Role of tissue and circulating microRNA and DNA as biomarkers in medullary thyroid cancer

Martina Chiacchiarini, Sofia Trocchianesi, Zein Mersini Besharat, Agnese Po, Elisabetta Ferretti

PII: S0163-7258(20)30239-4

DOI: https://doi.org/10.1016/j.pharmthera.2020.107708

Reference: JPT 107708

To appear in: *Pharmacology and Therapeutics*

Please cite this article as: M. Chiacchiarini, S. Trocchianesi, Z.M. Besharat, et al., Role of tissue and circulating microRNA and DNA as biomarkers in medullary thyroid cancer, *Pharmacology and Therapeutics* (2020), https://doi.org/10.1016/j.pharmthera.2020.107708

P&T #23708

Role of tissue and circulating microRNA and DNA as biomarkers in medullary thyroid cancer

Martina Chiacchiarini[1], Sofia Trocchianesi[2], Zein Mersini Besharat[1], Agnese Po[2], Elisabetta Ferretti[1]

[1] Department of Experimental Medicine, Sapienza University of Rome, 00161 Rome, Italy
[2] Department of Molecular Medicine, Sapienza University of Rome, 00161 Rome, Italy

Abstract

Medullary thyroid carcinoma (MTC) is a rare neuroendocrine tumor comprising hereditary or sporadic form with frequent mutations in the rearranged during transfection (RET) or RAS genes. Diagnosis is based on presence of thyroid tumor mass with altered levels of calcitonin (Ctn) and carcinoembryonal antigen (CEA) in the serum and/or in the cytological smears from fine needle aspiration biopsies.

Treatment consists of total thyroidectomy, followed by tyrosine kinase inhibitors (TKi) in case of disease persistence. During TKi treatment, Ctn and CEA levels can fluctuate regardless of tumor volume, metastasis or response to therapy.

Research for more reliable non-invasive biomarkers in MTC is still underway. In this context, circulating nucleic acids, namely circulating microRNAs (miRNAs) and cell free DNA (cfDNA), have been evaluated by different research groups. Aiming to shed light on whether miRNAs and cfDNA are suitable as MTC biomarkers we searched three different databases, PubMed, Scopus, WOS and reviewed literature. We classified 83 publications fulfilling our search criteria and summarized the results. We report data on miRNA and cfDNA that can be evaluated for validation in independent studies and clinical applications.

Abbreviations

MTC: Medullary Thyroid Carcinoma
hMTC: hereditary Medullary Thyroid Carcinoma
sMTC: sporadic Medullary Thyroid Carcinoma
fMTC: familial Medullary Thyroid Carcinoma
MEN: Multiple Endocrine Neoplasia
RET: REarranged During Transfection
FNA: Fine Needle Aspiration
Ctn: Calcitonin
CEA: Carcinoembryonic Antigen
IHC: Immunohistochemical
TKI: Tyrosine kinase inhibitor
miRNAs: microRNAs
cfDNA: cell free DNA
FF: Fresh frozen
FFPE: Formalin-fixed paraffin-embedded
NGS: Next generation sequencing

Keywords
Medullary thyroid cancer, medullary thyroid carcinoma, medullary thyroid neoplasm, biomarkers, microRNA, cfDNA, disease staging, therapeutic response

Corresponding author: Elisabetta Ferretti
E-mail: elisabetta.ferretti@uniroma1.it

Contents

1. Introduction

Medullary thyroid cancer (MTC) is a rare malignant thyroid tumor arising from the neuroendocrine parafollicular cells or C cells, accounting for 2-3% of thyroid cancers (Kim, Gosnell, & Roman, 2019).

MTC is hereditary (hMTC) in approximately 20-25% of cases and sporadic (sMTC) for the remaining 75-80%.

HMTC occurs in the context of multiple endocrine neoplasia (MEN) syndromes type 2 (MEN2A and MEN2B) or as a familial form (fMTC) due to germline mutations of REarranged During Transfection (RET) proto-oncogene, located on chromosome 10q11.2 (Eng, et al., 1996; R. Hofstra, et al., 1996). To date 76 pathogenic variations including mutations, duplications, insertions, or deletions have been identified in the RET oncogene, as reported in ClinVar ("ClinVar MTC RET," ; Landrum, et al., 2018). Strong genotype–phenotype associations, affecting the age of onset and tumor aggressiveness, have been reported for specific germline RET mutations (Wells Jr, et al., 2015).

RET codon M918T, codon C634F and codon A883F mutations are genotypes of highest or high risk patients while other RET mutations are genotypes of moderate risk patients (Wells Jr, et al., 2015).

Around half of sMTC present RET somatic mutations that associate with more aggressive disease, sMTCs with distant metastases in most cases (around 90%) harbor RET M918T mutation (Filetti, et al., 2019). Sequencing studies reported also RAS mutations (HRAS, KRAS or rarely NRAS) that are recurrent events in about 80% of cases (Agrawal, et al., 2013). sMTC patients are often diagnosed at a later stage with respect to hMTC (Wells Jr, et al., 2015).

Generally, patients with cancer limited to the thyroid gland have a 10-year survival rate of over 95%, while patients with local nodes metastases have a 75% decrease in survival rate that can diminish to 20-40% if distant metastases are found (Roman, Lin, & Sosa, 2006). Almost half of the patients are already in stage III or IV at diagnosis and approximately 30% of patients without detectable metastases at diagnosis will not be cured by surgery and will present progressive disease with appearance of metastases during the follow-up (Hadoux, Pacini, Tuttle, & Schlumberger, 2016). In summary, considering all MTC patients about 50% recur with still poor outcome (Hadoux, et al., 2016).

Currently, MTC diagnosis is performed with thyroid ultrasound screening, followed by fine needle aspiration (FNA), the standard procedure for differential diagnosis of thyroid nodules. The diagnostic accuracy of FNA in patients with MTC nodules is less than 50%, due to inadequate sampling and invasiveness of the procedure, that can hinder diagnosis and cause patient discomfort (Mon & Hodak, 2014). The main diagnostic biomarker is serum calcitonin (Ctn), accompanied by serum carcinoembryonic antigen (CEA), whose levels can correlate to tumor volume (Filetti, et al., 2019). Additionally, diagnosis includes immunohistochemical (IHC) assessment of Ctn, CEA and chromogranin markers and measurement of Ctn levels in the FNA washout fluid. The Union for International Cancer Control (UICC) TNM staging system is recommended for staging MTC patients and provides an estimated mortality risk (Brierley, Gospodarowicz, & Wittekind, 2017)

Standard treatment for MTC consists of total thyroidectomy and dissection of cervical lymph node compartments. In case of progression or recurrence, currently available treatments include the use of multi-tyrosine kinase inhibitors (TKI), which are not specific for RET inhibition (Filetti, et al., 2019). The two approved multi-TKI are vandetanib that targets RET, VEGFR, and cKIT, and cabozantinib, which targets RET, VEGFR, and EGFR (Kurzrock, et al., 2013; Priya, Dravid, Digumarti, & Dandekar, 2017). Both drugs are effective in delaying disease progression**,** but may not prolong survival and are associated with severe side effects for their wide spectrum of action (Romeo, et al., 2018).

Patient follow-up is carried out by assessing serum Ctn and CEA levels. If the doubling time of Ctn and CEA levels is less than 6 months, the disease is considered to be progressing. However, in patients treated with TKI, the levels of the two markers fluctuate significantly independent of response to treatment (Kurzrock, et al., 2013). Moreover, repeated measurements are required for a period of at least 2 years to obtain reliable estimates of the disease trend (Wells Jr, et al., 2015).

Since MTC progression is not always accompanied by the increase of serum biomarkers, the detection of recurrence during patients' follow up could be delayed until the appearance of

symptoms or a new lesion. This leads to clinicians missing an important window of opportunity where patients could be treated before recurrence is established.

In this context, reliable detection of progressive disease (PD) with novel non-invasive biomarkers is crucial in order to obtain prognosis stratification, differentiation of aggressive MTCs from indolent ones and evaluate TKI therapy effectiveness (Romeo, et al., 2018).

