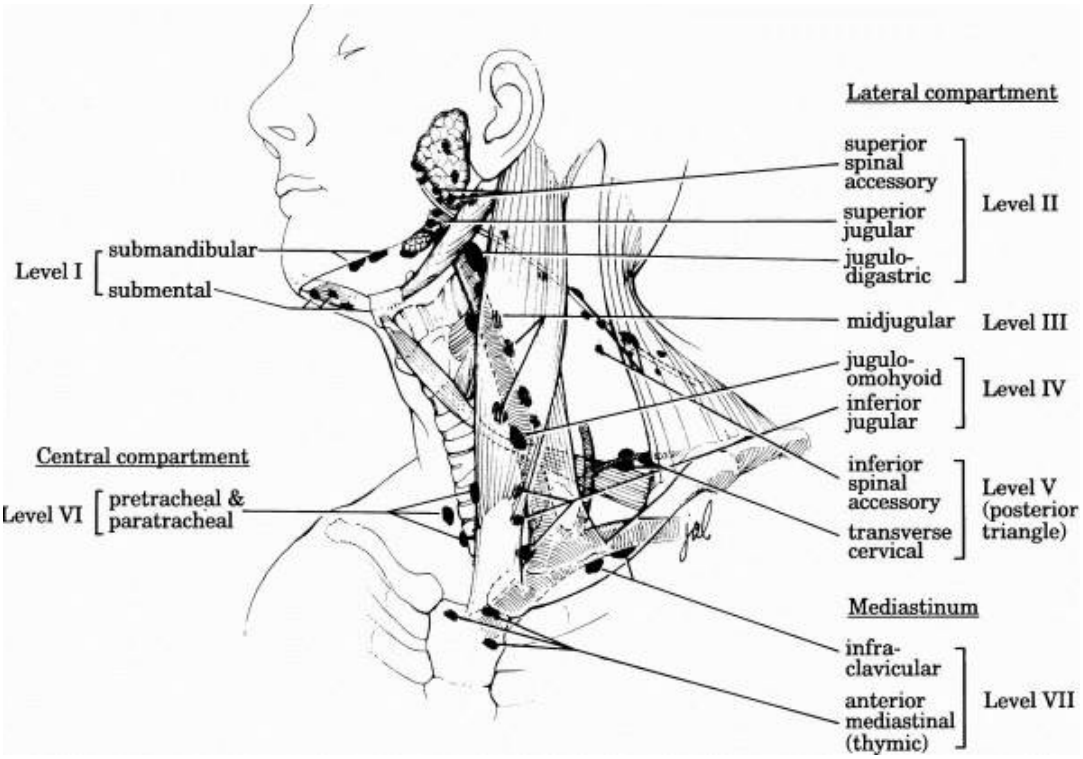

Figure 10: Lymph node compartments in the neck and upper mediastinum in surgical interventions of MTC **(60)**.

2.9 **Follow-up and prognosis**

After prophylactic thyroidectomy, the MTC patients are regarded to be cured and recurrent occurs in very few cases. In the post-operative period, calcitonin should be evaluated annually, after 60 months of follow-up, if no recurrence is observed then

these tests could be done less frequently (43,46). When MTC is limited to the thyroid gland and there is no evidence of nodal involvement then these patients have a very low risk of recurrence (46,63). But, nodal disease has been observed in many patients with MTC, which is associated with a very high risk of developing recurrent or persistent disease. So close post-operative follow up is very important (43,46,49). 2-3 months after the operation, follow-up should begin and a new baseline calcitonin and CEA level must be obtained. If calcitonin levels are undetectable after the operation then these patients should be evaluated and serum calcitonin should be measured every 6-12 months (46,49). In case if calcitonin levels rise but are <150 pg/ml, neck ultrasound must be done. Other imaging methods and options may be considered if the ultrasound delivers negative results. If calcitonin levels are >150 pg/ml then metastatic MTC should be assessed and neck ultrasound, CT, MRI, and bone scan must be done (46,49,53).

After a total thyroidectomy replacement of the thyroid hormone is needed; however TSH suppression is not indicated. Development of pheochromocytoma and hyperparathyroidism must be evaluated each year in hereditary MTC (43,46).

Generally, MTC patients have good prognosis and the 10-year survival rate is 75-80% (46,64-66). The extent of disease at the time of diagnosis is an important factor for the prognosis. About 50% of MTC patients have localized disease (limited to the thyroid gland) and a 10-year survival of 96% is observed in these patients (46,65). A 5-year overall survival rate of 76% is reported in MTC patients with regional disease (43,46,65). Distant metastases are present in 10-15% of MTC patients have distant metastasis at the time of diagnosis. These patients have a poor prognosis with a 10-year survival of 40% (43,46,65).

2.10 **Recurrence**

Most often recurrent or metastatic MTC cannot be investigated by the conventional imaging methods. Ultrasound, magnetic resonance imaging and CT usually fail to deliver information about recurrent lesions and the ^{131}I-MIBG (metaiodobenzylguanidine) or ^{123}I-MIBG and Octreoscan® (the most used

radiopharmaceuticals in NETs) have low sensitivity. Nowadays positron emission tomography CT with the use of ^{18}F-FDG (Fluorodeoxyglucose) is the best available method for the assessment of MTC patients, who have persistent high levels of calcitonin and it is said that the sensitivity of this method for the investigation of recurrent or residual MTC is 44.1%-85% (28,67).

But, some studies have indicated that ^{18}F-FDG has less sensitivity then ^{18}F-DOPA (flourin-18-L-dihydroxyphenylalanine), which means that ^{18}F-DOPA is better in the detection of recurrent MTC (28,68,69).

Vandetanib and cabozantinib (these two agents are presented in detail in the results section) are category 1 NCCN recommended drugs for recurrent or persistent MTC and locoregional or distant metastases. (28,55).

Recurrent disease is identified in about 50% of the MTC patients and metastases are seen in up to 20% (43,46,49). Residual and recurrent MTC can be evaluated and detected by calcitonin and stimulated calcitonin levels. This method of evaluation has a high sensitivity (43,45,46). In case if elevation of calcitonin level persists after the operation then metastatic MTC must be evaluated carefully (46,49). Significant risks correlate with neck reoperations so the recurrent MTC should only be operated when its advantages for the patient are high. These advantages are for example, achieving locoregional control or reduction of symptoms such as tracheal or oesophageal compression and pain. So, if symptomatic locoregional recurrence develops in MTC it should be operated surgically (46,49,70).

Neck ultrasound is the most sensitive method to investigate lymph node involvement in MTC. Distant metastases can be detected with CT and MRI. CT scans are more sensitive for the investigation of lung lesions while bone involvement is best detected with MRI scans (46).

If distant metastases are present in MTC then surgery should only be considered for palliation of symptoms. External beam radiation therapy or participation in clinical

trials with new available drugs may be offered to MTC patients, when the carcinoma cannot be resected (43,45,46,49).

2.11 **Chemotherapy and Radiation for MTC**

Low response rates are observed with cytotoxic chemotherapy and its effects last for shrot time. (24,28).

Doxorubicin was the therapy option of MTC, but low response rates are observed and it usually has significant toxicity (28,71).

Because medullary thyroid carcinoma originates from C cells, radioactive iodine is not effective in the therapy of MTC. External beam radiation therapy (EBRT) has been reported in MTC and its indications contain postoperative radiation in patients with residual disease and without distant metastases, extranodal MTC with soft tissue extension, mediastinal involvement, and high calcitonin levels (after the operation) (28,72).

EBRT has been reported in the therapy of patients with recurrent or metastatic MTC, but it is not used commonly. Patients with primary, histologically identified medullary thyroid carcinoma and total thyroidectomy with one or more lymph nodes removed between 1988 and 2004, were studied by Martinez *et al*. Surveillance, Epidemiology and End Results database was used in the mentioned study. It had 534 patients and 66 of these patients received EBRT. Univariate analysis of the study delivered that no significant improvement in overall survival was observed with EBRT ($P<0.14$). No improvement in overall survival was seen with EBRT in multi-variant analysis (28,73).

3 Aim of this work

The progress made in the therapy of MTC is undeniable, but the discovery of novel effective drugs is still very important, because all medical treatments do not lead to cure and cause transient disease control. Antiproliferative therapies have limited

efficacy, and new drugs that act against proproliferative cellular pathways are being studied. Over the last few years, there have been essential developments in the knowledge of the MTC molecular biology that caused relevant advances in the clinic.

In this context, cell lines from MTC portray a useful experimental model to study cell proliferation and tumour biology. Cell lines are crucial for the evaluation and establishment of new therapeutic agents. To date, various MTC-derived cell lines have been established and characterised, 9 at our Institute in Graz by Prof. Roswitha Pfragner and colleagues.

The aim of my work was to assemble the data provided about MTC, to introduce the tumour markers and the new drugs for MTC and to give an overview of the characteristics of the MTC cell lines.

4 Materials and Methods

This diploma is mainly based on the literature research. I collected the provided data about the MTC, which I found in PubMed. I tried my best to reduce the usage of books to a minimum. The literatures used in this diploma are about MTC, MTC cell lines, MTC tumour marker, and the new drugs for MTC (Cabozantinib and Vandetanib).

5 Results

5.1 MTC Marker

In contrast to hereditary MTC, early detection of sporadic MTC is difficult, making prognosis more unfavourable. Immunohistochemical staining with calcitonin is still routinely used to make a differential diagnosis of MTC. However, when metastatic MTC occurs without the expression of calcitonin, the diagnosis remains challenging.

Being a neuroendocrine tumour, MTCs express other tumour markers listed below.

5.1.1 **Calcitonin**

Calcitonin is a polypeptide, which has 32 amino acids and a seven-member disulfide ring at the carboxy terminal. Amino acid sequence in human calcitonin differs a lot from the porcine type; it contains leucine and isoleucine; in the amino-terminal, it has a seven-member ring, which includes just one amino acid substitution; and leucine and glycine are found in indistinguishable positions in the chain. Calcitonin plays its main role in the regulation of plasma calcium by a feedback mechanism. It prevents bone resorption, and reduces the amount of circulating calcium. Increased level of the circulating calcium in blood stimulates secretion of calcitonin and decreases the content and granulation of the C cells, but the complete physiologic importance is not known until now. The reduced level of circulating calcium stimulates the parathyroid glands and these glands may develop hyperplastic or possibly neoplastic changes, when calcitonin is secreted continuously (74).

In the therapy of particular manifestations of Paget's disease (in bone) calcitonin has been found useful. However porcine calcitonin is only used in the production of antibodies. This hormone is secreted in tumour excessively, in large amounts than that of the normal thyroid gland; which can be observed in venous drainage from the tumour and in the peripheral blood. This secretion of calcitonin in the neoplasm is not independent and may be enhanced by calcium infusion and other agents, particularly gastrin (calcium and gastrin stimulate the secretion of calcitonin) (74).

Calcitonin is an essential marker in the diagnosis of the tumour, because it can have increased levels in the blood either before or after stimulation; bioassay is expansively replaced by radioimmunoassay in the measurement of this marker. For the detection and diagnosis of very small tumours, the assay of calcitonin in the peripheral blood is most often used; without the stimulation of calcitonin secretion. It is very important for the persons who have high risk through familial relationship. In some cases, a provocative test may be necessary to detect the abnormal secretion of calcitonin by the tumour, in such cases calcium chloride infusion is commonly used (74).

Calcitonin is very important for the early detection and diagnosis of MTC. Because it is a sensitive marker for MTC, its early detection and then the surgical treatment may be beneficial in the improvement of the clinical prognosis of medullary thyroid cancer. In the diagnostic evaluation of patients who suffer from nodular thyroid disease, calcitonin should be measured. If serum calcitonin is elevated (>20pg/ml), stimulation testing should be done in order to improve the prognostic power for medullary thyroid carcinoma, especially in patients who have small nodules. Micro-MTC (<10mm) and C cell hyperplasia cannot be differentiated by the measurement of serum calcitonin. If stimulated calcitonin levels are higher than 100pg/ml then thyroidectomy should be offered due to the high inherent risk of medullary thyroid cancer. Very high levels of basal and stimulated serum calcitonin are indicative for medullary thyroid carcinoma and surgical management must be done (75).

5.1.2 **Carcinoembryonic antigen**

CEA is an oncofoetal cell surface glycoprotein. Because carcinoembryonic antigen is highly expressed in neoplasms and secreted to serum, it is most often used as tumour marker. It is a member of the immunoglobulin superfamily named CEA-related cell adhesion molecule (CEACAM) (76).

Members of CEACAM are identified in different cancers and cause cancer growth and invasion (76). The members of this family are: CEACAM1, CEACAM3, CEACAM4, CEACAM5 (CEA), CEACAM6, CEACAM7, and CEACAM8. The profiles of mRNA expression of CEACAM family were analysed in different tumour cell lines. The data showed that the mRNA expression patterns of CEACAMs in TT cells (one of the thyroid carcinoma cell lines; from MTC), were different from other tumour cell lines (76).

Most of the CEACAMs except for CEACAM8 were expressed by TT; this expression profile was different from other cell lines. CEACAM4 was only expressed in TT cells although it was not expressed by other thyroid carcinoma cell lines (76).

These data indicated that production of carcinoembryonic antigen and its related molecules in medullary thyroid cancer may be different from other tumour-based production of those molecules. Differentiation between MTC and other CEA-producing tumours would be possible through the expression of CEACAM4. (76).

Levels of the serum carcinoembryonic antigen and calcitonin play an effective role in surveillance of MTC patients, but are not associated with progressive or stable status of the disease.(76-78)

5.1.3 **Chromogranin A**

Neuroendocrine cells have typical secretory granules, called large dense–core vesicles. These granules contain specific peptide hormones (neuropeptides), and one or more Cg/secretogranin proteins (79,80). These are members of a unique family of secretory proteins, which have similarities in many biochemical characteristics as well as an unique presence in neuronal and neuroendocrine secretory granules (81,82). CgA was the first identified member of this family. It was first discovered in the catecholamine-containing chromaffin granules of the adrenal medulla (83). CgB and Sg II are other members of this family, which are well-characterized (81).

The best studied granin in humans is CgA. The human CgA molecule has 439 amino acids and contains 10 pairs of basic amino acids. CgA is a precursor of many peptides resulting from its enzymatic cleavage (84). It has an extended distribution and is also observed in some cells that cannot express CgB or Sg II (79,80). Endocrine cells of the anterior pituitary, parafollicular C cells of the thyroid, chief cells of the parathyroids, chromaffin cells of the adrenal medulla and islets cells of pancreas express CgA. Due to the fact that CgA is expressed in a great number of various NETs, its level in the circulation can be used as a ‘general’ marker for various NETs such as anterior pituitary tumours, parathyroid tumours, medullary thyroid carcinoma etc. CgA can be used as a marker in ‘non-functioning’ tumours, which may not have other suitable markers. It can also be evaluated when existing markers are unstable or rapidly fluctuating (e.g. as serotonin and catecholamine) (85).

5.1.4 **Chromogranin B**

CgB was obtained from a rat phaeochromocytoma cell line (86). The human CgB molecule has 657 amino acids and there are some structural similarities between CgB and CgA. CgB contains 15 pairs of basic amino acids and it can be processed in to several smaller peptides through proteolytic cleavage. CgB derived peptides such as secretolytin (CgB 614-626) (87), chrombacin (CgB 564-626) (88) and the fragment CgB 312-331 (89) have some biological functions. Some of the immunohistochemical studies indicate the expression of CgB in NETs. A statistically significantly poorer prognosis was observed in patients suffering from sporadic MTC, which contained few immunoreactive cells to a CgB antibody (90); the CgB amino acid sequences used for immunization were not described in that report, but the study shows that some sequences of the CgB molecule appear to be of prognostic value.

5.1.5 **Secretogranins II, III and V**

The Secretogranin (Sg) family has at least six established members (glycoproteins), named SgII to SgVII. The difference between Sg family and chromogranins is that Sgs lack the N-terminal hydrophobic disulphide-bonded loop (91). Secretogranin II, III and V have been found to be expressed in MTCs.

Secretogranin II

The human SgII molecule has 589 amino acids with nine pairs of basic amino acids. Secretogranin II is the main member of the Sgs, which may also be proteolytically processed to smaller peptides. The most important of these small peptides of SgII is secretoneurin (SgII 154-186), which may play a role in biological activities. Vallet *et al*. (92) showed immunoreactivity in pituitary gonadotroph tumours and non-functioning adenomas when they used an antibody raised against human recombinant SgII molecule, but in mammotroph tumours no such reaction was observed. Various types of neuroendocrine tumours, such as MTCs, carcinoids of the lung, duodenum and appendix, pancreatic neuroendocrine tumours, rectal NETs of

the L-cell type and Merkel cell carcinomas express SgII, but the expression of SgII is not observed in parathyroid adenomas (93-95).

Secretogranin III

Human SgIII is formed from 468 amino acids and seven pairs of basic amino acids (96). One function of SgIII is to bind with CgA (97); this CgA–sgIII complex appears to be important to intervene the targeting of CgA in the budding immature secretory granule (97,98). Nearly all NETs expressed SgIII. In the evaluated neuroendocrine tumours, the expressions of CgA, CgB, secretoneurin and SgIII were similar, except in phaeochromocytomas, which had only few SgIII cells (94).

Secretogranin V (neuroendocrine protein 7B2)

Human SgV has 185 amino acids and contains 3 pairs of basic amino acids as well (99). It has been reported that 3 smaller peptides are produced by cleavage of pro7B2 (100,101), but the biological characteristics of these smaller peptides have not been studied until now. There are some reports about the rare expression of SgV in MTCs (102,103).

5.1.6 Achaete-scute complex homolog 1

ASCL1 encodes a member of the basic helix-loop-helix family of transcription factors. Its overexpression is observed in NETs and NET derived cell lines (104-106). And it is believed that ASCL1 is involved in signalling pathways that cause growth and differentiation of MTCs (107,108).

5.1.7 Homo sapiens cut-like homeobox 1

Homo sapiens cut-like homeobox 1 (CUX1) belongs to the homeodomain family of DNA binding proteins (109).

Krug *et al*.(110) evaluated the effect of CUX1 on proliferation, resistance to apoptosis and angiogenesis. They studied these effects of CUX1 in murine and human pancreatic neuroendocrine tumours.

They evaluated the expression and effects of CUX1 with the help of Knockdown and overexpression strategies in Ins-1 and Bon-1 cells. Xenograft models and a genetically engineered mouse model of insulinoma (RIP1Tag2) were used (110). The RIP1Tag2 model indicates a model for the tumour progression, which was made to develop invasive neuroendocrine pancreatic islet tumours via hyperplastic and angiogenic precursor periods that closely outline the development of human disorder or disease (110,111).

RNA profiling and functional tube-forming assays in HMEC-1 cells were used to evaluate the angiogenesis regulation. At the end, they studied the expression of CUX1 in a tissue microarray of 59 human insulinomas and associated it with the provided clinical and pathological information (110).

Upregulation of CUX1 expression was observed during tumour progression in a time- and stage-dependent manner in the RIP1Tag2 model, and correlated with pro-invasive and metastatic features of human insulinomas. Tumour cell proliferation, tumour growth, resistance to apoptosis, and angiogenesis were increased by both endogenous and recombinant CUX1, which could be seen *in vitro* and *in vivo* (110).

5.1.8 **Mac-2 binding protein**

Mac-2 is a member of a family of beta-galactoside-binding proteins (M2BP = comparable to Galectin-3-binding). Mac-2 is associated with regulating cell-cell and cell-matrix interactions. The investigations showed that Mac-2 was elevated in the serum of patients, who suffered from NETs (112). Immunohistochemical staining of NET and normal tissue for Mac-2BP is exhibited in figure 11.

Mac-2BP (Mac-2 binding protein) levels were evaluated in serum samples from 47 patients and the control arm had 24 healthy subjects. Very high serum Mac-2BP levels were observed in the group of patients with NET (3.31 µg/ml; range, 0.82-10.66 µg/ml), which was not the case in the control group (2.30 µg/ml; range, 1.16-3.56 µg/ml; $p<0.001$). According to the primary site of NET, significant elevation of serum Mac-2BP was seen in NETs originating from the midgut (3.34 µg/ml; range, 0.82-10.66 µg/ml) in comparison with controls ($p<0.001$), and pancreatic NET (2.67 µg/ml;

range, 1.37-10.50 µg/ml) when brought in comparison with controls ($p<0.05$). MAC-2BP levels in patients with pancreatic NET and midgut NET varied not much. A positive correlation between CgA and Mac-2BP was seen in patients with midgut NETs, a spearman rank correlation was used ($r=0.36$, $p=0.013$), but no such correlation was observed in pancreatic NETs (113).

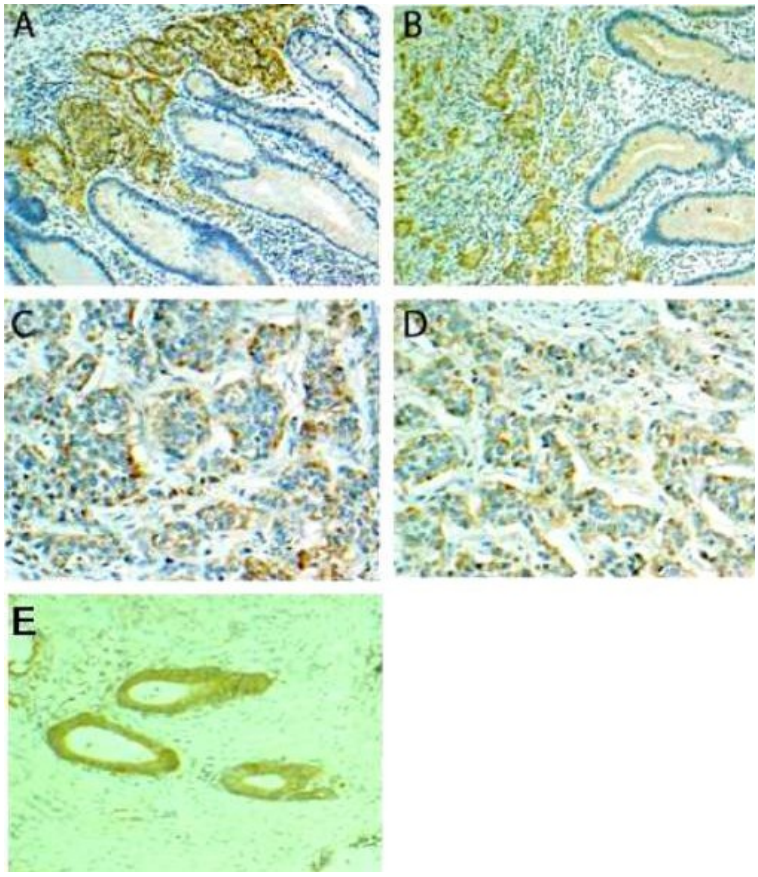

Figure 11: Immunohistochemical staining of neuroendocrine tumour and normal tissue for Mac-2 binding protein.(113) Anti-Mac-2BP antibody stained the fixed tissue sectoins (*brown*), and hematoxylin counterstained the nuclei (*blue*). *A*, ileal neuroendocrine tumour tissue exhibits mainly cytoplasmic staining of the clusters of neuroendocrine tumour cells. The neighbouring mucosa is presented normal with no visible staining. *Magnification*, ×200. *B*, midgut neuroendocrine tumour distinctly demonstrates staining of clusters of neuroendocrine tumour cells and negative staining of the neighbouring tissue. *Magnification*, ×200. *C*, pancreatic neuroendocrine tumour indicating cytoplasmic staining in the tumour cells. *Magnification*, ×400. *D*, ileal neuroendocrine tumour markedly containing neuroendocrine tumour and normal cells. Normal tissue shows no sign of epithelial staining, whereas adjoining neuroendocrine tumour cells are obviously stained. *Magnification*, ×400. *E*, gastric cancer control indicating moderate staining of gastric cancer cells with minimal staining of the background. *Magnification*, ×200 **(113)**.

Serum samples from patients and control subjects were taken in order to examine the usefulness of Mac-2BP levels, in the circulation, as marker for neuroendocrine

tumour. ELISA assessed the levels of serum Mac-2BP. Receiver operator characteristic (ROC) curves for serum Mac-2BP were designed to find out the cut-off values (for sensitivity and correlated specificity of the Mac-2BP assay) for all neuroendocrine tumours. ROC curves are a graphical method of measuring the quality of a diagnostic test by calculating and plotting sensitivity (true positive rate) against 1 –specificity (false positive rate). Authenticity of the test is assessed by the area under the ROC curve: an area of 1 shows a perfect test, and an area of 0.5 indicates a random association; usually, >0.75 is considered as a good marker. If the area under the curve (AUC) was 0.75, then it means generally that patient will have more abnormal Mac-2BP level than 75% of controls. When every patient had a more abnormal test result than every control then the test was perfect, and the corresponding AUC would equal 1. The area under the curve for all neuroendocrine tumours was 0.77, which indicates that Mac-2BP is a good marker of neuroendocrine tumours. Serum Mac-2BP ≥2.41 µg/ml was an immensely reactive marker (≥75%) for NETs; the correlated specificity associated to the control group was 59.3% (see Table II). The area under the curve was to some extent higher at 0.79 in midgut NET (Figure 12), and the sensitivity was at ≥75%, the specificity had the value of 70.8% (cut-off value >2.91 µg/ml) (113).

Assessment of serum Mac-2BP as marker for all GEP- NETs and midgut NETs has been demonstrated in the table below (Table.II).

Table II. Measurement of serum Mac-2BP as marker for all gastroenteropancreatic (GEP) neuroendocrine tumours and midgut neuroendocrine tumours **(113)**

Detection rate (sensitivity)	CI	Mac-2BP in all GEP NETs				Mac-2BP in midgut NETs			
		Cutoff	FPR	CI	LR	Cutoff	FPR	CI	LR
%	%	µg/ml	%	%		µg/ml	%	%	
47	32.1–61.9	>3.58	0	85.6–100		>3.59	0	85.6–100	
75	59.7–86.0	>2.41	41.7	22.2–63.4	1.80	>2.91	29.2	12.2–44.6	2.56
80	66.7–90.9	>2.06	58.3	37.4–78.2	1.37	>2.30	50.0	29.2–61.8	1.60
85	71.7–93.8	>1.97	58.3	36.4–77.2	1.46	>2.02	58.3	36.4–77.9	1.46
90	76.9–96.5	>1.72	66.6	45.3–84.3	1.35	>1.94	58.2	37.0–77.7	1.55
95	85.5–99.5	>1.65	70.2	45.3–84.3	1.35	>1.70	66.6	44.7–84.3	1.43

The sensitivity is the total true positive results divided by the true positive and false negative results. 100% − specificity is the false positive rate (FPR). CI shows the confidence interval. Likelihood ratio (LR) is calculated when sensitivity is divided by 1 − specificity. When Mac-2BP has a level >3.58 µg/ml, then it is 100% specific for neuroendocrine tumours **(113)**.

Due to the fact that this marker is not aimed for population screening but for obtaining information about the disease status of patients suffering from NET, a high specificity CI recognizing patients with disease is more beneficial than a high sensitivity. Mac-2BP obtains 100% specificity for recognizing disease in all of the patients suffering from neuroendocrine tumour, by the time that cut-off value was increased to >3.58 µg/ml, equivalent to a detection rate of 47% (Table. II).

Figure 12 shows assessment of serum Mac-2BP as NET marker with the related ROC curves and the corresponding AUCs.

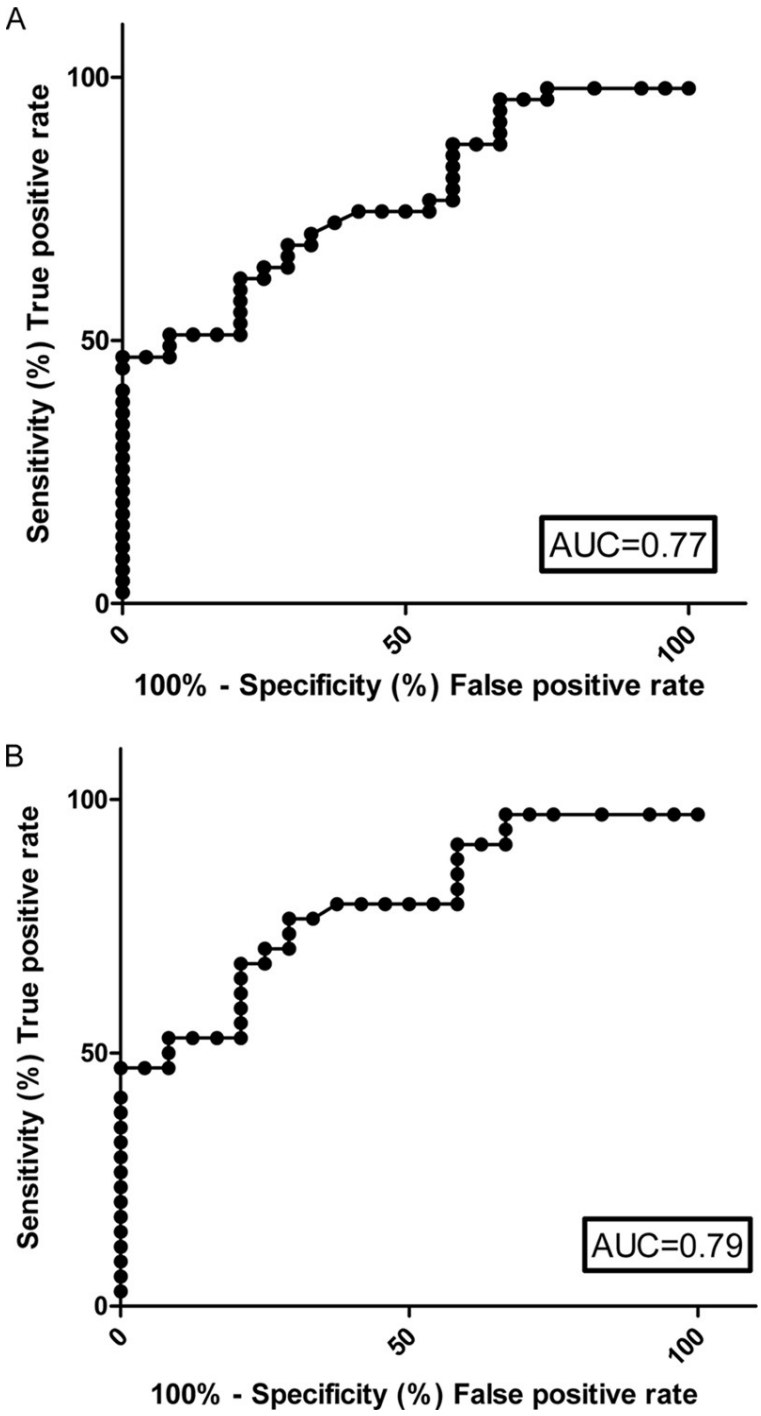

Figure 12: Evaluation of serum Mac-2BP as NET marker **(113).** ROC curves and the correlative AUC are presented for serum Mac-2BP levels in all types of neuroendocrine tumours (*A*) and serum Mac-2BP from patients with midgut primary neuroendocrine tumours (*B*) **(113)**.

5.1.9 **Enolase**

Enolases are metalloenzymes with multifunctional activity, both enolase1 (ENO1) and enolase2 (ENO2 or neuron-specific).

Circulating NSE and CgA were evaluated in 128 patients, who suffered from NET. The aim of this trial was the comparison of the sensitivity and specificity of these two markers, the investigation of factors correlated with elevated serum levels of NSE and CgA, and the certification of usefulness of these markers in the follow-up of these patients (114).

As it is mentioned before, NSE (Cispack NSE, Cis Bio International, Gif-sur-Yvette, France: normal<12.5 μg 1^{-1}) and chromogranin A (CgA-Riact, Cis Bio International, normal <100 μg 1^{-1}) were measured in 128 patients, who had no renal insufficiency. 99 patients had gastroenteropancreatic (GEP) NET, 19 were with MTC and 10 had phaeochromocytoma. The control arm had 53 patients with non-NET. In 48 (38%) and 76 (59%) of the 128 NET patients, had elevated serum NSE and CgA levels. It was proven that in all groups of patients with NET, CgA was more sensitive than NSE. A specifity of 73% and 68% was observed for NSE and CgA (114).

3 out of 8 controls had positive NSE immunostaining with elevated CgA levels, but all cases had negative immunostaining for CgA and synaptophysin. High CgA levels were markedly correlated with the presence of other secretions (P=0.0001) and a heavy tumour burden (P=0.001). High NSE levels were remarkably correlated with poor tumour differentiation (P=0.01). 6 patients with NET were followed for 11-37 months and CgA proved to be a better marker. (114).

5.2 **Tyrosine kinase inhibitors in the therapy of MTC**

5.2.1 **Vandetanib (Caprelsa) in the therapy of MTC**

Vandetanib is a TKI and has low molecular weight. It has shown effective prohibition of *RET in vitro*. *In vivo* phosphorylation and signalling of the *RET* /MEN2B oncoprotein were stopped by vandetanib and it inhibited growth of two human cancer cell lines that had spontaneous *RET* rearrangements (28,115). Vandetanib is active

against epidermal growth factor receptor (EGFR), which is not seen in cabozantinib (28).