MicroRNAs (miRNAs) are 18–25 nucleotide long non-coding RNAs that act as post-transcriptional regulators of gene expression. MiRNA "signatures/profiles" have been reported as specific in several cellular contexts (Besharat, et al., 2018) and diseases (Catanzaro, et al., 2018; Catanzaro, et al., 2017; Esquela-Kerscher & Slack, 2006; Iorio & Croce, 2012; Miele, et al., 2013) as well as in response to therapy (Chan, Prado, & Weidhaas, 2011; Gasparri, et al., 2017; Gasparri, et al., 2018; Skinner, et al., 2014). Previous studies reported the aberrant expression of specific miRNAs in MTC tissues, suggesting these miRNAs as candidate biomarkers. Interestingly, miRNAs that can be released from cells and found in the blood stream are referred to as circulating microRNAs. In this setting, the use of circulating nucleic acids as biomarkers is referred to as "liquid biopsy" (De Smaele, Ferretti, & Gulino, 2010; Macías, et al., 2018), including the detection of miRNA as well as cfDNA released from cancer cells, which can be measured through the detection of cancer specific mutations or measurement of specific tumor features in plasma or serum samples. The first study reporting the possible use of circulating miRNAs as biomarkers in tumors was conducted by Sozzi et al. in lung cancer (Sozzi, et al., 2014).

An increasing number of studies have demonstrated the diagnostic and prognostic value of cfDNA in tumors (Polivka Jr, Pesta, & Janku, 2015), including MTC (Cote, et al., 2017).

The aim of this review is to give the reader a detailed overview of up to date knowledge of miRNA and DNA, including mutational status and methylation, as potential biomarkers in MTC, reporting their investigation in tissue and liquid biopsy samples (Figure 1).

2. Search strategy/characteristics of included studies

A systematic review of the literature was conducted using the following key words for the miRNAs section: [(MTC OR Medullary Thyroid Carcinoma) AND (MicroRNA OR MicroRNAs)]; for the cfDNA section, we performed multiple search strategies: [(MTC OR Medullary Thyroid Carcinoma) AND (DNA) AND (Biomarker OR Biomarkers)], [(MTC OR Medullary Thyroid Carcinoma) AND (DNA) AND (Methylation)], [(MTC OR Medullary Thyroid Carcinoma) AND (Next Generation Sequencing)] on the search engines of the databases "Pubmed", "Scopus", and "WOS".

Exclusion criteria were:

i) commentaries/seminars/reviews/consensus statements; ii) case report/book/editorial; iii) articles not available; iv) non-English language articles; v) studies reporting non-quantitative data or not focused on MTC; vi) articles containing data from less than five patients. Duplicate publications were eliminated through the JabRef software. The last search was performed on 4 June 2020.

Two researchers, evaluating independently the titles and abstracts of the identified articles, performed the initial screening. A third evaluator was consulted when agreement between the two researchers could not be reached.

The initial literature search identified a total of 1366 articles and a manual search of bibliographies yielded a further 17 articles resulting in a total of 1383. Removal of duplicates led to a final number of 403 papers. After reviewing titles, abstracts and full texts, 83 papers fulfilling the above mentioned search criteria were included (all studies are listed in Tables 1-9 and Supplementary Table 1). Figure 2 depicts the PRISMA flow diagram and the number of studies included in the different phases of the review.

The 83 studies included in the review were published between 1992 and 2020 and $p<0.05$ was considered to identify statistically significant results.

Several aspects need to be taken into consideration before the review of the included studies. There are a number of challenges that are a direct product of the nature of MTC and others that are due to the analyses performed in these studies.

The biological variability of MTC includes factors, similar to other types of cancer, such as age, gender and ethnicity. However, specific features of MTC such as the lack of control tissue, the rarity of this type of cancer and the fact that it is frequently analyzed and compared to other types of thyroid cancer render more difficult the establishment of a reference point and result in the lack of a true control.

The variability caused by the different analyses performed in these studies results from the different techniques used by each group and the different experimental designs that were applied, a problem that is also present in other types of cancer.

In order to address these points, technical details including extraction and evaluation methods are reported in the Supplementary section.

3. miRNAs in MTC tissues and FNA samples

Studies on miRNAs from tissue and FNA samples are summarized in Tables 1 and 2 respectively.

3.1 miRNAs in MTC formalin-fixed paraffin-embedded (FFPE) and fresh-frozen (FF) samples

We report here an overview of the studies that investigated the miRNA expression profile of MTC tissue samples in comparison to healthy thyroid or respect to metastatic spread. We also report, where available, the comparison with other diagnostic and prognostic biomarkers. The cohorts that were investigated consisted of formalin-fixed paraffin-embedded (FFPE) and fresh-frozen (FF) samples, depending on the types of samples that were available for each research group. Since each sample type necessitates different processing to obtain the miRNA expression profile this information has been reported throughout this section.

Abraham et al. were the first to investigate miRNAs in MTC: they observed high levels of miR-9*, miR-183 and miR-375 in sMTC and hMTC FF samples and validated their expression in a separate cohort of FFPE samples. MiR-183 and miR-375 were up-regulated and miR-9* was down-regulated in sMTC versus hMTC. High levels of miR-183 and miR-375 predicted lateral lymph node metastasis and were associated with residual disease, distant metastases, and mortality (D. Abraham, et al., 2011). In another study, miR-9*, miR-183 and miR-375 expression levels were correlated in primary tumors and matched lymph node metastasis (Gundara, et al., 2014)

One of the most studied oncomiRs is miR-21, described in different types of tumors (Bautista-Sánchez, et al., 2020). MiR-21 was found up-regulated in MTC with respect to normal thyroid

tissues, showing an inverse correlation with the tumor suppressor Programmed Cell Death 4 (PCD4) (Mian, et al., 2012; Pennelli, et al., 2015). The expression of miR-21 positively correlated with Ctn levels, lymph node metastases, advanced stage and biochemically persistent disease (Pennelli, et al., 2015).

The correlation between miR-21 and metastatic spread was confirmed, as miR-21 and miR-183 levels were higher in MTC with lymph node involvement (LNI) with respect to those without LNI. Therefore, miR-21 levels were proposed as a marker for the extent of surgery to cervical nodes, along with the abundance of desmoplastic stroma. (Aubert, et al., 2018).

Interestingly, miR-21 showed no differential expression in metastatic versus primary tumors (Chu, Hardin, Schneider, Chen, & Lloyd, 2017).MiR-375 is another oncomiR whose high expression correlated with poor prognosis in a variety of tumors (Huang, et al., 2015; Zhengyan Wang, Hong, Gao, & Feng, 2013; X. Zhang, et al., 2011). High levels of miR-375 were reported in MTC (Hudson, et al., 2013) and correlated with tumor size, frequency of thyroid capsule infiltration, risk of lymph node metastases and mortality (D. Abraham, et al., 2011; Galuppini, et al., 2017; Gundara, et al., 2014). YAP1 (yes-associated protein 1) was identified as a potential target of miR-375 (Galuppini, et al., 2017; Hudson, et al., 2013) .

MiR-224 has been proposed as biomarker since its low levels correlate with high levels of Ctn and worse prognosis (Cavedon, et al., 2017; Mian, et al., 2012).

Several reports identified miRNAs as features of MTC, using normal thyroid as the control tissue that however includes several other different types of cells apart from C cells.

Mian et al. reported the high expression of miR-127, miR-224, miR-154, miR-323, miR-370, miR-9* and miR-183 in both hMTC and sMTC tissues compared to normal thyroid (Mian, et al., 2012).

Chen et al. reported that miR-9-3p was up-regulated in MTC compared to normal tissues, and that high expression of miR-9-3p in TT cells was correlated with decreased growth and enhanced apoptosis via targeting Bladder Cancer-associated protein (BLCAP) (Y. Chen, Zhang, Zhao, Zhao, & Zhang, 2017).

Recently, miR-34a and miR-144 were evaluated and compared to the normal adjacent tissue specimens. Through the receiver operating characteristic curve (ROC curve) analysis, it was demonstrated that high levels of two miRNAs had significant diagnostic value (p=0.001) (Shabani, et al., 2018).

Jiang et al. re-analyzed two miRNA profiles from GEO database and used 22 pairs of MTC and para-carcinoma tissues as a validation cohort. MiR-31-3p resulted to be the most significantly dysregulated. Functional analysis showed that overexpression of miR-31-3p inhibited MTC cell proliferation in vitro and in vivo and negatively regulated RASA2 (RAS P21 Protein Activator 2) (Jiang, Shi, Zhu, Wei, & Li, 2019).

Santarpia et al. identified a 10-miRNA signature associated with sMTC metastases, using matched primary and metastatic samples. A strong point of this study is that the evaluation was performed using two different methods and the validation was performed on an independent cohort of MTC. Bioinformatics approaches revealed potential miRNA targets and molecular signaling involved in metastatic MTC pathways, e.g., TGFb signaling pathway (Santarpia, et al., 2013).

The following reports investigated the link between miRNAs and the known driver genes RET and RAS. On multivariate analysis, miR-224 positively correlated with RAS mutation status in sMTC

(Cavedon, et al., 2017). MiR-127 expression was lower in sMTC carrying the somatic RET mutation compared to wild-type RET(Mian, et al., 2012).

Ehyaei et al. investigated the relationship between Ctn and miR-323 levels in patients with or without RET mutation, reporting no significant difference between the two (Ehyaei, et al., 2017). It has been demonstrated that RET can be targeted by miRNAs and this finding might help in developing a therapeutic approach for the treatment of RET-activated MTC. Specifically, Duan et al. showed that high levels of RET mRNA and protein were accompanied by remarkably low levels of miR-129-5p in MTC tissues compared to the matched normal tissue. The biological role of miR-129-5p was investigated in TT and MZ-CRC-1 cells, that both carry RET mutations (Duan, Hao, Liu, Zhang, & Zhang, 2014), where miR-129-5p was able to bind directly to the 3'UTR of RET mRNA, repressing RET expression, inhibiting cell growth, inducing cell apoptosis, and reducing cell invasion in vitro (Duan, et al., 2014).

Integration of methylation and miRNA expression data from MTC samples carrying RET M918T mutation, showed that miR-10a, miR-30a, and-200c expression levels were negatively correlated with their promoters' methylation. Pathway enrichment analyses of these miRNAs uncovered JAK/Stat pathway involvement in RET M918T MTCs (Mancikova, et al., 2017).

3.2 miRNAs in MTC fine needle aspiration (FNA) samples

FNA samples can provide information similar to those of MTC tissue, however it is a "difficult" type of sample due to the limitations in collecting a sufficient amount and quality of cells, which raises challenges in the miRNA expression profile analysis.

Since FNA is already in use for thyroid cancer diagnosis, the development of a reliable microRNAs signature would improve the diagnostic path.

Titov et al. analyzed thyroid cancer FNA samples of different histotypes and proposed high levels of miR-151a as a potential biomarker able to distinguish MTC from benign neoplasms and papillary thyroid carcinoma (Titov, et al., 2016).

MiR-375 high levels were reported in MTC FNA samples confirming data from tumor tissue (Lithwick-Yanai, et al., 2017). Recently, an algorithm was developed for the differential diagnosis of FNA samples on the basis of a small set of molecular markers: indeed, low levels of miR-146b, −155, −31, and -551b and high levels of miR-375 characterize MTC in comparison to PTC (Titov, et al., 2019).

4. Circulating miRNAs as biomarkers in MTC

MiRNAs detectable in the blood are referred to as *"circulating miRNAs"* and have been proposed as biomarkers for diagnosis, prognosis and prediction of therapeutic response (De Smaele, et al., 2010; Mitchell, et al., 2008; Schwarzenbach, Nishida, Calin, & Pantel, 2014). Circulating miRNAs can be obtained with minimally invasive approaches (e.g. liquid biopsy), are easily quantified and their expression is stable in the blood (Mitchell, et al., 2008).

Although the origin of circulating miRNAs is still controversial, previous reports have demonstrated that miRNAs up-regulated in tumor tissues may be detected in plasma and can correlate with clinicopathological features and patients' prognosis (Schwarzenbach, et al., 2014).

To date, there are only 3 studies investigating miRNA expression levels from MTC patients-derived plasma or serum samples, summarized in Table 3.

Romeo et al. identified high levels of circulating miR-375 in MTC respect to sex- and age-matched healthy controls and reported its correlation with distant metastases and reduced overall survival (OS) (Romeo, et al., 2018). Of note, plasma miR-375 levels failed to distinguish patients with partial response or stable disease respect to those with disease progression. Interestingly, they also reported this miRNA as the most significantly upregulated in both primary and metastatic tissue samples compared to non-neoplastic thyroid.

Shabani et al. investigated plasma levels of miR-144 and miR-34a, identified previously in tissue studies**,** in RET-mutant and RET-wild type MTC patients and in healthy controls. Both were expressed at higher levels in patients with MTC than in controls and in RET mutant versus RET wild type patients. However, ROC curve analysis reported no significant value for miR-144 and miR-34a as circulating MTC biomarkers (Shabani, et al., 2020).

Zhang et al. proposed miR-222-3p and miR-17-5p as potential diagnostic biomarkers in MTC. These miRNAs were identified as highly expressed in serum of MTC patients compared to patients bearing benign nodules and healthy controls and ROC curves analyses confirmed their high diagnostic accuracy (A. Zhang, et al., 2019).

5. Bioinformatics studies identifying miRNAs in MTC

In silico bioinformatics miRNAs analyses were included in this review, since an integrate bioinformatics approach could help in the understanding of MTC multifactorial aspects. Up to date, there are 4 microarray Gene Expression Omnibus (GEO) (GSE40807, GSE97070, GSE27155, GSE67742] datasets that were re-interrogated in the following studies, listed in Table 4.

MiR-375 was the focus of few studies, given its high levels in MTC -as observed in (D. Abraham, et al., 2011; Galuppini, et al., 2017; Gundara, et al., 2014; Huang, et al., 2015; Hudson, et al., 2013; Lithwick-Yanai, et al., 2017; X. Zhang, et al., 2011) -and its potential functional role in the disease.

Shi and colleagues showed that the PPI (protein-protein interaction) network revealed potential miR-375 gene targets involved in several crucial pathways, such as PI3K/Akt signaling pathway, pathways in cancer and MAPK signaling pathway (Shi, et al., 2017). Functional enrichment analysis of target genes in pan-cancer (including MTC) showed specific biological function of miR-375: amino acid biosynthetic, metabolic process and Cysteine and methionine metabolism. In particular, VASN, MAT2B, HERPUD1, TPAPPC6B and TAT were listed as probable gene targets of miR-375 (Zeng, Liang, Lan, Zhu, & Liang, 2018).

Other studies identified different subsets of deregulated miRNAs. MiR-1, miR-9-5p, miR-96-5p and miR-590-5p were identified as up-regulated in MTC compared to healthy controls; enriched pathways related to these miRNAs included the mitogen activated protein kinase (MAPK) signaling and k light polypeptide gene enhancer in B-cells 1 (NF-KB1) pathway (Fu, et al., 2017).

In a different study miR-375, miR-127-3p, miR-429 were up-regulated and miR-199b-5p and miR-199a-3p was down-regulated in MTC. Pathways enriched by the deregulated miRNAs included adherent junctions, kinase, protein binding, non-canonical Wnt signaling pathway and RNA transport (L. Zhang, Lu, Liu, Zhang, & Peng, 2019).

6. Evaluation of miRNA functional role in MTC cell models and xenografts

Although the investigation of the biological role of miRNAs in MTC is not the focus of this review, we report here previous studies related to the biological effects of miRNAs identified as potential biomarkers. The studies summarized below describe miRNAs in MTC *in vitro* and *in vivo* models and can be found in Table 5. Notably, this type of data may be useful to uncover new therapeutic targets amid active pathways in MTC.