A phase I/II trial was organized for children (5-12 years) and adolescents (13-18 years) with medullary thyroid carcinoma. The aim of this trial was to describe a recommended dose and evaluate anti-tumour activity of vandetanib. The starting dose of vandetanib was 100mg/m^2/d given orally, which was continued for 28 day treatment cycles. The dose could be increased to 150 mg/m^2/d after 2 cycles. RECIST (v1.0) quantified radiographic response to vandetanib; biomarker response was quantified as well. For the measurement of Biomarker response post-treatment serum calcitonin and carcinoembryonic antigen levels were compared to baseline, and clinical benefit was assessed by patient reported outcome. 16 patients with locally advanced or metastatic medullary thyroid cancer were given vandetanib for a median of (range) 27 (2-52) cycles. Eleven patients continued the protocol therapy. The primary dose-limiting toxicity was diarrhoea. The confirmed objective partial response rate, in subjects with M918T *RET* germline mutations (n=15), was 47% (exact 95% CI, 21%, 75%). In twelve subjects, biomarker partial response was confirmed for calcitonin, and it was confirmed in eight subjects for carcinoembryonic antigen (116).

The corresponding radiographic Objective Responses in MEN2B and MTC are shown in the figure below (figure 13).

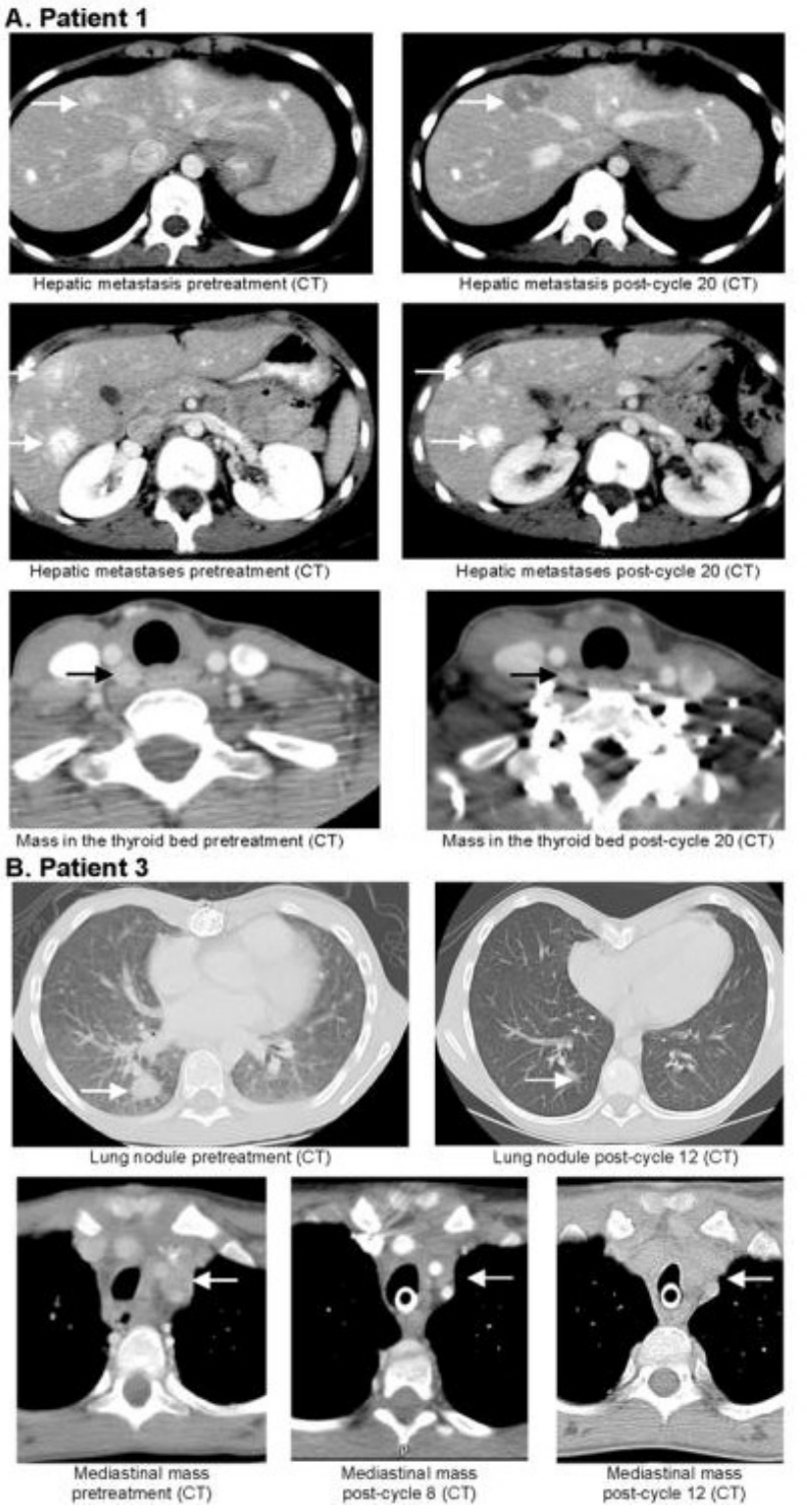

Figure 13: Radiographic Objective Responses in Children and Adolescents with MTC and MEN2B **(116)**.

(A) Computer tomography of chest and neck in Patient 1 (15 year old female) and (B) Computer tomography of chest in Patient 4 (11 year old male) **(116)**.

At the beginning vandetanib was developed as an oral small-molecule tyrosine kinase which prevented the vascular endothelial growth factor receptor 2 (VEGFR2) (IC_{50}=40 nmol/L) and it was also found that it prohibits vascular endothelial growth factor receptor 3 (VEGFR3) (IC_{50}=110 nmol/L) and epidermal growth factor receptor EGFR (IC_{50}=500 nmol/L) (117,118). After that preclinical studies demonstrated that vandetanib inhibited *RET* mutant forms (MEN2A and MEN2B) and *RET* translocations, which exist in papillary thyroid cancer (118,119). The activities of vandetanib are demonstrated in figure 14, and figure 15 shows the detailed intreaction of tumour cells with VEGF proteins and the VEGF receptors and the action of Vandetanib against them.

Three phase I trials of patients with treatment-refractory solid tumours, in which vandetanib was used as a single-agent, assured the recommended phase II dose of 300 mg/d. The dose-limiting toxicities (DLT) were diarrhoea, hypertension, and rash (118,120-122). Asymptomatic QTc prolongation was reported in two of the studies (118,120,121). In one trial of 36 patients, only one partial response was seen (this patient had medullary thyroid cancer) (118,122). Taken together, the phase I and preclinical studies furnished motivation and reason for the clinical development of vandetanib in MTC (118).

Two phase II single-arm, open-label studies established efficacy of vandetanib in hereditary MTC. The first phase II study had 30 patients with hereditary, unresectable, or metastatic MTC, who received vandetanib at 300mg/d (118,123). Six partial responses (20%) were seen and stable disease was observed in 53% of the patients; median progression-free survival (PFS) was 27.9 months. Most of the patients experienced at least one adverse event, which contained diarrhoea, rash, fatigue, and nausea. A second phase II study enrolled 19 patients, who received vandetanib at 100 mg/d, with allowance for postprogression increase to 300 mg/d (118,124). 16% was partial response rate and 53% was stable disease rate, which confirms the anti-tumour activity of low-dose vandetanib (118).

The pivotal ZETA trial (118,125) caused the approval of vandetanib for advanced progressive MTC by the U.S. FDA in April 2011. This international, randomized, double blind phase III trial enrolled 331 patients with unresectable, locally advanced, or metastatic MTC (hereditary or sporadic). Patients were randomized 2:1. These patients received vandetanib 300 mg daily (n=231) or placebo (n=100) until disease progression. PFS was the primary endpoint, and objective response rate (ORR), disease control rate (DCR) at 24 weeks, duration of response, overall survival, biochemical response, time to worsening of pain, safety, and tolerability were secondary endpoints. Disease progression was not included in eligibility criteria, but measurable tumour at baseline and a calcitonin level of at least 500 pg/ml were required. Most patients had nonhereditary MTC (90%) and metastases (95%). 40% had received prior systemic therapy. Calcitonin or carcinoembryonic antigen (CEA) doubling time of ≤ 24 months was present in 51% of patients for calcitonin and 31% of patients for CEA (Calcitonin or CEA doubling time of ≤ 24 months is a marker, which is associated with more aggressive disease) (118,126).

Efficacy of vandetanib was observed in all evaluable endpoints in the ZETA trial except survival. At a median follow-up of 24 months, median PFS was not attained with vandetanib (calculated ~30 months) and was 19.3 months with placebo [HR, 0.46; 95% (CI), 0.31-0.69; P<0.001].(118,125). 83% was the 6-month PFS rate in the vandetanib group and in the placebo group 63%. Vandetanib demonstrated a higher ORR than placebo (45% vs. 13%; P<0.001) and higher DCR (87% vs. 71%; P=0.001). Among the13 patients in the placebo arm who responded, 12 did so after treatment with open-label vandetanib. Data about overall survival was immature at the time of analysis and no significant difference was revealed (HR, 0.89; 95% CI, 0.48-1.65). Vandetanib had a better biochemical response rate (complete response defined as normalization of serum levels; partial response defined as ≥50% decrease from baseline in serum calcitonin and CEA for ≥4 weeks) than placebo (69% vs. 3% for calcitonin, P<0.0001; 52% vs. 2% for CEA; P<0.0001). Vandetanib had a delay in time to worsening of pain (HR, 0.61; 95% CI, 0.43-0.87; P=0.006); however, other health related quality-of-life measures were not judged (118).

RET genotype was correlated with response to vandetanib in the ZETA trial. Tissue was attained for *RET* genotyping in 297 of 298 patients, who had sporadic medullary thyroid carcinoma. Somatic *RET* mutations were diagnosed in 155 patients (52%), and they were absent in 8 patients (2.7%) (118,125). A large population of patients was classified as *RET* mutation status “unknown” (n=135; 45.3%) because of the insufficient tumour DNA, which could fulfil stringent testing criteria. This condition made subgroup analysis by *RET* mutation status for PFS and ORR in ZETA inconclusive. It was interesting to observe that a higher response rate to vandetanib was seen in patients with sporadic MTC tumours having a somatic M918T mutation (54.5%; 55 of 101) in comparison to patients with sporadic MTC tumours without a somatic M918T mutation (32%; 33 of 103). In all prespecified subgroups, vandetanib appeared to be active (118,126).

Not many treatment options exist for patients with unresectable or metastatic MTC. In metastatic disease, a watch-and-wait approach is likely in asymptomatic patients with low tumour burden, who have no evidence of tumour progression on periodic restaging, because patients with indolent metastatic disease may survive for many years without systemic therapy. Clinical trial enrolment is encouraged for patients with progressive or symptomatic metastatic disease and high tumour burden, by the National Comprehensive Cancer Network (NCCN). When no trial enrolment is done then the systemic therapy with TKIs is preferred over cytotoxic and chemotherapy. Cytotoxic and chemotherapy are associated with high toxicity and low efficacy. The first-line systemic therapy of choice is vandetanib (118,127).

When to begin with vandetanib therapy is less clear. Vandetanib should be used only in symptomatic or rapidly progressive MTC, which is determined by clinical judgment. To guide patient selection, no validated predictors of response to vandetanib exist. Subgroup analyses recommend that patients with sporadic MTC tumours having the aggressive M918T mutation and patients with CEA doubling times ≤24 months compared with those with longer doubling times, will have greater benefit from vandetanib (54% vs. 37%) (118,126). It is critical to evaluate the treatment risk-to-benefit ratio and to monitor the toxicities (118).

It is important to monitor and correct ECG, serum potassium, calcium, magnesium, and thyroid-stimulating hormone levels. Synchronous use of drugs that prolong the QT interval or cause torsade de pointes should be avoided, because the risk for torsade de pointes may be protracted given the vandetanib's long half-life ($t_{1/2}$=19 days) (118). Vandetanib cause an increase in thyroid stimulating hormone in most of the patients (78%) and about 50% of these patients will require increase in thyroid replacement dose. Due to the toxicity profile, vandetanib should not be given for asymptomatic, indolent disease (118).

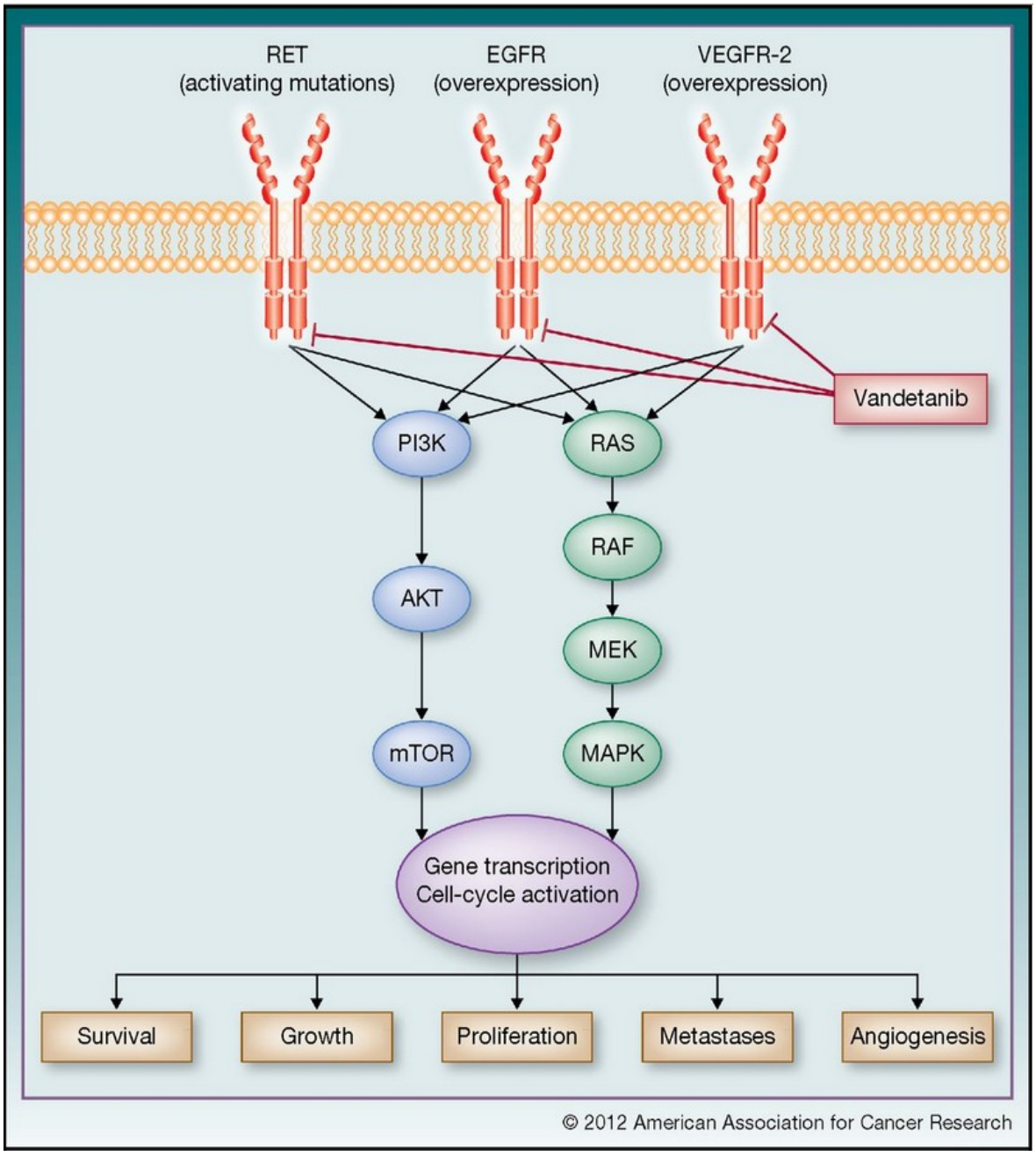

Figure 14: Activity mechanism of vandetanib in MTC **(118)**.