MTC cell lines available include human TT (RET C634W mutant) and MZ-CRC-1 (RET M918T mutant) cell lines, which were derived from primary and metastatic MTC respectively. Human normal thyroid follicular NThy-ori 3.1 cell line was also used in experiments, even though the follicular cells are not the cell of origin of MTC, therefore the results from the analyses using this cell line remain elusive.

MiR-9-3p is dysregulated in several types of cancer (Zawistowski, et al., 2013) and its upregulation in TT and MZ-CRC-1 was associated to enhanced cell death *in vitro* due to inhibition of autophagic flow and apoptotic increase (Gundara, et al., 2015).

Using an approach combining transcriptome analyses of miR-375 activation and inhibition in NThy-ori 3.1 and TT cells together with data exploration of the Cancer Cell Line Encyclopedia (CCLE) transcriptome database, SEC23A was proposed as a miR-375 target. Interestingly, low levels of SEC23A may improve the efficacy of vandetanib (Lassaile, et al., 2016).

Spitschak et al. identified a novel mechanism for MTC aggressiveness, reporting that miR-182 was activated by M918T and C634W RET mutations in an NF-κB-dependent manner in NThy.RET M918T and TT cells. In turn, miR-182 directly targeted HES1, hindering the tumor suppressive HES1/Notch1 pathway (Spitschak, Meier, Kowtharapu, Engelmann, & Pützer, 2017).

Ye et al. identified downregulation of miR-149-5p and upregulation of GIT1 in 36 MTCs, showing that these features correlated with distant metastases and shorter overall survival, and that miR-149-5p inhibited GIT1 expression in vitro in MTC cells, regulating cell proliferation and invasion (Ye & Chen, 2019).

Joo et al. recently identified a RET-regulated miRNAs profile using the inhibition of RET activity in MTC cells through either RET siRNA or cabozantinib. MiR-153-3p resulted as the most upregulated after RET inhibition and it exerts a tumor suppressive role via inhibition of mTOR signaling, and its low expression has already been described in other cancer types (Shan, Shen, Wang, He, & Duan, 2015; Zhongli Wang & Liu, 2016; Xia, et al., 2015; Yuan, et al., 2015). Additionally, in vivo results showed that intravenous delivery of miR-153-3p using targeted EDV nanocells in mice bearing MTC xenograft reduced tumor volume and sustained tumor stabilization in combination with cabozantinib treatment (Joo, et al., 2019).

7. Genetic landscape of germline and somatic mutations in MTC

Mutations associated to MTC have been investigated by several studies, proposing them as potential biomarkers of diagnosis and prognosis and more details are reported in this paragraph. The studies that investigated DNA mutations in MTC tissue and peripheral blood leukocytes are summarized in Table 6.

The most common mutated gene is RET, followed in frequency by RAS and mutations in the two genes are generally mutually exclusive.

RET encodes for a tyrosine kinase receptor and its mutations activate signaling pathways regulating tumor development and progression. These mutations are distributed within six exons ex 10 and 11 (in cysteine codons 609, 611, 618, and 620) and ex 13, 14, 15 and 16 (in noncystein codons such as 804, 883 and 918) (Gagel, Robinson, Donovan, & Alford, 1993; Santarpia, et al., 2013; Ye & Chen, 2019).

Romei and colleagues analyzed blood and tumor DNA of 19 sMTC and 6 parathyroid adenoma patients. Their results revealed that the somatic mutations found in sMTC could affect not only exon 16 but also exon 11 and were both associated with less favorable clinical results (Romei, et al., 1996). Other authors confirmed that in sMTC M918T mutation is the most frequent, ranging from 50 to 79% depending on the cohorts investigated, and that its conferred a worse prognosis (Dvorakova, et al., 2008; Elisei, et al., 2008; Schilling, et al., 2001). Moura et al reported that in sMTC in addition to exon 16 also exon 15 mutation was associated with worse prognosis, while patient carring other RET mutations had more indolent course, and those with no RET mutations had an intermediate risk (M. Moura, et al., 2009).

All hereditary MTC (hMTC) carrying germline RET mutations in MEN2A and fMTC, where first described mutations in exons 10 and 11 (codon 634), while in MEN2B mutation in exon 16 (M918T) (Bergant, et al., 2006; Eng, et al., 1996; R. M. Hofstra, et al., 1994; Mulligan, et al., 1994; Patocs, et al., 2006).

Codon 618 (exon 10) was the most frequent RET mutation in 79 subjects from 10 unrelated Saudi families with hMTC and MEN2A (Qari, 2013), while in a cohort of Iranian patients mutations in codons 611, 618 and 620 (exon 10) were found in fMTC and no exon 10 mutation in MEN2 was identified (Yeganeh, Sheikholeslami, Behbahani, Farashi, & Hedayati, 2015).

Komminoth and colleagues analyzed RET point mutations from 16 hMTC and 16 sMTC, correlating their findings with clinico-pathological features and follow-up. They concluded that it was possible to identify subjects at risk for MEN2A, fMTC and MEN 2B, with genetic screening (Komminoth, et al., 1995).

Gene expression profiles correlate with the type of genetic mutation rather than the sporadic or hereditary form of MTC. Ameur et al analyzed gene expression profiles in RET 634, RET 918 and RET wild-type MTCs and results showed that, independently of their inherited or sporadic status, MTC bearing the RET 918 mutation clustered together with RET wild-type MTC with distant metastases, whereas RET wild-type MTC without metastases segregated with RET 634 MTC (Ameur, et al., 2009). Lairmore and colleagues showed that early thyroidectomy in kindred members positive for RET mutations was beneficial to the lives of individuals (Lairmore, Frisella, & Wells, 1996). Indeed, a study of a retrospective cohort of MTC patients showed that hMTC patients had lower age at presentation, smaller tumor, lower local recurrence rate, lower preoperative Ctn levels than in sMTC patients but no statistical difference in overall survival and disease-free survival (D. T. Abraham, et al., 2011). A later study on a Turkish cohort reported the possible limitations of late prophylactic thyroidectomy in the lifespan (Aydoğan, et al., 2016).

Investigations were carried out to identify activating mutations in genes other than RET and the most commonly mutated gene was RAS.

In a series of RET-wild type and RET-mutant sMTCs, somatic mutations of HRAS and KRAS were detected in 56% and 12% of cases respectively. Interestingly, only one of the 40 RET-mutant sMTC

had a mutation in HRAS, suggesting that the activation of the proto-oncogenes RAS and RET represented alternative genetic events (M. M. Moura, Cavaco, Pinto, & Leite, 2011).
This observation was later confirmed in a large multi-centric Italian cohort that also reported a less aggressive behavior in patients carrying the RAS mutation (Ciampi, et al., 2013).
Romei and colleagues examined the genetic profile of 70 advanced and metastatic MTC and correlated the type of mutation with the outcome. RET mutations, mainly M918T, were the most prevalent. K or HRAS mutations were present in 6.2% of cases and were mutually exclusive with RET. In RET and K/HRAS wild type tumors, authors failed to identify other mutations. Moreover, the correlation analysis revealed a positive correlation between an unfavorable prognosis and a multiple number of mutations in RET (Romei, et al., 2016).
Grubbs and colleagues investigated in a cohort of 62 sMTCs the loss of CDKN2C, associated with tumorigenesis in multiple types of cancer (Chinnam & Goodrich, 2011; Di Fiore, D'Anneo, Tesoriere, & Vento, 2013; Knudsen & Wang, 2010; Zhu, Lu, & Zhao, 2015). The somatic loss of CDKN2C was associated with distant metastases and the reduction of overall survival (Grubbs, et al., 2016).
Mian and colleagues reported that the combination of somatic RET analysis with Ki-67 staining evaluation could identify patients with aggressive tumors (Mian, et al., 2011).
The search for more mutations in MTC yielded poor or no results. Herfarth and colleagues failed to find mutations in exons 4-9 of the TP53 gene (Herfarth, et al., 1997). Mutations of the MET oncogene were investigated and a germline point mutation, T1010I, was identified in one patient (Wasenius, et al., 2005). In another study, the p18 somatic inactivating mutations were identified together with the activation of RET mutations in MTC (van Veelen, et al., 2009)
BRAF mutations were also evaluated and in almost studies none mutation were found (M. M. Moura, et al., 2011) with the exception of one patient carrying BRAF V600E mutation (Cho, et al., 2014).
No mutations in the TERT gene and promoter were found (Bae, et al., 2016; Romei, et al., 2016) as well as no rearrangement of the ALK gene (Bae, et al., 2016).

7.1 DNA alterations in MTC reported by Next generation sequencing

Next-generation Sequencing (NGS) techniques allowed concurrent analysis of the whole genome, exome or a subset of genes of interest with the generation of more data with respect to methods like Sanger sequencing, that analyze one DNA fragment at a time (Aziz, et al., 2015; Sanger, Nicklen, & Coulson, 1977).
All studies concerning NGS analysis in MTC are summarized in Table 7.
Whole genome sequencing (WES) and exome sequencing allowed the investigation of genes that were not previously linked specifically to MTC.
WES was performed in a cohort of Taiwanese sMTC patients with and without RET mutations and new somatic mutations in BICD2 (BICD Cargo Adaptor 2), DLG1 (Discs Large MAGUK Scaffold Protein 1), FSD2 (Fibronectin Type III And SPRY Domain Containing 2), IL17RD (Interleukin 17 Receptor D), KLHL25 (Kelch Like Family Member 25), PAPPA2 (Pappalysin 2), PRDM2 (PR/SET Domain 2), PSEN1 (Presenilin 1), SCRN1 (Secernin 1), TTC1 (Tetratricopeptide Repeat Domain 1) and PDE4DIP (Phosphodiesterase 4D Interacting Protein) were identified (Chang, et al., 2018).

Exome sequencing showed that, besides RET, HRAS and KRAS, relatively few mutations were described, including MDC1 (Mediator of DNA Damage Checkpoint 1) or ATM (ATM Serine/Threonine Kinase), suggesting a potential role for the DNA damage pathway in RET-negative MTC (Agrawal, et al., 2013).

Hybrid-capture-based comprehensive genomic profiling was performed on 512 consecutively submitted thyroid carcinomas. The majority of MTCs harbored RET M918T alterations and 3 MTCs had novel RET insertions/deletions in exons 6 and 11. Many of these MTC patients with novel RET alterations experienced clinical benefit from vandetanib treatment (Borre, et al., 2017).

Several gene panels were used for investigations of MTC mutational status, either pan-cancer or thyroid cancer related.

Most reports confirmed the prevalence of RET mutations (Kato, et al., 2017; Pitt, et al., 2016), followed by HRAS and KRAS mutations and that these mutations were mutually exclusive (H. Chen, et al., 2018; Simbolo, et al., 2014; Wei, LiVolsi, Montone, Morrissette, & Baloch, 2016).

No mutations within NRAS, TP53, and BRAF genes were detected (Guo, et al., 2019) and mismatch repair system was not affected, independently of stromal desmoplasia or RET (Ingenwerth, Goetz, Schmid, & Theurer, 2020). Rare mutations were identified in ATM (Simbolo, et al., 2014).

Targeted sequencing was used to correctly diagnose apparently sporadic MTCs carrying the novel germline RET S409Y mutation that was demonstrated to be pathogenic and associated with a lower penetrance of MTC than that for the C618Y and C634Y mutations (Qi, et al., 2019).

The first NGS thyroid cancer specific panel was ThyroSeq, which targeted 284 mutational hot spots in 12 cancer genes. The most common mutation was RET M918T, followed by HRAS Q61K, KRAS G12R and HRAS G13R (Nikiforova, Wald, Hsu, Durso, & Nikiforov, 2013). No EIF1AX mutation were identified in MTC (Karunamurthy, et al., 2016).

The latest updated version of ThyroSeq panel includes recently discovered thyroid tumors related genetic markers and enable the analysis of copy number alterations and showed that the main type of genetic alteration that occurred in the MTC was single nucleotide variations (SNVs) (80%) and copy number alterations (CNAs) (27%) (Nikiforova, et al., 2018).

The efficacy of an NGS panel (Thyroline) in predicting the classification of benign and malignant thyroid nodules and lymph node metastasis status was compared to the results obtained from ultrasound and showed that The predictive value of NGS was higher than the ultrasound and that the sensitivity of NGS was consistently >70% in MTC cases (Ke, et al., 2018).

A recent study by Ciampi and colleagues analyzed the largest series of sMTC so far, highlighting the bad prognostic role of RET mutations and consolidated the favorable prognostic role of RAS mutations. The mutations identified in sMTC samples were mostly RET and RAS-related: only 3/148 patients harbored mutations in independent genes: CHK2 W114, EIF1AX G135A, and TSHR I630L. For the first time, it was shown that the variant allele frequency (VAF) represents an additional prognostic marker in the group of RET-mutated sMTC. Indeed, larger tumors harbored mutations with a higher VAF value and it was also correlated with a worse outcome (Ciampi, et al., 2019).

The RET M918T mutation was detected with significantly higher frequency in metastatic versus non-metastatic MTC (56.52% *vs* 10.00%). However, no significant correlation was found between

the presence of a RET mutation in the primary tumor and treatment response at 3 months (Tiedje, et al., 2016).

8. DNA Methylation in MTC

It has been demonstrated that DNA methylation plays an important role in the regulation of gene expression. Indeed, hypo and hypermethylation across the genome contribute to changes in gene expression (Moarii, Boeva, Vert, & Reyal, 2015). Variations in the global DNA methylation levels have been observed in different cancer types and may be a potential source of non-invasive cancer biomarkers (Esteller, 2008; Feinberg & Tycko, 2004; Witte, Plass, & Gerhauser, 2014).
All the studies concerning DNA methylation in MTC are summarized above in Table 8.

8.1 DNA methylation in MTC formalin-fixed paraffin-embedded (FFPE) and fresh-frozen (FF) samples

Hypermethylation of cancer-related gene promoters in the MTC were thought to be a relative rare event, occurring less frequently compared to follicular adenomas and goiters, suggesting that other genetic events could be more important for MTC development (i.e. RET mutations) (Schagdarsurengin, Gimm, Dralle, Hoang-Vu, & Dammann, 2006).

Recently, genome-wide DNA methylation profiling was performed to assess more than 27,000 CpGs in the largest MTC series reported to date. Unsupervised hierarchical cluster analysis of MTC samples, divided the sample set into 2 main clusters: one including RET M918T mutation-related samples and the second including the majority of RET C634 and wild-type cases. These data are in line with gene expression results by Ameur et al. [paragraph 7. Genetic landscape of germline and somatic mutations in MTC] (Ameur, et al., 2009) and suggested that methylation profile relates to RET mutational status (Mancikova, et al., 2017).
Although TERT mutations were not identified in MTC (Bae, et al., 2016; Romei, et al., 2016) , telomerase activation was detected in approximately 50% of sMTCs (Liu, et al., 2014). A recent study highlighted that the hypermethylation of the TERT promoter played an important role in the induction of TERT transcription and expression during MTC development, as seen in other human malignancies (Castelo-Branco, et al., 2013; Guilleret, et al., 2002). Furthermore, the results showed that a higher methylation index at TERT promoter was significantly associated with both shorter overall and disease-free survivals in sMTC patients (N. Wang, et al., 2016).
Hypermethylation of promoters of tumor suppressor genes was investigated: methylation of RASSF1 (Ras Association Domain Family Member 1) was investigated in different thyroid cancer and results showed that it was higher in the aggressive forms of thyroid cancer, including MTC and undifferentiated thyroid cancer when compared to the more benign PTC. RASSF1A inactivation was detected in all stages of thyroid carcinoma (Schagdarsurengin, et al., 2002).
Spry1 (Sprouty RTK Signaling Antagonist 1) promoter resulted frequently methylated in MTC tissue samples and the methylation status was inversely correlated to Spry1 expression. Functional studies showed that deletion of Spry1 caused precancerous lesion preceding MTC in mice (Macià, et al., 2012).