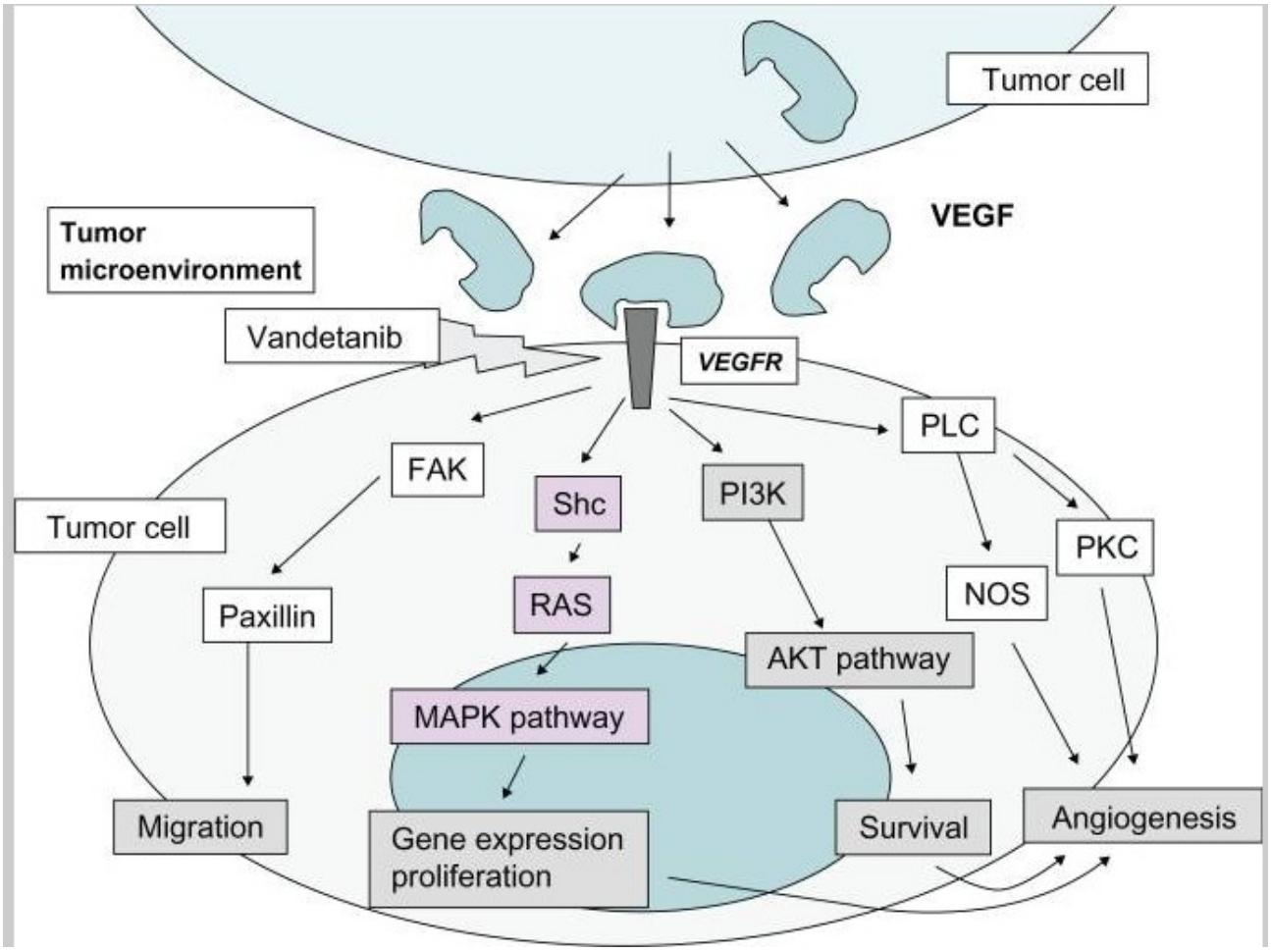

Figure 15: The action of vandetanib againt VEGF and detailed intreaction of tumour cells with VEGF proteins and the VEGF receptors **(128)**.

It was a landmark event to approve vandetanib as a systemic treatment for patients with unresectable or metastatic MTC and it symbolizes a new standard of care for these patients. Improvements in progression free survival (PFS) and overall survival are possible and may be obtained when vandetanib is combined with either chemotherapy agents or other targeted treatments. The high cost of a new TKI can limit the number of patients who may have access to this medication (128).

Tumours exposed to vandetanib may also develop resistance. It is speculated that there may be many reasons for this resistance which could include new molecular abnormalities involving the *RET* or other receptors such as loss of expression,

genomic amplification or the activation of alternative signalling pathways. Further work needs to be done to clarify which of these is most important. It might be possible to overcome or delay resistance mechanisms when vandetanib and other drugs are combined (128).

5.2.1.1 Adverse events of Vandetanib

Due to the fact that most of the patients who respond to vandetanib wil receive this TKI for long periods of time, all care providers, which include community healthcare physicians and nurses as well, should have a good understanding of the drug's safety profile and its potential impact on quality of life and compliance of these patients (129).

Before it is initiated with the vandetanib therapy, a review of past medical history, current comorbidities, and medications should be carried on, emphasizing the potential interactions and effects on vandetanib-related adverse events. As it is mentioned before, there is controversy in the literature about the optimal time to initiate with treatment in patients having advanced medullary thyroid carcinoma (129,130). In order to identify the optimal time of starting with vandetanib treatment, it might be helpful to know the size and number of tumour foci and the rate of change of tumour volume during watchful waiting (129). The rate of change in serum levels of calcitonin and/or carcinoembryonic antigen are also of importance, but are not to be considered in isolation (129).

Patients who receive vandetanib may develop a rash (Figure 16). The incidence of all-grade and high-grade rash/folliculitis in association with vandetanib 300 mg was 46% (95%CI 40.6-51.8%) and 3.5% (95% CI 2.5-4.7%). This result was reported in a systemic review of trials involving 2,961 patients with different tumours, inclusive of MTC (129,131). The mechanism of the rash has not been fully explained, but most seen rashes, especially those, that appear as follicular pustules, are probably because of the anti-EGFR effect of vandetanib, as anti-EGFR agents have association with acute and subacute folliculitis (129,132). The effect of vandetanib which blocks EGFR may activate follicular hyperkeratosis, leading to follicle

obstruction and inflammatory response (129,133). These lesions may be superinfected as well (129).

Photosensitivity, xerosis, finger clefts, paronychia, genital skin reactions, subungual splinter hemorrhages, and blue-gray macules are other cutaneous adverse events of vandetanib (129,132). All patients, treated with vandetanib, have photosensibility, even through glass behind closed windows. This photosensibility should be prevented by protection against any sun exposure. Blue-gray macules can have variable size and are most often on the face, scalp, or trunk. They have similarities with the pigmented macules seen on the skin and cornea of patients, who receive amiodaron (129,134). These blue-gray macules appear after several months of treatment with vandetanib and usually disappear after the treatment is interrupted. Mucositis, erythrodysesthesia, and hand-foot skin reactions occur rarely and are most often minor (129,132). Serious skin reactions such as Steven-Johnson syndrome and erythema multiforme have been reported in literature, but are rare (129,135). Folliculitis and blue-gray macules in patients with MTC under the treatment of vandetanib are shown below (figures. 17 and 18).

Most of the dermatologic AEs are manageable, but before starting vandetanib treatment, it is important to discuss the potential development of skin reactions with patients, begin with preventive measures, and provide relieve to the anxiety of the patients saying that these dermatologic AEs can usually be managed effectively. An assessment of mucosal and skin surface is important whenever patients attend clinic. Strict photoprotection (e.g. use of a broad-spectrum UVA/UVB sunscreen with sun protection factor of 30 or higher, avoidance of any sun exposure by cloth protection) and avoidance of products that dry the skin (e.g. soaps, alcohol-based or perfumed products) are the key management points, and have to be discussed with the patients. The emergence of rash can be captured by early monitoring and in complicated cases collaboration with a dermatologist may be needed (129).

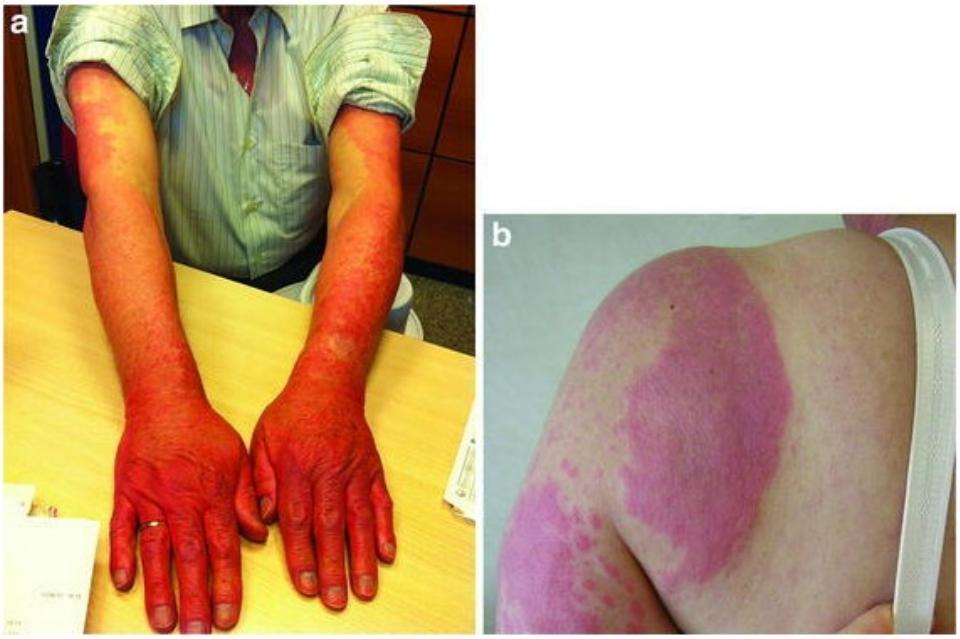

Figure 16: Rash observed with vandetanib treatment in metastatic MTC. **a** Rash is present on hands and forearms; **b** Rash caused by photosensitivity on upper extremity and back **(129)**.

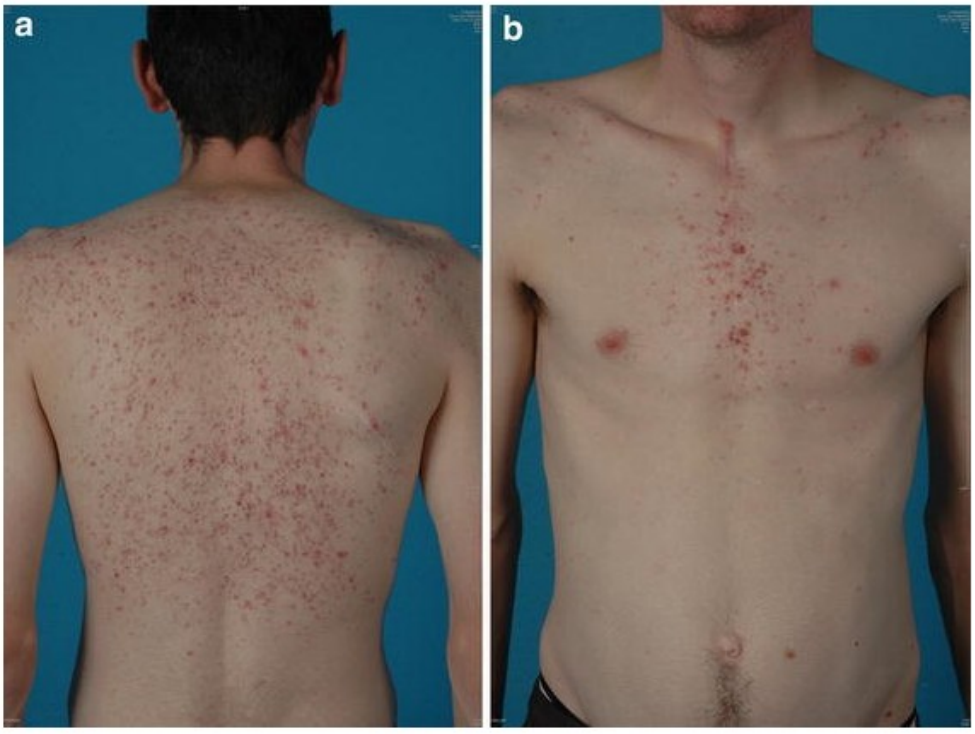

Figure 17: Folliculitis in a male patient treated with vandetanib. **a** Folliculitis on back; **b** folliculitis on front **(129)**.

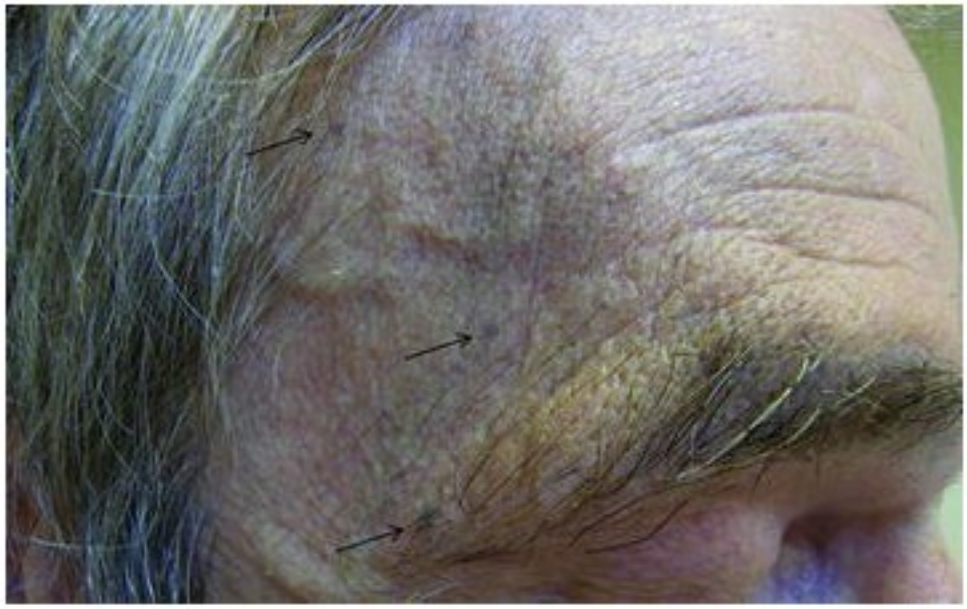

Figure 18: Blue-gray macules on the forehead of a patient treated with vandetanib. *Arrows* show small spots on a greyish background **(129)**.

Patients taking vandetanib may have QTc prolongation and increased blood pressure. According to a systemic review and meta-analysis of 3,154 patients who received vandetanib, the incidences of all-grade and high-grade hypertension were 24.2% (95% CI 18.1-30.2%) and 6.4% (95% CI 3.3-9.5%) (129,136). A higher incidence of all-grade events with vandetanib therapy was also seen in patients with MTC, which was not the case by patients with non-small cell lung cancer (NSCLC) and non-MTC/NSCLC tumours receiving vandetanib.The relative risk was 1.36 (95% CI 1.05-1.76, p=0.02) and 2.06 (95%CI 1.26-3.3, P=0.004), probably because of longer treatment exposure and higher doses of vandetanib in MTC (129,136).

Current guidelines recommend early detection and effective management of hypertension. Close monitoring of blood pressure during the first months of treatment is also recommended. Pre-existing hypertension has to be managed accurately before treatment is stated. This management is done according to the current guidelines, such as those from the fifth joint task force of the 'European Society of Cardiology' (they define hypertension as blood pressure ≥140/90 mmHg) (129,137). Under vandetanib treatment, angiotensin–converting-enzyme (ACE) inhibitors are the

most commonly prescribed anti-hypertensive drugs. If hypertension is not controlled with angiotensin-converting-enzyme inhibitors, then calcium antagonists and beta-blockers can also be helpful. The patients themselves may monitor their blood pressure 1-3 times per month allowing close control of hypertension without the need for special hospital visits (129).

The term “corrected” QT interval may not be understood properly and may cause misunderstanding. It relates to the QT interval but modifies heart rate. Definitions for QTc interval prolongation differ in the literature, and prolongation is divided into absolute (e.g. >500 ms) or relative (e.g. >30 ms change from baseline in QTc interval). Most commonly, an interval of above 480 ms is prolonged (129).

The QTc interval is best measured manually, than relying on automatic measurements of standard ECG machines, for the treatment with QTc-prolonging drugs (129,138). Use of QT nomogram is an alternative method, in which a QT interval-heart rate pair that plots above an “at-risk” line demonstrates that the patients is at risk of torsade de pointes (129,139).

QTc prolongation above 450 ms has association with a risk of ventricular arrhythmias (e.g. TdP, syncope, and sudden death). This risk rises when the duration of prolongation is increased. Most often in the first 3 months of treatment with vandetanib, first QT prolongations take place (129).