8.2 DNA methylation in MTC Peripheral blood Leukocytes (PBL)

While the methylation status of tumor tissue can be analyzed only after surgery or an invasive biopsy, methylation of PBL can be considered as "liquid biopsy". A first report found no association between global methylation levels and calcitonin levels, tumor size or presence of metastatic disease in MTC patients. Despite this, sMTC patients had a higher level of methylation when compared to hMTC (Ceolin, Goularte, Ferreira, Romitti, & Maia, 2018). A more recent study evaluated the methylation status of RET from peripheral blood leukocytes of MTC patients. It emerged that in RET wild type MTC patients the RET promoter was hypomethylated and RET gene expression was elevated. The authors concluded that the methylation status of RET promoter could be considered as a new potential prognostic, diagnostic and therapeutic marker in MTC (Shakiba, Movahedi, Majd, & Hedayati, 2019).

DNA methylation was also investigated in MTC cell models with a specific focus on the combination of demethylating agents with drugs and the results reported in the supplementary section and Supplementary Table 1, suggest that novel combinatory treatments strategies could prove promising for MTC patients.

9. Cell-free DNA (cfDNA) as biomarker in MTC

An increasing number of studies have demonstrated the potential use of cell-free DNA (cfDNA) as a potential biomarker in cancer for diagnosis, prognosis, and follow-up (Bronkhorst, Ungerer, & Holdenrieder, 2019).

Up to date, 3 studies investigated cfDNA in MTC and are reported in Table 9.

The first study demonstrated that two mutations in the ALU gene, $cfDNA_{ALU83}$ and $cfDNA_{ALU244}$, were detectable in different kind of Thyroid tumors including MTC, anaplastic thyroid cancer, synchronous medullary and follicular thyroid cancers, follicular adenomas and papillary thyroid cancers and that these markers had high ability to discriminate healthy individuals from cancer patients (Zane, et al., 2013).

A second study demonstrated that the detection of RET M918T mutation in cfDNA in a cohort of sMTC strongly correlated with worse overall survival and more accurately predicted a worse outcome than calcitonin doubling time (Cote, et al., 2017).

More recently, mutation variants of RET, BRAF and HRAS were identified in a cohort of mixed thyroid tumors including MTC and later detected as cfDNA in plasma samples of matched patients. ctDNA was detected in at least one plasma timepoint in 67% of patients and the detection rate was highest in MTC compared with other thyroid tumors. Authors demonstrated that cfDNA was an earlier biomarker of disease respect to Ctn (Allin, et al., 2018).

In summary, these studies point out to cfDNA as an early biomarker for MTC progression.

10. Conclusions

The search for biomarkers to aid diagnosis, prognosis and response to therapy in MTC is still underway. Indeed, besides established markers such as Ctn and CEA, recent technological advances have allowed the generation of a wealth of new data regarding nucleic acids in MTC.

MiRNAs have emerged as a powerful tool in oncology. MiRNAs have been investigated in MTC in the last 10 years, in order to identify miRNA expression profiles and potential biomarkers of MTC with a specific focus on advanced stage disease. Interestingly, miR-375 and miR-21 were the most frequently up-regulated miRNAs in MTC tumor samples.

Starting from 1992, many studies investigated DNA mutations characterizing MTC, leading to a mutational panorama focused mainly on RET protooncogene, which was mutated in about 40% of sMTC and about 95% of hMTC. Further investigations identified RAS mutations in about 35% of sMTC patients Specific RET mutations (M918T, C634F and A883F) conferred worse prognosis. Recent studies with NGS allowed the examination of gene panels, however few and very rare novel mutations were reported.

Methylation studies have also been carried out to obtain a better understanding of MTC, however the lack of uniformity in methylation analysis techniques is one of the reasons that still hinders the use these tools in the clinical setting yet (Draht, et al., 2016; Shakiba, et al., 2019).

The relatively recent identification of circulating tumor cell-free DNA (cfDNA) and miRNA, set the basis for the use of liquid biopsy in MTC. CfDNA offers a very specific tool for detection of the residual disease or the early identification of metastases. Limitations include the limited fraction of circulating tumor-derived DNA among DNA derived from non-transformed cells, that depends on the tumor mass, the intrinsic DNA shedding of the tumor, clearance of circulating DNA and the possibility that the identified mutation could be sub-clonal (Schilling, et al., 2001). In addition, current detection techniques have a limit of detection of 0.01% (Chin, et al., 2019).

Circulating miRNAs have been recently identified as a potential tool for diagnosis and prognosis. As opposed to cfDNA, circulating miRNAs analyses do not rely on the identification of mutations, but on the differential expression of miRNAs in patients with respect to healthy control or among clinically distinct patient groups, therefore some tumor cell-free DNA limitations are not applicable.

Precision medicine is an innovative branch still at its infancy, aiming at the identification of robust biomarkers and, in the case of MTC, at improving patient care and management that includes the identification of biomarkers for diagnosis, prognosis, differentiating aggressive MTCs from indolent ones, and response to therapy.

As reported in this review, only few data on cfDNA and miRNAs in MTC are reported but they are promising; therefore more studies are necessary to shed light on these issues and identify novel and robust biomarkers that can enter the clinical setting.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by AIFA (Agenzia Italiana del Farmaco) grant, Proposal code: TRS-2016-00001141 and Ateneo 2018 grant.

Figure legends

Figure 1. Overview of medullary thyroid carcinoma
Medullary thyroid carcinoma (MTC) is a rare tumor of the thyroid gland, arising from C cells. 75% of MTC are sporadic and 25% are hereditary, including those developed in the context of MEN2A and MEN2B symdromes.
Diagnosis of MTC is currently based on clinico-pathological data shown on the left, in blue boxes. The ultrasound is the primary and non invasive screening, followed by a Fine Needle Aspiration (FNA) biopsy that allows to perform a cytological investigation of cells of the nodule, providing a differential diagnosis among the different types of thyroid tumors. Serum levels of calcitonin and carcinoembryonal antigen are well established biomarkers for MTC diagnosis and follow up. Therapy includes total thyroidectomy, followed by kinase inhibitors treatment in case of metastatic disease or recurrence/progression. This review focuses on the role of nucleic acids as potential biomarkers. Indeed microRNA expression profiles and DNA mutations have been investigated in MTC tissues and, more lately, in liquid biopsies, in search for biomarkers of diagnosis, prognosis, relapse and response to therapy.

Figure 2. PRISMA flow diagram illustrating the study selection process. From the 1383 initially identified studies, 980 duplicates were removed. 403 records were screened by title, abstract and full text, leading to the inclusion of 83 studies.

Table legends

Table 1. Characteristics of studies on miRNAs analyzed from MTC tissue samples
P values are listed to correspond to the order of miRNA reference in the microRNAs column

Table 2. Characteristics of studies on miRNAs analyzed from FNA samples
P values are listed to correspond to the order of miRNA reference in the microRNAs column
n.a. not applicable

Table 3. Characteristics of studies on circulating miRNAs analyzed from MTC liquid biopsy
P values are listed to correspond to the order of miRNA reference in the microRNAs column

Table 4. Characteristics of studies on miRNAs from bioinformatics analyses
P values are listed to correspond to the order of miRNA reference in the microRNAs column

Table 5. Characteristics of studies on miRNAs including vitro and in vivo data
P values refer to the findings described in details

Table 6. Characteristics of studies on DNA mutations from FFPE, FF samples and peripheral blood leukocytes

Table 7. Characteristics of studies reporting DNA alterations using Next Generation Sequencing on FFPE and FF samples

Table 8. Characteristics of studies reporting DNA methylation data conducted on tissue samples, peripheral blood leukocytes and lymphoblast cell line AC

Table 9. Characteristics of studies reporting cfDNA data from liquid biopsy samples

Supplementary Table 1. Characteristics of studies reporting DNA methylation data conducted

References

Abraham, D., Jackson, N., Gundara, J. S., Zhao, J., Gill, A. J., Delbridge, L., Robinson, B. G., & Sidhu, S. B. (2011). MicroRNA profiling of sporadic and hereditary medullary thyroid cancer identifies predictors of nodal metastasis, prognosis, and potential therapeutic targets. *Clinical Cancer Research, 17*, 4772-4781.