The treatment with vandetanib should be stopped if the QTc interval is longer than 500 ms and when the QTc interval returns to 450 ms then a reduced dose of vandetanib can be given (129). It is very important to record a baseline ECG with QTc measurement before starting with vandetanib, and it should not be given to patients with a baseline QTc longer than 450 ms (value may differ, which depends on local product information). Mean QTc-prolongation is 30 ms, during the treatment with vandetanib. QTc should be monitored at least once every month for the first 3 months. After initiating vandetanib treatment if any new cardiologic abnormality is detected then the therapy should be stopped and the patient should be referred to a cardiologist. In some clinical situations, close attention to the QTc and monitoring it

may be a requirement (129). When preventive measurements are applied, the QTc interval prolongation is most often no problem. These measurements include avoidance of drugs, which can prolong the QTc interval, and correcting hypothyroidism, hypokalaemia, hypomagnesaemia, and hypocalcaemia (129). Vandetanib is contraindicated with some of the drugs and it is recommended not to combine them (Table.III)

Table III. Selection of drugs, that are contraindicated and not recommanded with vandetanib **(129)**.

Contraindicated	Not recommended
Arsenic	Amisulpride
Cisapride	Chlorpromazine
Class IA and III antiarrhythmics	Halofantrine
Intravenous erythromycin	Haloperidol
Mizolastine	Lumefantrine
Moxifloxacin	Methadone
Toremifene	Metoclopramide
	Ondansetron
	Pentamidine
	Sulpiride
	Zuclopenthixol

5 HT3 antagonists are generally associated with the potential of prolonging the QTc interval. Ondansteron should be avoided in patients, who are treated with vandetanib,

specially those who have cardiovascular problems and a high risk of drug-induced torsade de pointes. When Ondansteron was given to the patients with cardiovascular disease who had one or more risk factors for torsade de pointes, after administration, the QTc interval was increased by about 19ms for 120 min (129,140). two studies of patients under chemotherapy showed that palonosetron induced no severe rhythmic disorders or ECG changes (129,141). And no statistically significant increase in median QT minimum value was observed with palonosteron (129,142). Because of the benificial effects mentioned above Palonesetron is an alternative antiemtic therapy . But attenton must be paid to its associative use with other agents that prolong the QTc interval or when it is administered to patients who have or can develop QTc prolongation. Due to the fact that large bowel transit time may be prolonged by palonosteron, patients who have diarrhea under chemotherapy could have advantage from this drug (129,143).

Quality of life in patients with MTC can be reduced because of diarrhea, which can be caused by the disease or treatment. Diarrhea can be the main reason of dose reduction or discontinuation of therapy. Patients may also develop dehydration with electrolyte problems and/or potentially life-threatening complications because of the increased QTc interval under vandetanib therapy. Antibiotic therapy of folliculitis has worse effects on Diarrhea (129).

In MTC, production of hormones that stimulate gastrointestinal motility can cause diarrhea, which may worsen with vandetanib treatment. And partial relief can be obtained if agents that slow peristaltic movements such as loperamid or mild opioids such as codeine are used. There is no evedence that can proof the effect of somatostatin analogs against diarrhea in patients with MTC (129).

Awareness and education about gastrointestinal tolerability is a key part of patient education (129). Hypokalemia can also cause increase in QTc interval, therefore, serum electrolytes must be monitored regularly. Vandetanib therapy should be withheld, when the patients develop sever gastrointestinal symptoms and it can be given again when these symptoms improve. After discontinuation of treatment, if the

severe diarrhea still persist then a stool- work-up should be done so that organic causes are excluded (129).

Generalized adverse events (AEs) can affect the quality of life, but these effects can differ. Many patients can maintain normal activities, others have the experience of exhausting fatigue that causes dose limitation or treatment interruption (129,144). Fatigue contains emotional, physical, and/or cognitive tiredness and can be a disturbing and constant AE. This symptom is most often multifactorial and the underlying burden of disease, hypothyrioidism, anemia, depression, sleepdisturbances, or pain can cause it. It can be difficult to address fatigue. Fatigue is managed primarily with support; however, it is necessary to diagnose treatable causes which lead to it. To optimize emotional and social support, patients should be evaluated for depression and applicable treatments such as avoidance of drugs that may increase QTc interval should be introduced to them (129).

Visceral fat and muscle body content are increased by vandetanib in patients with MTC, which is not the case in other TKIs (129,145). Because of this effect, patients on vandetanib treatment should be encouraged to continue with a normal social and professional life, and try to participate in sports activities. It is important to notice that pregnancy is a contraindication for vandetanib and effective contraception is essential for all patients (129).

In a multidisciplinary care team, an experienced specialist nurse is in an incomparable position to assist the progress of early detection, intervention, and mangement of adverse events which are associated with vandetanib. Because some AEs are typically observed in the first 3 months of treatment with vandetanib, it is essential to have close patient contact in this period. For the first 6-8 weeks the patients may have clinic visits every 2 weeks and ECG, serum electrolyte monitoring, and review of AEs could be done (129).

Specialist nurses not only provide patient education, but face difficulties in obtaining AE information from patients too. Some patients feel more comfortable when they discuss AEs with a nurse rather than a physician, but not all patients are aware of the

significance of reporting AEs. Some patients may not contact the specialist nurse or physician if they have AEs during treatment. Therefore it is essential that patients are informed, most of the time by the specialist nurse, of symptoms and the value of reporting about these AEs at the beginning. Patients should become a list of drugs that are to be avoided during the treatment with vandetanib so that any care provider can confirm whether a drug is contraindicate or not and , in case of suspicion, contact the clinic. It is also important to inform patients of the significance of reporting to their specialist nurse and/or physician of any associative medication started by other clinicians for additional disorders and side effects. These reports from the patients should be carefully documented and cross-checked to avoid possible dangerous and complicated interacitons between the given drugs (129).

The logistical effect of geographical distance on patient visits must also be considerd. Because MTC is a rare disease, it is possible that the patients live far from the clinic. So contact via telephone may be necessary as a replacement for a clinic visit. In some cases, instead of a doctor's appointment, an experienced oncology nurse can visit the patient. A telephone given by the clinic, internet video calls, and call center are some of the alternative options for the communictaion with the patients, according to the clinic's policies and resources. Oncology nurses have a very important role in the treatment of patients with MTC (129).

5.2.2 **Cabozantinib and the treatment of MTC**

Cabozantinib or XL 184 is an oral tyrosine kinase inhibitor. It is also known as a multikinase inhibitor and is active against mesenchymal epithelial transition (MET), vascular endothelial growth factor 2 (VEGFR2), and rearranged during transfection (*RET*). Activation of these receptors is associated with both development and progression of medullary thyroid cancer (24,28,71). The activity mechanism of cabozantinib and other TKIs (vandetanib, axitinib, crizotinib, motesanib, and sunitinib) are summarised in figure 19 and the inhibitory activity of cabozantinib is compared with other tyrosine kinase inhibitors (TKIs) in Table IV.

Cabozantinib was approved by the US Food and Drug Administratoin (Cometriq®, Exelixis, Inc., San Francisco, CA, USA) in November 2012 for metastatic medullary thyroid carcinoma. It is orally bioavailable and it is important not to eat for 2 hours before taking cabozantinib and 1 hour after it. The recommended dose of cabozantinib is 140 mg/d (one 80 mg capsule and three 20 mg capsules) and it is metabolised in the liver via cytochrome P450 (CYP) 2C8 (28,146).

The safety profile of cabozantinib is acceptable and its half-life is 91.3±33.3 hours. Its half-life supports once-daily dosing, and it has efficacy in patients who have experienced progression on other therapies, inclusive of other TKIs (such as vandetanib) (28,146,147).

Sennino *et al*. made comparison between the effects of cabozantinib, which is able to prevent VEGFR and c-MET, and prohibition of VEGFR, in which a selective antibody was used, in pancreatic neuroendocrine tumours. It was assured by this study that tumour growth is reduced by selective inactivation of vascular endothelial growth factor (VEGF) but it causes greater invasiveness and metastases. Inhibition of both VEGFR and c-MET reduces tumour growth, decreases invasiveness and metastasis, and increases host survival (28,148).

Selectivity of cabozantinib against approximately 270 human kinases was tested by Yakes *et al*. They were able to indicate that cabozantinib had an antiangiogenic effect rather than a cytotoxic effect: it blocked endothelial cell tubule formation *in vitro*. Cabozantinib prolonged tumour hypoxia and apoptosis at 8 and 4 hours after the first and second applicaton by interrupting tumour vasculature and causing tumour and endothelial cell death. The effect of cabozantinib on metastasis in comparison with that of other VEGFR2-targeting therapies was also studied in preclinical models, and prevention of metastasis was observed (28,149).

The expression of the multidrug resistance1 (MDR1) gene in MTC can lead to resistance against cytotoxic agents. TKIs have shown good results in MTC unresponsive to chemotherapy and radiation. Compensatory signalling pathway causing cell growth can be activated, when just a single tyrosine kinase receptor is

inhibited. Multitargeted tyrosine kinase inhibitors (TKIs), such as cabozantinib, have been developed to avoid such resistance. Some patients cannot benefit from these therapies because of the specific *RET* mutations that cause resistance (*RET* V 804 leads to resistance against vandetanib). Cabozantinib has no reports of such resistance until now (28,41).

Elisei *et al*. performed the largest study of cabozantinib for medullary thyroid carcinoma in a randomized Phase III study (28,150). A double-blind trial in 330 patients with metastatic MTC was organized by them and they compared cabozantinib 140 mg/d with placebo. Documented radiographic evidence of disease progression and no former systemic cancer therapy 4 weeks before enrolment were required, but the patients could have had previous TKIs in their care history. Patients were randomized 2:1 (cabozantinib to placebo) and stratified by age and privious TKI treatment. Progression-free survival (PFS) was the primary end point of the trial (28,150).

55 years was the median age of the patients. Lymph nodes (79.9%), liver (69.4%), lung (53%), and bone (51.1%) were the central sites of metastases. Two or more sites were involved in more than 85% of patients. *RET* mutation was positive in about 50% of patients, with M918T being the leading *RET* mutation. *RET* mutation status was not known in 38.7% of the patients in this trial (28,150).

An improvement in median progression-free survival (PFS) was observed in the cabozantinib group (11.2 months) versus placebo (4.0 months), which was statistically significant and the stratified hazards ratio was 0.28. All group stratifications (age, previous treatment, and *RET* mutation status) had improvement in PFS. For the cabozantinib group, Kaplan-Meier estimates of the percentage of patients progression-free and alive at one year were 47.3% and for the patients in the placebo arm 7.2%. 28% was the response rate in cabozantinib group (0% for placebo) (28,149).

Side effects were significant, which required dose reductions in 79% of patients and interruption of therapy in 16%. Nausea, diarrhoea, hypocalcaemia, palmar-plantar

erythrodysesthesia, hypertension, pulmonary embolism, weight loss, loss of appetite, and fatigue were the reported AEs. 69% and 33% of patients reported grade 3 or 4 AEs, and the most common adverse events were diarrhoea (15.9%) and palmar-plantar erythrodysesthesia (12.6%). Clinically relevant increase in QTc interval was not observed in the trial (28,150). Table V demonstrates the schedule of cabozantinib dose reduction for patients experiencing toxicity.

Warnings regarding perforation/fistula and haemorrhage are mentioned for cabozantinib. 3% of patients reported gastrointestinal fistulas and 3% told about haemorrhage, with haemoptysis and/or gastrointestinal bleeding. Signs of bleeding should be monitored in patients under the treatment. Cabozantinib should be withheld at least 28 days before surgery because it can cause wound healing complications (28).

Thrombotic events (venous thromboembolism 6%, arterial thromboembolism 2%), treatment-emergent hypertension (61%), osteonecrosis of the jaw (1%), proteinuria (2%), and reversible posterior leukoencephalopathy syndrome are other adverse events reported in patients under cabozantinb treatment. Palmar-plantar erythrodysesthesia syndrome was reported in 50% of patients, in one trial. In the same trial grade 3 reaction was observed in 13% (28).

History of hepatic impairment is a contraindication for cabozantinib. Interactions of cabozantinib with CYP3A4 inhibitors (such as ketoconazole, clarithromycin, and ritonavir) and CYP3A4 inducers (such as phenytoin, rifampin, and phenobarbital) have been reported (28,151).

If these medications (CYP3A4 inhibitors such as ketoconazole) are necessary and important then the dose of cabozantinib should be reduced 40-mg. And if the use of CYP3A4 inducers such as phenytoin is necessary, then the dose of cabozantinib should be increased by 40 mg (152).

Surgery as the gold standard therapy for MTC will not be replaced by cabozantinib, but it is an option in the treatment of MTC when surgery cannot be done. However,

patients must take many capsules a day, which can create a noticeable pill burden and may lead to noncompliance. The monthly calculated cost of almost $12,000 may also be a limitation of cabozantinib therapy (152).

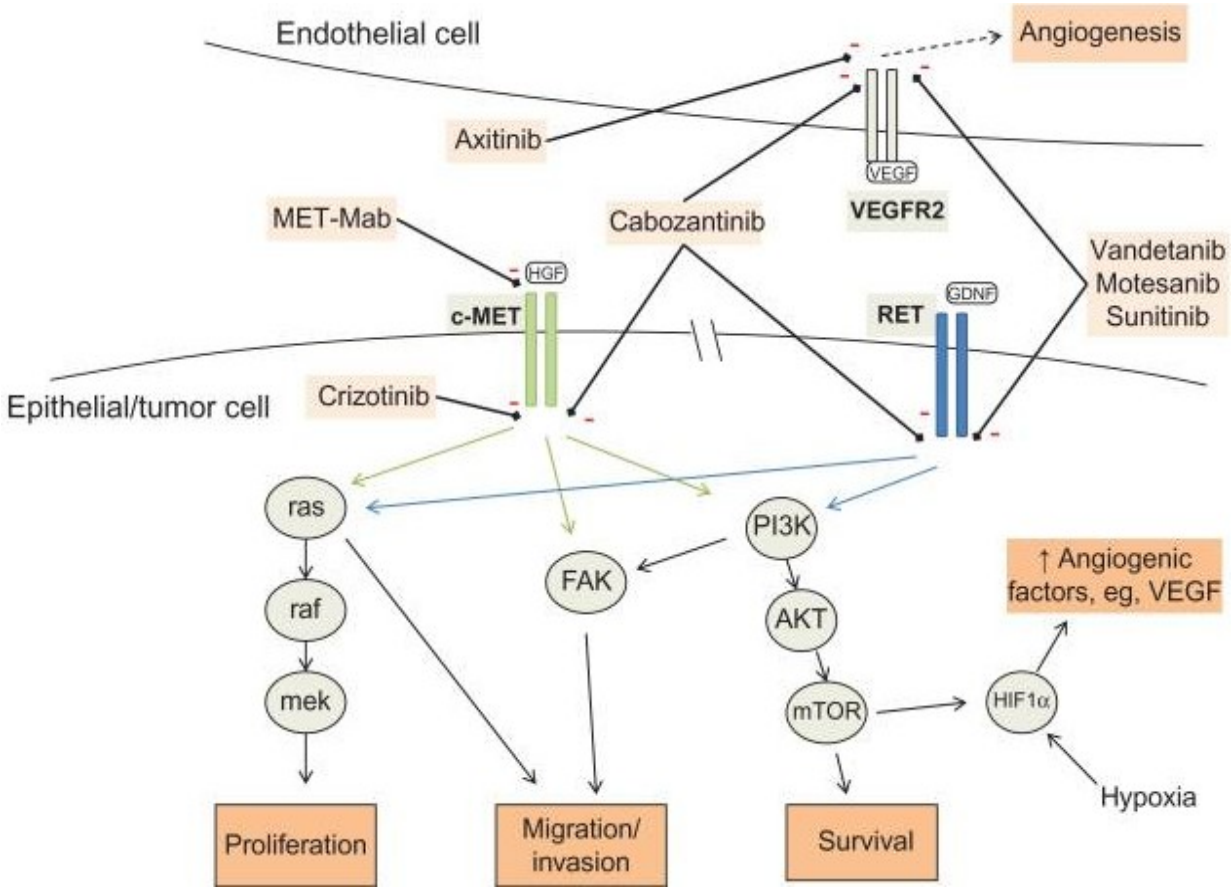

Figure 19: Molecular pathways of c-MET, *RET*, and VEGFR2, with targets of TKIs **(24)**.

Note: Dashed arrow shows molecular signal transduction pathways.PI3K, phosphoinositol-3-kinases; FAK, focal adhesion kinase; HIF1α, hypoxia-induced factor 1 alpha; mTOR, mammalian target of rapamycin; GDNF, glial-cell-line-derived neurotrophic factor; HGF, hepatocyte growth factor; VEGFR2, vascular endothelial growth factor receptor 2 **(24)**.

Table IV. Inhibitory activity of cabozantinib compared with that of vandetanib, motesanib, sunitinib, and axitnib **(24)**.