Abraham, D. T., Low, T.-H., Messina, M., Jackson, N., Gill, A., Chou, A. S., Delbridge, L., Learoyd, D., Robinson, B. G., & Sidhu, S. (2011). Medullary thyroid carcinoma: long-term outcomes of surgical treatment. *Annals of surgical oncology, 18*, 219-225.

Agrawal, N., Jiao, Y., Sausen, M., Leary, R., Bettegowda, C., Roberts, N. J., Bhan, S., Ho, A. S., Khan, Z., & Bishop, J. (2013). Exomic sequencing of medullary thyroid cancer reveals dominant and mutually exclusive oncogenic mutations in RET and RAS. *The Journal of Clinical Endocrinology & Metabolism, 98*, E364-E369.

Allin, D., Shaikh, R., Carter, P., Thway, K., Sharabiani, M., Gonzales-de-Castro, D., O'Leary, B., Garcia-Murillas, I., Bhide, S., & Hubank, M. (2018). Circulating tumour DNA is a potential biomarker for disease progression and response to targeted therapy in advanced thyroid cancer. *European Journal of Cancer, 103*, 165-175.

Ameur, N., Lacroix, L., Roucan, S., Roux, V., Broutin, S., Talbot, M., Dupuy, C., Caillou, B., Schlumberger, M., & Bidart, J.-M. (2009). Aggressive inherited and sporadic medullary thyroid carcinomas display similar oncogenic pathways. *Endocrine-related cancer, 16*, 1261-1272.

Aubert, S., Berdelou, A., Gnemmi, V., Behal, H., Caiazzo, R., D'herbomez, M., Pigny, P., Wemeau, J. L., Carnaille, B., & Renaud, F. (2018). Large sporadic thyroid medullary carcinomas: predictive factors for lymph node involvement. *Virchows Archiv, 472*, 461-468.

Aydoğan, B. İ., Yüksel, B., Tuna, M. M., Başaran, M. N., Kocaeli, A. A., Ertörer, M. E., Aydın, K., Güldiken, S., Şimşek, Y., & Karaca, Z. C. (2016). Distribution of RET mutations and evaluation of treatment approaches in hereditary medullary thyroid carcinoma in Turkey. *Journal of clinical research in pediatric endocrinology, 8*, 13.

Aziz, N., Zhao, Q., Bry, L., Driscoll, D. K., Funke, B., Gibson, J. S., Grody, W. W., Hegde, M. R., Hoeltge, G. A., & Leonard, D. G. (2015). College of American Pathologists' laboratory standards for next-generation sequencing clinical tests. *Archives of Pathology and Laboratory Medicine, 139*, 481-493.

Bae, J. S., Kim, Y., Jeon, S., Kim, S. H., Kim, T. J., Lee, S., Kim, M.-H., Lim, D. J., Lee, Y. S., & Jung, C. K. (2016). Clinical utility of TERT promoter mutations and ALK rearrangement in thyroid cancer patients with a high prevalence of the BRAF V600E mutation. *Diagnostic pathology, 11*, 21.

Bautista-Sánchez, D., Arriaga-Canon, C., Pedroza-Torres, A., De La Rosa-Velázquez, I. A., González-Barrios, R., Contreras-Espinosa, L., Montiel-Manríquez, R., Castro-Hernández, C., Fragoso-Ontiveros, V., & Álvarez-Gómez, R. M. (2020). The promising role of miR-21 as a cancer biomarker and its importance in RNA-based therapeutics. *Molecular Therapy-Nucleic Acids*.

Bergant, D., Hocevar, M., Besic, N., Glavac, D., Korosec, B., & Caserman, S. (2006). Hereditary medullary thyroid cancer in Slovenia–genotype-phenotype correlations. *Wiener Klinische Wochenschrift, 118*, 411.

Besharat, Z. M., Abballe, L., Cicconardi, F., Bhutkar, A., Grassi, L., Le Pera, L., Moretti, M., Chinappi, M., D'Andrea, D., Mastronuzzi, A., Ianari, A., Vacca, A., De Smaele, E., Locatelli, F., Po, A., Miele, E., & Ferretti, E. (2018). Foxm1 controls a pro-stemness microRNA network in neural stem cells. *Scientific reports, 8*, 3523.

Borre, P. V., Schrock, A. B., Anderson, P. M., Morris III, J. C., Heilmann, A. M., Holmes, O., Wang, K., Johnson, A., Waguespack, S. G., & Ou, S. H. I. (2017). Pediatric, adolescent, and young adult thyroid carcinoma harbors frequent and diverse targetable genomic alterations, including kinase fusions. *The oncologist, 22*, 255.

Brierley, J. D., Gospodarowicz, M. K., & Wittekind, C. (2017). *TNM classification of malignant tumours*: John Wiley & Sons.

Bronkhorst, A. J., Ungerer, V., & Holdenrieder, S. (2019). Early detection of cancer using circulating tumor DNA: biological, physiological and analytical considerations. *Critical Reviews in Clinical Laboratory Sciences*, 1-17.

Brooks-Wilson, A. R., Smailus, D. E., & Goodfellow, P. J. (1992). A cluster of CpG islands at D10S94, near the locus responsible for multiple endocrine neoplasia type 2A (MEN2A). *Genomics, 13*, 339-343.

Castelo-Branco, P., Choufani, S., Mack, S., Gallagher, D., Zhang, C., Lipman, T., Zhukova, N., Walker, E. J., Martin, D., & Merino, D. (2013). Methylation of the TERT promoter and risk stratification of childhood brain tumours: an integrative genomic and molecular study. *The lancet oncology, 14*, 534-542.

Catanzaro, G., Besharat, Z. M., Miele, E., Chiacchiarini, M., Po, A., Carai, A., Marras, C. E., Antonelli, M., Badiali, M., Paso, A., Mascelli, S., Schrimpf, D., Stichel, D., Tartaglia, M., Capper, D., von Deimling, A., Giangaspero, F., Mastronuzzi, A., Locatelli, F., & Ferretti, E. (2018). The miR-139-5p regulates proliferation of supratentorial paediatric low-grade gliomas by targeting the PI3K/AKT/mTORC1 signalling. *Neuropathology and Applied Neurobiology*.

Catanzaro, G., Sabato, C., Russo, M., Rosa, A., Abballe, L., Besharat, Z. M., Po, A., Miele, E., Bellavia, D., Chiacchiarini, M., Gessi, M., Peruzzi, G., Napolitano, M., Antonelli, M., Mastronuzzi, A., Giangaspero, F., Locatelli, F., Screpanti, I., Vacca, A., & Ferretti, E. (2017). Loss of miR-107, miR-181c and miR-29a-3p Promote Activation of Notch2 Signaling in Pediatric High-Grade Gliomas (pHGGs). *International journal of molecular sciences, 18*, 2742.

Cavedon, E., Barollo, S., Bertazza, L., Pennelli, G., Galuppini, F., Watutantrige-Fernando, S., Censi, S., Iacobone, M., Benna, C., & Vianello, F. (2017). Prognostic impact of miR-224 and RAS mutations in medullary thyroid carcinoma. *International journal of endocrinology, 2017*.

Ceolin, L., Goularte, A. P. P., Ferreira, C. V., Romitti, M., & Maia, A. L. (2018). Global DNA methylation profile in medullary thyroid cancer patients. *Experimental and molecular pathology, 105*, 110-114.

Chan, E., Prado, D. E., & Weidhaas, J. B. (2011). Cancer microRNAs: from subtype profiling to predictors of response to therapy. *Trends in molecular medicine, 17*, 235-243.

Chang, Y.-S., Chang, C.-C., Huang, H.-Y., Lin, C.-Y., Yeh, K.-T., & Chang, J.-G. (2018). Detection of molecular alterations in taiwanese patients with medullary thyroid cancer using whole-exome sequencing. *Endocrine pathology, 29*, 324-331.