Target	Cabozantinib IC_{50} (nM)21	Vandetanib IC_{50} (nM)32	Motesanib IC_{50} (nM)33	Sunitinib IC_{50} (nM)34	Axitinib IC_{50} (nM)35
VEGFR-2	0.035	40	3	9	0.2
c-Met	1.3–14.6	–	–	4000	–
RET	5.2	100	59–2500	50	–
c-Kit	4.6	>20000	8	1	1.7
Flt3	11.3	–	–	30–250	<1000
AXL	7	–	–	–	–
Tie2	14.3	2500	–	–	–

Notes: a range shows that IC_{50} differs according to receptor mutation status. A hyphen indicates that no IC_{50} was reported for that receptor tyrosine kinase. VEGFR-2, Vascular endothelial growth factor receptor 2; IC_{50}, concentration at which 50% of maximal inhibition occurs; nM, nanomolar **(24)**.

Table V. Schedule of Cabozantinib dose reduction for patients who experience toxicity **(152)**.

Table. Cabozantinib Dose Reduction Schedule for Patients Experiencing Toxicity[a]

	Current dose: 140 mg	Current dose: 100 mg	Current dose: 60 mg
1st occurrence[a]	Hold until return to baseline, then decrease to 100 mg daily		
2nd occurrence[a]		Hold until return to baseline, then decrease to 60 mg daily	
3rd occurrence[a]			Hold until return to baseline, then resume 60 mg daily
4th occurrence[a]			Discontinue therapy

[a]Grade 4 hematologic toxicity or ≥ grade 3 or intolerable grade 2 nonhematologic toxicity.

5.2.3 **Other TKIs tested in MTC**

Molecular pathways targeted by multikinase inhibitors in refractory thyroid cancer are illustrated in figure 20.

Sorafinib is an oral TKI and has small-molecule. It is active against VEGFR2, VEGFR3, *RET*, and BRAF. In a trial of 16 patients with advanced medullary thyroid cancer (stratified into hereditary or sporadic MTC), who were treated with sorafenib 400 mg twice/d, 1 patient gained a partial response and 14 achieved stable disease

(4 had stable disease, which lasted longer than 15 months). The median PFS was 17.9 months (28,42,153,154).

Motesanib is a tyrosine kinase inhibitor that is able to block VEGFR1, VEGFR2, and VEGFR3. Schlumberger *et al.* studied, 91 patients with MTC who were treated with 125 mg of motesanib daily. In this trial 2 patients had partial remission and in 81% stable disease was observed. The median PFS was 48 weeks (28,42,153,155).

Sunitinib is a TKI, which inhibits VEGFR (1, 2, and 3), *RET*, and *RET* /PTC subtypes 1 and 3. Sunitinib 37.5 mg/d was given to patients with FDG-avid, advanced thyroid cancers (7 medullary thyroid cancer and 28 differentiated thyroid cancer) by Goulart *et al*. In this study a RECIST response was achieved in 3 of 6 patients with MTC (28,42,153,156).

Axitinib is another TKI, which prohibits the VEGF receptor. In phase II trial of axitinib for advanced thyroid carcinoma that had 11 medullary thyroid carcinoma patients (18% of the study patients), a partial response rate of 18% and a stable disease rate (>24 weeks) of 27% were reported by Cohen *et al.* (28,42,153,157).

In a trial by de Groot *et al.*, (28,158) 15 patients with confirmed medullary thyroid cancer were treated with 600 mg/d of imatinib. 4 months was median duration of treatment and no objective responses were observed. 9 patients with MTC were treated by Frank-Raue *et al.* (28,159). For a median duration of 13 months, they used 600 mg/d of imatinib. Stable disease at 3 months was observed in 7 patients, but at 12 months only 1 remained stable. The median PFS was 6 months and no clinical responsens were seen (28,42,158,159).

An *in vitro* Trial which compared four TKIs provided the result that cabozantinib is the most effective TKI in MEN2A MTC and vandetanib most effective in MEN2B MTC (28,160). Patients who have experienced progression in vandetanib therapy have shown response to cabozantinib treatment, but the opposite sequence has not yet been studied. As a conclusion it can be said that cabozantinib is the first therapy

developed to improve PFS in progressive MTC and is an effective drug to help patients with MTC (28,71).

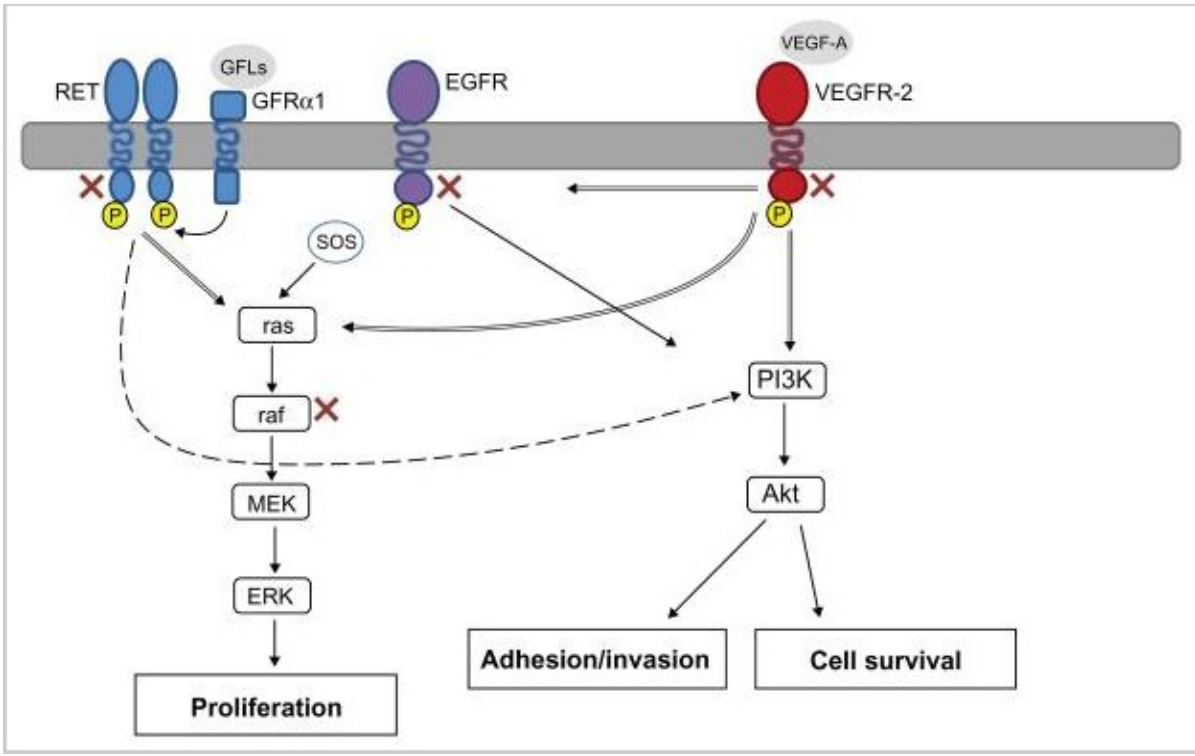

Figure 20: Pathways that are targeted by multikinase inhibitors in unmanageable thyroid cancer. EGFR, epidermal growth factor; ERK, extracellular signal-regulated kinase; GFL, glial cell line-derived neurotrophic factor family of ligands; GFRα, glial cell line-derived neurotrophic factor family α coreceptor; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; PI3K, phosphatidylinositol 3′ kinase; *RET*, rearranged during transfection; VEGF-A, vascular endothelial growth factor A; VEGFR2, vascular endothelial growth factor receptor-2 **(153)**.

5.2.4 **Future uses and indications**

Cabozantinib and vandetanib are the options for therapy in advanced stage of MTC or its recurrence (which are most often not surgically manageable). Both of the mentioned drugs have been approved by US Food and Drug Administration. The 2 phase III studies (ZETA and EXAM) investigated about these drugs. Trying to find out the use of vandetanib and cabozantinib these two trials varied markedly and cannot easily be brought into comparison. Median PFS was longer in the vandetanib group of the ZETA trial (28,161) (30.5 months, 11.2 months longer than placebo) than in the cabozantinib group of the EXAM trial (28,162) (11.2 months, 7.2 months longer than

placebo). The placebo arms varied significantly from each other as well. The ratio of median PFS with cabozantinib's placebo arm was 2.80, and it was 1.58 with vandetanib's placebo arm, which shows that cabozantinib has greater benefit. It is to be noticed that one of the important differences between vandetanib and cabozantinib is that cabozantinib does not increase QTc interval significantly, and patients with a history of heart disease, arrhythmia, or QT prolongation should be treated with cabozantinib. (28).

In order to consider the differences or similarities between 4 TKIs, the efficacy of cabozantinib, vandetanib, sunitinib, and axitinib was compared by Verbeek *et al*. Three different cell lines, i.e., MTC-TT derived from a sporadic medullary thyroid carcinoma having a C634W *RET* mutation, MZ-CRC-1 extracted from a metastatic sporadic medullary thyroid cancer harbouring a M918T *RET* mutation, and TPC-1 derived from papillary thyroid cancer having a *RET* /PTC-1 rearrangement were used in this trial. All of the above mentioned four TKIs showed a dose-dependent decrease of cell proliferation. Cabozantinib was the most potent inhibitor of MTC-TT and TPC-1, and MZ-CRC-1 was significantly inhibited by vandetanib. This *in vitro* trial indicates that mutation-specific therapy could bring advantage in the therapy of MTC (28,160).

Because new pathways that take part in the pathogenesis of medullary thyroid cancer are clarified and drugs against them are developed, patients may have a survival benefit when these drugs are combined. The Ras-Raf-MEK-ERK pathway and its interaction with the PI3K-AKT-mTOR pathway are involved in the development of sporadic MTC. Everolimus is active against the PI3K/AKT/mTOR pathway and has shown anti-tumour efficacy in medullary thyroid carcinoma (28,41). Studying the facts about cabozantinib (causes apoptosis) and everolimus (causes cell cycle arrest and senescence), it might be possible in the future to combine these two drugs and have survival benefit in medullary thyroid carcinoma. (28,149,163). But more investigation must be done (28).

The inhibitory effect of the leukemia inhibitory factor in human MTC xenografts in mice was reported by Starenki *et al*. To achieve this goal, bacterially produced

recombinant leukemia inhibitory factor was used. Growth suppression, activation of the JAK/STAT pathway, and downregulation of *RET* and E2F1 expression in tumours were shown in this study. This cytostatic form of *RET* inhibition is cytokine-mediated and is capable of making a good combination with the approved therapies that already exist. In the treatment of special neurologic problems such as amyotrophic lateral sclerosis, leukemia inhibitory factor has been used earlier and it has been shown to be relatively nontoxic (28,164).

A new family of drugs known as withanolides have also shown effect on MTC in preclinical investigations. Withaferin A is the most common withanolide, and it decreased MTC tumour mass *in vivo* (in a metastatic mouse model) (28).

Withanolides caused inhibition of MTC cell growth through induction of apoptosis, promotion of cell cycle arrest in MTC cells, prevention of clonogenic growth, and suppression of *RET* and the AKT/mTOR pathway activation, as was reported by Samadi *et al*. These effects of withanolides may play a role in the future therapy of MTC but further investigation is needed (28,165). Inhibition of clonogenic growth in MTC cells by withanolides is demonstrated below (Figure 21).

The knowledge about the molecular biology of MTC has made noticeable advances, but surgery will be the most effective treatment in the future as well. TKIs inhibiting *RET* have their effect and have shown efficacy, but the absence of complete responses in treatments with single agents indicates the need for more active monotherapy options or discovery of effective combinations. Until now no major combination trials and studies have been published (28,166).

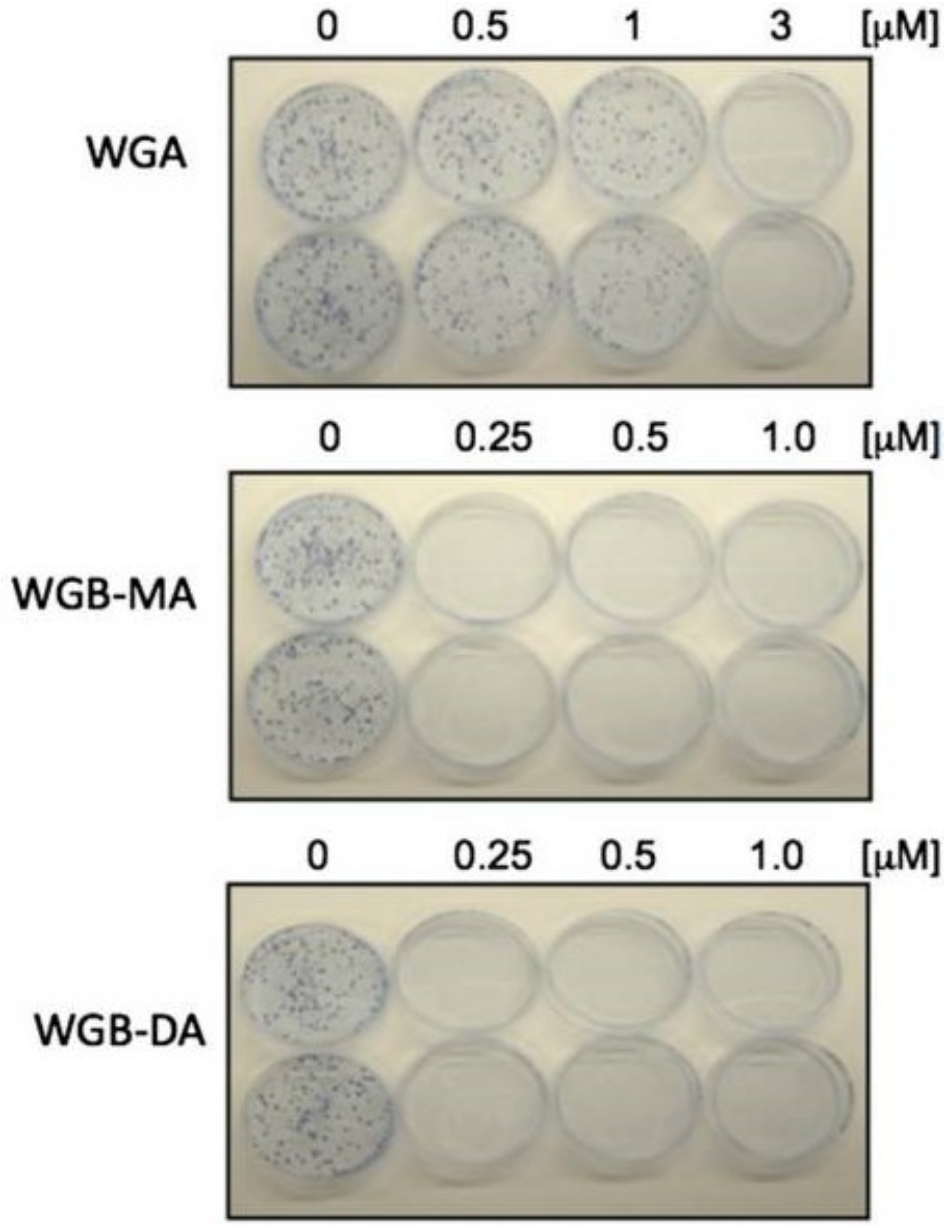

Figure 21: Novel withanolides prevent clonogenic growth of MTC cells. Prevention of clonogenic growth formation by WGA at 1 μmol/L drug concentration; WGB-MA and WGB-DA prevented growth at only 250 nmol/L concentrations. This shows that the effect of withanolides on MTC is not easily reversible **(165)**.

5.3 **Cell Lines**

During the period of 1986 until now, 9 different MTC cell lines have been established by Roswitha Pfragner. The human MTC cell lines established in our laboratory are shown in the Table VI. MTC cells are cultured in Ham's F12:M199 medium (1:1) (BioWhittaker, Lonza Verviers, Belgium) with 10% fetal bovine serum (FBS) [Biochrom (Berlin, Germany)]. At the beginning the cell number was 2 x 10^5 cells/ml at 37°C in a humidified atmosphere. It contained 5% CO_2 without antibiotics.

Table VI shows an overview of the MTC cells, the results from array-CGH, M-FISH and SCID are presented in table VII, and table VIII demonstrates their immunocytochemical characterisation.

Table VI. Overview of MTC cell lines established by Prof.Roswitha Pfragner and colleagues in Graz.

MTC cell line	Tumour stage at the time of surgery	Sex/age	Hereditary or sporadic	Somatic *RET* mutation	Primary tumour/metastasis
BOJO	pT4N1M1	Male/69	Sporadic	0 mutation	LN-metastasis
HEVE-II	pTxN1MX	Female/44	Sporadic	0 mutation	LN-metastasis
SHER-I	pT4N1MX	Male/72	Sporadic	0 mutation	Primary tumour
SINJ	pT2N1M0	Male/28	Sporadic	0 mutation	
OEE-III	pT2aN1bM1	Female/53	Sporadic	M918T	LN-metastasis
GRS-IV	pT4N1M1	Male/55	Sporadic		LN-metastasis
GRS-V	pT4N1M1	Male/55	Sporadic		LN-metastasis
RARE	pT4N1MX	Female/53	Sporadic		LN-metastasis
MTC-SK	pT4N1MX	Female/51	Sporadic		LN-metastasis

Table VII. Array CGH, M-FISH, and SCID of MTC cell lines.

MTC CELL LINE	Array CGH	M-FISH	SCID
BOJO	Gain of chromosome 5 –q and loss of chromosome 9p	Unbalanced translocation between chromo. 5 and 9	
HEVE-II	Balanced profile	Exclusion of balanced structural chromosomal rearrangements such as translocation	Tumour growth
SHER-I	Balanced profile	Exclusion of balanced structural chromo. rearrangements such as translocation	Tumour growth
SINJ	Balanced profile	No success in the preparation of metaphase	
OEE-III	Many gains and losses	Multiple rearrangements	Tumour growth

Table VIII. Neuroendocrine properties of MTC-cell lines (Immunocytochemistry) **(167)**.

Cell line	CT	CGRP	GRP	SRIF	5-HT	NSE	PHE5	LK2H10	ER	Pgr
BOJO	++	++	n.d.	n.d.	n.d.	+++	++	n.d.	n.d.	n.d.
GRS-IV	++	++	++	-	-	++	++	+++	+	+
GRS-V	+	+	++	-	-	+	++	++	++	++
MTC-SK	++	+++	+++	-	-	+++	+++	n.d.	+/++	+++
SINJ	+/++	+/++	++	-	-	++	++	+++	+	+
OEE-III	++	n.d.	n.d.	(+)	n.d.	+++	+++	n.d.	++	n.d.
RARE	n.d.	++	+	n.d.	n.d.	+++	n.d.	++	(+)	n.d.
SHARE	+	n.d.	+			+		-		

CT= Calcitonin, **CGRP=** calcitonin gene- related peptide, **GRP=**gastrin releasing peptide, **SRIF=**somatistatin, **5-HT=**serotonin, **NSE=**neuron-specific enolase; **PHE5=**chromogranin clone PHE5, **LK2H10=**chromogranin A and related peptides clone LK2H10, **ER=**estrogen receptor, **Pgr=**progesterone receptor, tumour=tumourigenicty, +=weak, ++=moderate, +++=strong, n.d. = not done **(167)**.

It has been reported that maintenance of telomere length is an absolute requirement for unlimited growth in human tumour cells. In about 85% of cases, this requirement is obtained by reactivation of telomerase, which is the enzyme that elongates telomeres. In exceptional cases such as (MTC), telomerase activity (TA) is low or undetectable (168).

Very low telomerase activity observed in MTC cell derived from different patients is a requirement for prolonging their replicative life span. Assuring the high relevance of telomerase for tumour development, these details draw attention to the importance of low telomerase activity: although this low TA is not sufficient for telomere stabilisation, it allows medullary thyroid carcinoma cells to reach more population doublings. This increases cell numbers of MTC and the risk of accumulating mutations, which might support medullary thyroid cancer to become clinically significant (168).

5.3.1 **BOJO**

This cell line was established form a sporadic tumour, derived from Lymph node metastases of a 69 year old man, and the tumour stage at the time of surgery was pT4N1M1. No *RET* mutation was found.

A gain in chromosome 5q and a loss in chromosome 9p material were shown in array-CGH for BOJO. M-FISH exhibited an unbalanced translocation between chromosomes 5 and 9. The results of Array-CGH and M-FISH are comparable with each other (169).

According to the neuroendocrine characteristics the BOJO cell line produces calcitonin (CT) and calcitonin gene-related peptide (CGRP) in high amounts. Gastrin releasing peptide (GRP), somatostatin (SRiF) and serotonin (5 HT) are not defined in BOJO cell line until now. It produces neuron specific enolase (NSE) in higher amounts and the chromogranin clone (PHE5) is produced in high amounts by it. The expression of chromogranin A and related peptides clone (LK2H10), oestrogen receptor (ER) and progesterone receptor (Pgr) has not yet been determined in BOJO cell line. Figure 26, picture A shows the morphology of BOJO cell line.

5.3.2 **HEVE-II**

HEVE-II was established from a sporadic tumour of a female patient. Her age was 44. It was derived from lymph node metastasis. Tumour stage at the time of surgery was pTxN1MX. No *RET* mutation was found and it had a balanced array-CGH profile.

M-FISH included no balanced structural chromosomal rearrangements such as translocations in HEVE-II. To assure the tumourigenicity of this cell line, HEVE-II cells ($3x10^7$per mouse) were injected subcutaneously into SCID-mice. About four weeks after injection, tumour growth was observed, which was at the injection site. Additional experiments for the balanced HEVE-II cell line were performed. Cells from the xenotransplant were brought under cultivation and the experiment was done again, i.e., other mice were injected with cells from the first mouse xenotranspant and so second xenotranplants were generated. Cells from this second transplant showed

tumour growth with the same speed as the first transplant. Array-CGH was done for both the first and second xenotrasplants and it delivered the result that array-CGH profiles remained balanced (169). In Figure 26, picture B represents the morphology of HEVE-II cell line.

5.3.3 **SHER-I**

The mentioned cell line was established from a sporadic tumour of a 72 year old male patient. It was derived from primary tumour. Tumour stage at the time of surgery was pT4N1MX. No *RET* mutation was found and array-CGH profile was balanced.

M-FISH included no balanced structural chromosomal rearrangements such as translocations in SHER-I. To establish the tumourigenicity of this cell line, SCID (Severe combined immunodeficiency)-mice were injected with SCHER-I cells ($3x10^7$ per mouse). Tumour growth at the injection site, was seen about one month after injection. No metastases were seen and the tumour exulcerated when its diameter was >10mm (169). Figure 22 shows the tumourigenicity of SHER-I.

Neuron specific enolase (NSE), calcitonin (CT) and bombesin (GRP) are produced by SHER-I. Calcitonin gene-related peptide (CGRP) has not yet been defined in SHER-I cell line. This cell line produces no chromagranin A (CgA) (167). In figure 26, picture C the morphology of SHER-I is shown.

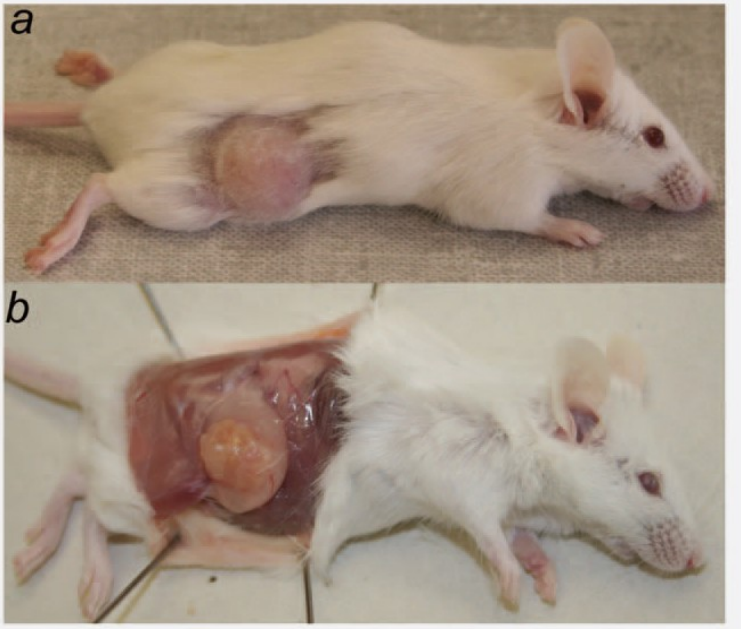

Figure 22: a: Tumourigenicity of SHER-I, one month after injection b: Subcutaneous tumour at the injection site **(169)**.

5.3.4 **SINJ**

The above cell line was established form a sporadic tumour. Tumour stage at the time of surgery was pT2N1M0. The Patient was 28 year old man and no *RET* mutation was fond.

Array-CGH delivered a balanced profile for this cell line. The preparation of metaphases with quality sufficient for detailed analysis was not successful (169). SINJ produces calcitonin (CT), calcitonin gene related peptide (CGRP) and gastrin releasing peptide (GRP), but it cannot produce somatostatin (SRiF) and serotonin (5HT). This cell line produces high amounts of neuron specific enolase (NSE) and chromogranin clone (PHE5). SINJ produces chromogranin A and related peptides clone (LK2H10) in higher amounts. It has oestrogen receptor(ER) as well as progesterone receptor (Pgr) (167).

Morphology and immunocytochemical properties: Best primary cell earnings were given with mechanical disaggregation and cold trypsinization, but with warm

trypsinization many cells were injured. From the beginning of the cultivation period, the liability to grow in suspension was observed. Only a small quantity of cells adhered. This sticking population of cells contained mainly stromal cells and a small number of tumour cells, which could not grow. The non–sticking population turned into a continuous cell line. These cells form spherical and irregular clusters and have diameters from 4 to 8 µ. An inoculum of $2x10^5$ cells/ml is doubled in 3 days, on condition that the medium is renewed 3 times per week. Tumour cells with irregularly shaped nuclei were shown with electron microscopy of the original tissue. Most of these tumour cells were with one nucleolus only. Only a small number of cells had neuroendocrine granules. Increase in rough endoplasmatic reticulum was seen. The cells from the cultures were morphologically similar to the original tissue, with the exception that they had no increase in endoplasmatic reticulum. The cytoplasm had very few neuroendocrine granules (170).

Tumourigenicity: Tumours in 2 out of 20 Fox Chase SCID-nu/nu mice were developed, 14 weeks after inoculation. These tumours were soft nodules and had different cell populations. These cell populations had adherent cells i.e. fibroblasts and keratinocytes from the host mouse and non-adherent cells, derived from the human MTC cells. Non-adhesive cells, from the human MTC cells could be passaged *in vitro* similar to the original cell line. In the nu/nu-BALB/c group, no tumour has been found so far (170).

Cytogenetic findings: After cultivation of 8 weeks chromosomes were prepared from spontaneously detached cells. SINJ was diploid with no persistent abnormalities, but a deletion of band 6p21.1 was questionable and a clonal deletion of band 11q14 or 11q22 took place with increasing frequency in later passages. In all analysable metaphases (8/8) of a tumour derived from a transplant of the SINJ into Fox Chase SCID- nu/nu mouse, the interstitial deletion of 11q was present [46, XY, del (11) (q 14.1q21 or q21q22.3)] (171).

Flow cytometry: 25 of 27 clones were near diploid. The other 2 clones were noticeably aneuploid and had a DNA index of 1.8. Relatively constant S-phases of about 13% were observed by them. The S-phases differed from 7.1 to 32.2% in the

near diploid clones. These findings were assured as diploid, later hypodiploid/hypotetraploid karyotypes by the cytogenetic analyses *(170).*

SV40 hybridization: No hybridization signal was achieved, which shows that the cell line SINJ does not include SV sequences (170). The morphology of SINJ could be seen in picture D of figure 26.

5.3.5 **OEE-III**

OEE was established from a sporadic tumour of a 53 year old female patient, derived from lymph node metastasis and the tumour stage at the time of surgery was pT2aN1bM1. Somatic *RET* status had a mutation (M918T).

OEE-III exhibited many rearrangements with M-FISH and multiple gain and losses in Array-CGH: The high number of copy number changes in this cell line may demonstrate that it was produced from an advanced stage of the disease. In order to assure the tumourgenicity of this cell line, SCID-mice were injected subcutaneously with OEE-III cells ($3x10^7$ per mouse). Tumour growth at the injection site was seen, about one month after injection. No metastases were observed and the tumour exulcerated when it obtained the diameter of >10mm. Judging whether the tumour genome transformed in the Xenotransplants, copy number changes in tumours from Xenotransplants from the OEE-III with many changes, were assessed. Practically an identical array-CGH profile with DNA from the mouse tumour was achieved when correlated to the original cell line profile (169).

OEE-III produces high amount of calcitonin (CT). It can also produce somatostatin (SRiF). Calcitonin gene- related peptide (CGRP), gastrin releasing peptide (GRP) and serotonin (5HT) are not defined in this cell line. OEE-III produces higher amounts of neuron specific enolase (NSE) and chromogranin clone (PHE5). Chromogranin A and related peptides clone (LK2H10) and progesterone receptor (Prg) are not yet defined in this cell line. OEE-III has high amounts of oestrogen receptor (ER) (167).

5.3.6 **MTC-SK**

We were able to establish this cell line from a sporadic tumour of a 51 year old female patient. It was derived from lymph node metastasis and the tumour stage at the time of operation was pT4N1Mx.

MTC-SK-cell line produces high amounts of calcitonin (CT). It produces higher amounts of calcitonin gene- related peptide (CGRP) and gastrin releasing peptide (GRP) as well. It cannot produce somatostatin (SRiF) and serotonin (5HT). Neuron specific enolase (NSE) and chromogranin clone (PHE5) are produced in higher amounts by MTC-SK cell line. It is not defined if it produces chromogranin A and related peptides clone (LK2H10). MTC-SK cell line has oestrogen receptor (ER) and can express higher amounts of progesterone receptor (Pgr) (167).

Establishment and Growth Properties of the MTC-SK: Tumour tissue of a human medullary thyroid carcinoma at the early stage formed monolayers of epithelioid cells. From these sticking cells, single cells and cell spheroids disconnected impulsively (MTC-SK cell line), as it is reported before (172).This cell line was found in suspension culture. The tumour cell spheroids included morphologically uniform non-necrotic cells. Prior to cell counting the spheroids were easily detached by aspirating with a pipette. The doubling time for an inoculum of $5x10^5$cell/ml is 2, 4 days. Adding nerve growth factor (NGF), epidermal growth factor (EGF) and dexamethasone to the medium did not impact the growth properties of the cells. Inclusion of bombesin strengthened the formation of tumour cell spheroids (173).

Ultrastructure: Studies of ultrastructure confirmed the maintenance of the characteristic tumour cell morphology in the MTC-SK. The original tissue had capacity for only a moderate number of neuroendocrine granules. The cultured cells had even fewer granules. The granules presented relatively uniform electron-dense cores and small halos. They had large nuclei, one or two prominent nucleoli, and an irregular profile. Increased number of Golgi complexes was observed. A rough endoplasmic reticulum was, in many cytoplasmic areas, well developed. The cisternas were wide and had dilated regions. There were no cellular junctions between the cells of the

spheroids (173). Figures 23 and 24 indicate the immunocytochemistry (detection of GRP and CGRP) of MTC-SK.

In situ hybridization: ISH of tumour tissue sections indicated, intense reactions with CT and GRP probes and the clear visualization of CGRP mRNA in tumour cells. Negative results were achieved from experiments localizing SRIF mRNA. Visualization of specific mRNAs was approved by hybridization of the CT, CGRP and GRP probes to MTC-SK cells, but SRIF mRNA was not detectable. Expression of Ha-ras, c-myc, and N-myc in both primary tumour and its lymph node metastases by in situ hybridization and Northern blot analyses are studied and published (174). In situ hybridization of MTC-SK is demonstrated in figure 25.

Chromosomal Findings: In MTC-SK, 42 of 125 metaphases contained telomerase activity. 32 of these rearrangements concerned 11p. 5 out of 125 metaphases had different translocations, 4 involving 11p. A supernumerary chromosome was present in 3 of 125 metaphases. A normal chromosomal constitution was found in 75 of 125 (60%) of the metaphases. A centromeric instability of chromosome 16 with different degrees of despiralization was observed in 10% of metaphases and 2% of the metaphases showed somatic pairing of the two homologous chromosomes 16. Lymphocyte cultures were done one year after the patient underwent surgical irradiation and therefore, have limited significance. Approximately 100% of the metaphases included structural chromosomal abnormalities containing dicentric and ring chromosomes, translocations, chromatid and isochromatic breaks, quadriradial figures, and double minutes. However, terminal chromosomal association of chromosome 11, ter rea ($11p^{p}$) and ter rea (11q; 16q), were seen in 2 of 20 metaphases, as observed in the MTC-SK. At that time bone metastases were developed and these two cells may have been tumour cells. Normal chromosomal constitution was seen in skin fibroblasts (173).

SV40 hybridization: No hybridization signal was achieved; indicating that the cell line MTC-SK does not include SV40 sequences (170). In figure 26, picture E the structure of MTC-SK cell line has been represented (together with other MTC cell lines).

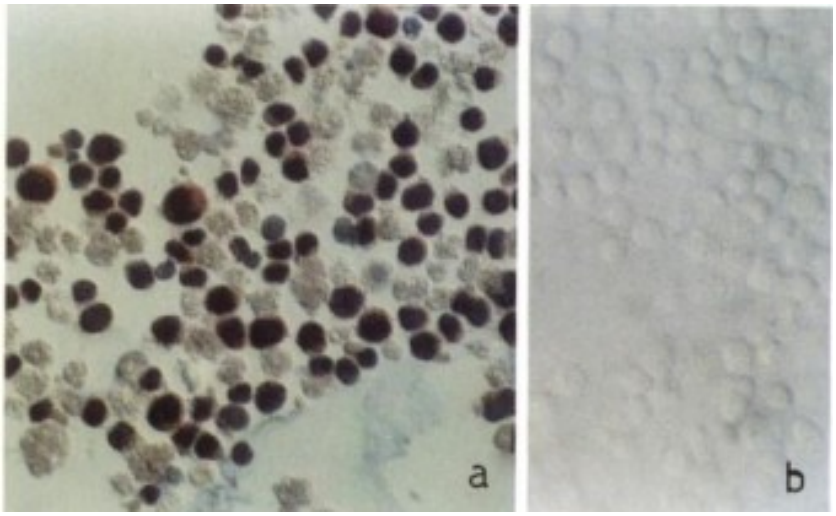

Figure 23: (a) Immunocytochemical detection of gastrin releasing peptid (GRP) in MTC, interference contrastx2,000; (b) Negativ control, interference contrast x2,240 **(173)**.

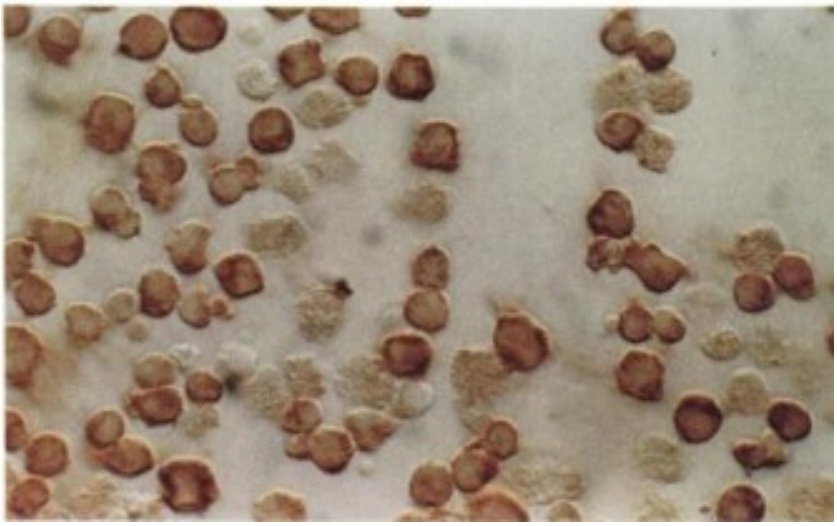

Figure 24: Immunocytochemical detectoin of Calcitonin gene-related peptide in MTC , interference contrast x 2,250 **(173)**.

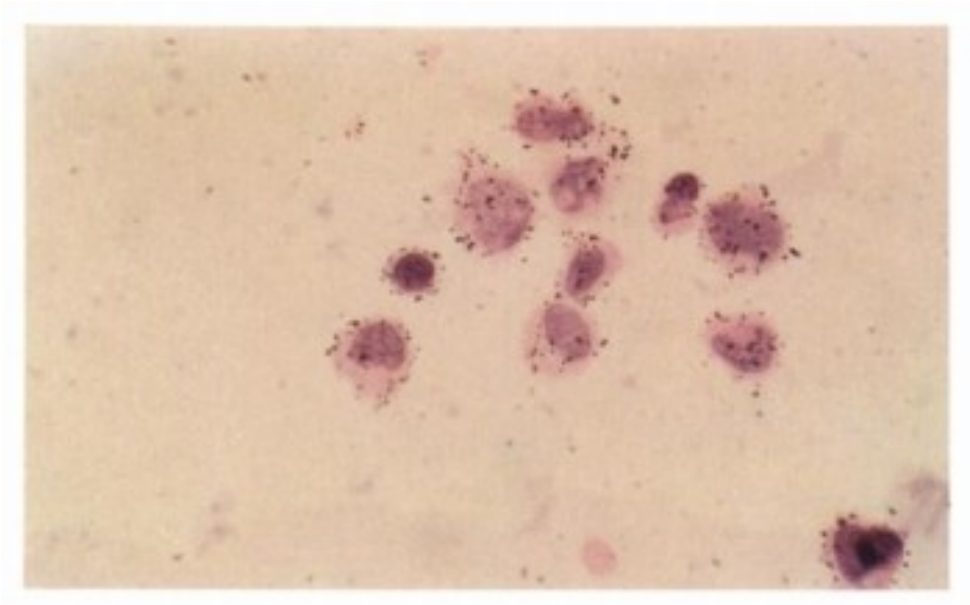

Figure 25: MTC-SK: In situ hybridization detection of GRP mRNA **(173)**.

5.3.7 **GRS-IV**

GRS-IV was established from a sporadic tumour of a male patient at the age of 55. It was derived from the lymph node metastasis and the tumour stage at the time of surgery was pT4N1M1.

High amounts of calcitonin (CT), calcitonin gene- related peptide (CGRP) and gastrin releasing peptide (GRP) are produced by GRS-IV. It cannot produce somatostatin (SRiF) and serotonin (5HT). Neuron specific enolase (NSE) and chromogranin clone (PHE5) are produced in high amounts by GRS-IV. This cell line produces chromogranin A and related peptides clone (LK2H10) in higher amounts. GRS-IV cell line expresses oestrogen receptor (ER) and progesterone receptor (Pgr) (167). Figure 26, picture F displays the morphology of GRS-IV.

5.3.8 **GRS-V**

The mentioned cell line was established from a sporadic tumour of a male patient at the age of 55. It was derived from the lymph node metastasis and the tumour stage at the time of surgery was pT4N1M1.

GRS-V produces calcitonin (CT), and calcitonin gene- related peptide (CGRP). Gastrin releasing peptide (GRP) is produced in high amounts by GRS-V. It cannot produce somatostatin (SRiF) and serotonin (5HT). Neuron specific enolase (NSE) is as well produced by this cell line. Chromogranin A and related peptides are produced in high amounts by GRS-V cell line. The mentioned cell line has oestrogen receptor(ER) and progesterone receptor (Pgr) in high amounts (167). The structure of GRS-V is represented in Figure 26, picture G.

5.3.9 **RARE**

RARE could be established from a sporadic tumour of a female patient at the age of 53. It was derived from the lymph node metastasis and the tumour stage at the time of surgery was pT4N1Mx.

It is not defined if this cell line produces calcitonin (CT), somatostatin (SRiF), serotonin (5HT), and chromogranin clone (PHE5). It cannot express the progesterone receptor (Pgr). RARE-cell line produces high amounts of calcitonin gene- related peptide (CGRP). It can also produce gastrin releasing peptide (GRP). RARE is capable of producing neuron specific enolase (NSE) in higher amounts. Chromogranin A and related peptides (LK2H10) is produced in high amounts by RARE cell line (167). The mentioned cell line can express oestrogen receptor (ER). A picture of RARE has been represented in Figure 26, picture H.

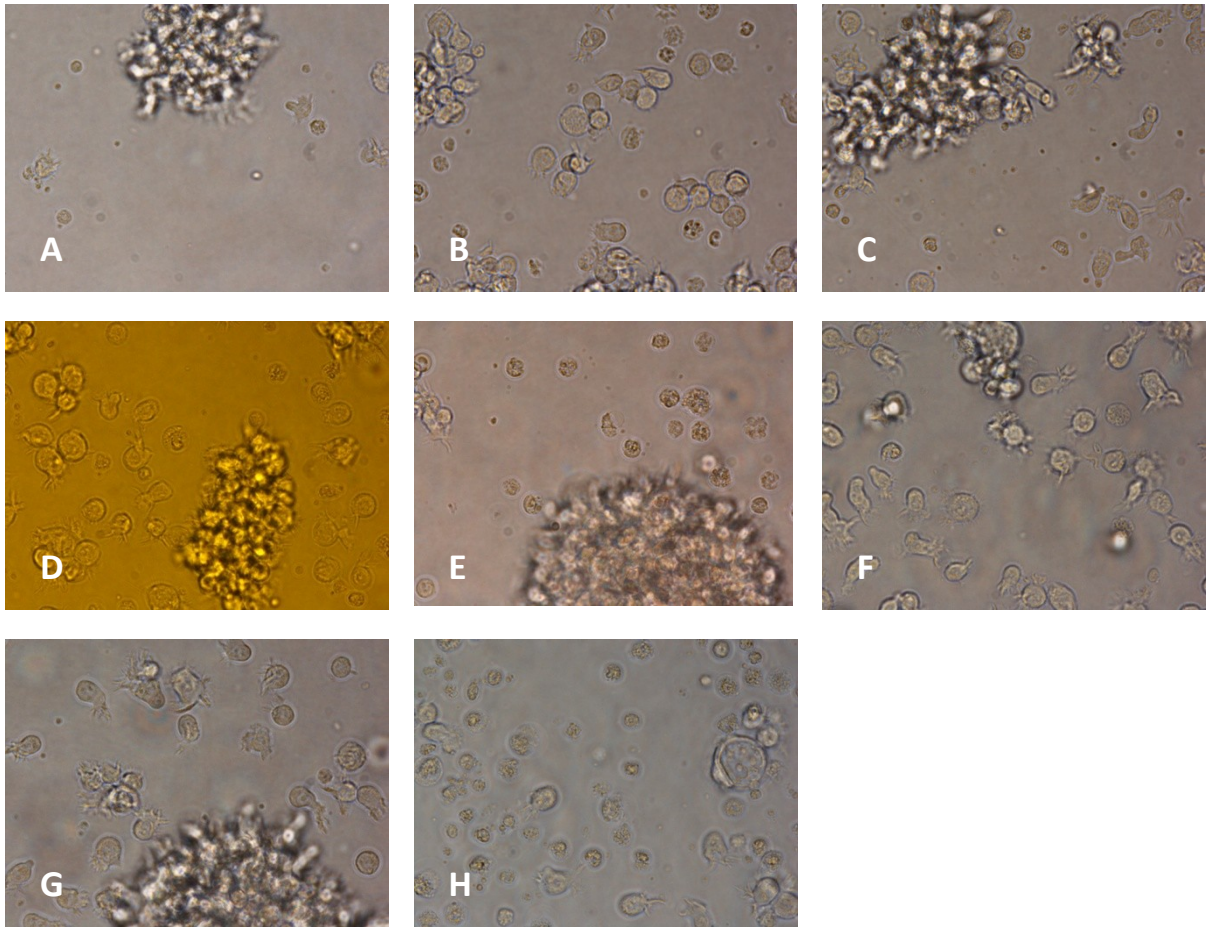

Figure 23: Morphology of MTC Cell Lines, A: BOJO, B: HEVE-II, C: SHER-I, D: SINJ, E: MTC-SK, F: GRS-IV, G: GRS-V, H: RARE

6 Discussion

The rarity of medullary thyroid cancer (30 MTCs / year in Austria), the very slow growth, and the very small size of the samples are some of the reasons for the lack of continuous cell lines.

Continuous cell lines are of enormous importance to provide new experimental models, which can then be used in testing of anticancer drugs, so for this reason I collected information, which is present about the MTC cell lines and MTC tumour marker.

There are very few literatures about the continuous cell lines of MTC. I collected the information about the 9 MTC cell lines established in Graz by Prof. Roswitha Pfragner and colleagues. All these cells were established from sporadic tumours. BOJO, HEVE-II, SHER-I and SINJ have no somatic *RET* mutation. OEE-III has M918T *RET* mutation and the research about the existence of *RET* mutation in other cell lines is not yet done. Just SHER-I was derived from primary tumour, but all other cell lines were derived from lymph node (LN)-metastasis.

During my search for information about the cell lines, I have come through the fact that more effort must be done to further characterize these cell lines to ensure that the cells can serve as a suitable model for MTC research. This would make the development of therapeutic strategies of the MTC much easier.

As it is mentioned before, medullary thyroid cancers are resistant to radioactive iodine therapy because they do not concentrate radioactive iodine, which differentiates them from other malignancies of the thyroid (175). The primary therapy for MTC is surgery, which is often curative and the size of the primary tumour and the extent of nodal and distant metastases is very important for the surgery planning. In all tumours less than grade T4 with negative nodes or subcentimeter central compartment nodes, total thyroidectomy with a dissection of level VI central neck lymph nodes is recommended (12,175,176). Preoperative radiologic evaluation of nodal involvement is crucial for the treatment (12,177). If an advanced metastatic disease is present then a more palliative approach is recommended. In order to preserve speck and swallowing

function and to improve the life quality of patients with MTC, aggressive surgery is not usually performed (12).

In patients with germline *RET* mutations, the prophylactic thyroidectomy is mainly recommended (12,176), and the type of MEN syndrome and the *RET* mutation are decisive for the time of surgery. For instance, total thyroidectomy during the first year of life is required for infants with MEN 2B and aggressive mutations at codons 883,918 or 922, whereas surgery can be delayed up to the age of 5 for patients with MEN 2A and less aggressive mutations for example, at codons 609 or 611(176).

In metastatic MTC, treatment depends on the extent of distant metastases. For resectable liver lesions or solitary brain metastases surgery may be offered, but because MTC metastases are usually multifocal, these events are infrequent. Symptomatic bone metastases may be treated with external beam radiation (178). When the patient's main metastatic load is in the liver, and it is detected at an early stage then chemoembolization is sometimes useful (179).

Knowledge of molecular cytogenetics of medullary thyroid cancer has helped to develop novel targeted therapies. As it is indicated before, MTC mutations cause abnormal activation of the *RET* kinase receptor, which results in excessive cell growth and reduced apoptosis. More knowledge about the genetics of MTC has led to several clinical trials that involved tyrosine kinase inhibitors (sorafenib, sunitinib, vandetanib, motesanib, and cabozantinib) with the rationale and thoughts that they can block the molecular pathways which cause this tumour, such as VEGFR or *RET* activity and thereby prevent tumour progression (180-182).

The results from EXAM show that cabozantinb has significant activity in Patients with MTC, inclusive of those previously treated with cytotoxic and targeted therapies. The results from the phase III trial raise the possibility of an effective treatment for MTC. Increased toxicity is a cost of multikinase inhibition, because each pathway plays a role in the normal function and homeostasis. Adverse events for cabozantinib are similar to those with other tyrosine kinase inhibitors, indicating that cabozantinib will be tolerated similar to the tyrosine kinase inhibitors that are already being used in clinical practice (24).

Other tyrosine kinase inhibitors have also indicated important activities in treating patients with MTC. Vandetanib, although not being active against MET, is an anti-VEGF, anti-*RET*, and anti-EGFR agent. Progression-free survival was prolonged significantly with vandetanib compared with placebo in a phase III trial of patients with MTC. The hazard ratio was 0.46 (95% confidence interval 0.31-0.69; P<0.001) (24,183).

The activity of vandetanib shows that EGFR inhibition may have a role in the treatment of MTC. EGFR activation is recognized to have importance in malignant progression of several tumours, and overexpression and activation of EGFR and VEGFR2 have been shown in MTC metastases (24,184). But the prevention and inhibition of EGFR is in association with upregulation of MET, which causes tumour escape (24,185). Dual inhibition with the usage of erlotinib (an EGFR inhibitor) and cabozantinib has been indicated to have effect in tumour cell lines that show resistance to each of the agent alone. If this combination would have superior results in humans, is yet to be tested (24).

Overall efficacy may be reduced, when MET is targeted without any knowledge of its mutation or amplification status. Onartuzumab (MetMAb, Genentech, South San Francisco, CA), is a monoclonal antibody that directly inhibits hepatocyte growth factor-MET binding. Onartuzumab, significantly prolonged overall survival in patients who harboured high levels of mesenchymal-epithelial transition, but those with low levels of mesenchymal-epithelial transition performed worse than the control arm when it was used in NSCLC (24). Because these results are not from a phase 3 trial, the indication of cabozantinib will remain: should cabozantinib be reserved only for MTC, which expresses high levels of c-MET or harbours specific mesenchymal-epithelial transition mutations? Or is the success and effect of cabozantinib in relation with its ability to inhibit the formation of “escape pathways” before they develop and before significant amplification of mesenchymal-epithelial transition occurs, and at the same time attacking VEGFR and *RET*? The activity of cabozantinib in a number of other solid tumours regardless of MET status shows that the latter will be the case (24).

Ultimately, the role that cabozantinb and vandetanib play in the management and therapy of patients with medullary thyroid cancer might be more efficient when they are combined with other therapeutic agents, but the research in this field is yet to be done.

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