Chen, H., Luthra, R., Routbort, M. J., Patel, K. P., Cabanillas, M. E., Broaddus, R. R., & Williams, M. D. (2018). Molecular profile of advanced thyroid carcinomas by next-generation sequencing: characterizing tumors beyond diagnosis for targeted therapy. *Molecular cancer therapeutics, 17*, 1575-1584.

Chen, Y., Zhang, S., Zhao, R., Zhao, Q., & Zhang, T. (2017). Upregulated miR-9-3p promotes cell growth and inhibits apoptosis in medullary thyroid carcinoma by targeting BLCAP. *Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics, 25*, 1215-1222.

Chin, R.-I., Chen, K., Usmani, A., Chua, C., Harris, P. K., Binkley, M. S., Azad, T. D., Dudley, J. C., & Chaudhuri, A. A. (2019). Detection of solid tumor molecular residual disease (MRD) using circulating tumor DNA (ctDNA). *Molecular diagnosis & therapy*, 1-21.

Chinnam, M., & Goodrich, D. W. (2011). RB1, development, and cancer. In *Current topics in developmental biology* (Vol. 94, pp. 129-169): Elsevier.

Cho, U., Oh, W. J., Bae, J. S., Lee, S., Lee, Y. S., Park, G. S., Lee, Y. S., & Jung, C. K. (2014). Clinicopathological features of rare BRAF mutations in Korean thyroid cancer patients. *Journal of Korean medical science, 29*, 1054-1060.

Chu, Y.-H., Hardin, H., Schneider, D. F., Chen, H., & Lloyd, R. V. (2017). MicroRNA-21 and long non-coding RNA MALAT1 are overexpressed markers in medullary thyroid carcinoma. *Experimental and molecular pathology, 103*, 229-236.

Ciampi, R., Mian, C., Fugazzola, L., Cosci, B., Romei, C., Barollo, S., Cirello, V., Bottici, V., Marconcini, G., & Rosa, P. M. (2013). Evidence of a low prevalence of RAS mutations in a large medullary thyroid cancer series. *Thyroid, 23*, 50-57.

Ciampi, R., Romei, C., Ramone, T., Prete, A., Tacito, A., Cappagli, V., Bottici, V., Viola, D., Torregrossa, L., & Ugolini, C. (2019). Genetic Landscape of Somatic Mutations in a Large Cohort of Sporadic Medullary Thyroid Carcinomas Studied by Next-Generation Targeted Sequencing. *iScience, 20*, 324-338.

. ClinVar MTC RET. In.

Cote, G. J., Evers, C., Hu, M. I., Grubbs, E. G., Williams, M. D., Hai, T., Duose, D. Y., Houston, M. R., Bui, J. H., & Mehrotra, M. (2017). Prognostic significance of circulating RET M918T mutated tumor DNA in patients with advanced medullary thyroid carcinoma. *The Journal of Clinical Endocrinology & Metabolism, 102*, 3591-3599.

De Smaele, E., Ferretti, E., & Gulino, A. (2010). MicroRNAs as biomarkers for CNS cancer and other disorders. *Brain research, 1338*, 100-111.

Di Fiore, R., D'Anneo, A., Tesoriere, G., & Vento, R. (2013). RB1 in cancer: different mechanisms of RB1 inactivation and alterations of pRb pathway in tumorigenesis. *Journal of Cellular Physiology, 228*, 1676-1687.

Draht, M. X., Smits, K. M., Jooste, V., Tournier, B., Vervoort, M., Ramaekers, C., Chapusot, C., Weijenberg, M. P., van Engeland, M., & Melotte, V. (2016). Analysis of RET promoter CpG island methylation using methylation-specific PCR (MSP), pyrosequencing, and methylation-sensitive high-resolution melting (MS-HRM): impact on stage II colon cancer patient outcome. *Clinical Epigenetics, 8*, 44.

Duan, L., Hao, X., Liu, Z., Zhang, Y., & Zhang, G. (2014). MiR-129-5p is down-regulated and involved in the growth, apoptosis and migration of medullary thyroid carcinoma cells through targeting RET. *FEBS letters, 588*, 1644-1651.

Dvorakova, S., Vaclavikova, E., Sykorova, V., Vcelak, J., Novak, Z., Duskova, J., Ryska, A., Laco, J., Cap, J., & Kodetova, D. (2008). Somatic mutations in the RET proto-oncogene in sporadic medullary thyroid carcinomas. *Molecular and cellular endocrinology, 284*, 21-27.

Ehyaei, S., Hedayati, M., Zarif-Yeganeh, M., Sheikholeslami, S., Ahadi, M., & Amini, S. A. (2017). Plasma Calcitonin Levels and miRNA323 Expression in Medullary Thyroid Carcinoma Patients with or without RET Mutation. *Asian Pacific journal of cancer prevention: APJCP, 18*, 2179.

Elisei, R., Cosci, B., Romei, C., Bottici, V., Renzini, G., Molinaro, E., Agate, L., Vivaldi, A., Faviana, P., & Basolo, F. (2008). Prognostic significance of somatic RET oncogene mutations in sporadic medullary thyroid cancer: a 10-year follow-up study. *The Journal of Clinical Endocrinology & Metabolism, 93*, 682-687.

Eng, C., Clayton, D., Schuffenecker, I., Lenoir, G., Cote, G., Gagel, R. F., Van Amstel, H. K. P., Lips, C. J., Nishisho, I., & Takai, S.-I. (1996). The relationship between specific RET proto-oncogene mutations and disease phenotype in multiple endocrine neoplasia type 2: International RET Mutation Consortium analysis. *Jama, 276*, 1575-1579.

Esquela-Kerscher, A., & Slack, F. J. (2006). Oncomirs—microRNAs with a role in cancer. *Nature Reviews Cancer, 6*, 259-269.

Esteller, M. (2008). Epigenetics in cancer. *New England Journal of Medicine, 358*, 1148-1159.

Feinberg, A. P., & Tycko, B. (2004). The history of cancer epigenetics. *Nature Reviews Cancer, 4*, 143-153.

Filetti, S., Durante, C., Hartl, D., Leboulleux, S., Locati, L., Newbold, K., Papotti, M., & Berruti, A. (2019). Thyroid cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Annals of Oncology, 30*, 1856-1883.

Fu, X., Fang, J., Lian, M., Zhong, Q., Ma, H., Feng, L., Wang, R., & Wang, H. (2017). Identification of microRNAs associated with medullary thyroid carcinoma by bioinformatics analyses. *Molecular medicine reports, 15*, 4266-4272.

Gagel, R. F., Robinson, M. F., Donovan, D. T., & Alford, B. R. (1993). Clinical review 44: Medullary thyroid carcinoma: recent progress. *The Journal of Clinical Endocrinology & Metabolism, 76*, 809-814.

Galuppini, F., Bertazza, L., Barollo, S., Cavedon, E., Rugge, M., Guzzardo, V., Sacchi, D., Watutantrige-Fernando, S., Viamonte, F., & Mian, C. (2017). MiR-375 and YAP1 expression profiling in medullary thyroid carcinoma and their correlation with clinical–pathological features and outcome. *Virchows Archiv, 471*, 651-658.

Gasparri, M. L., Bardhi, E., Ruscito, I., Papadia, A., Farooqi, A. A., Marchetti, C., Bogani, G., Ceccacci, I., Mueller, M. D., & Panici, P. B. (2017). PI3K/AKT/mTOR Pathway in Ovarian Cancer Treatment: Are We on the Right Track? *Geburtshilfe und Frauenheilkunde, 77*, 1095.

Gasparri, M. L., Besharat, Z. M., Besharat, R. A., Ruscito, I., Nirgianakis, K., Farooqi, A. A., Papadia, A., Ferretti, E., Benedetti Panici, P., & Mueller, D. M. (2018). Current knowledge of miRNAs as biomarkers in Breast Cancer. In S. Fayyaz & A. A. Farooqi (Eds.), *Recent Trends in Cancer Biology: Spotlight on Signaling Cascades and Micrornas: Cell Signaling Pathways and Micrornas in Cancer Biology*: Springer.

Grubbs, E. G., Williams, M. D., Scheet, P., Vattathil, S., Perrier, N. D., Lee, J. E., Gagel, R. F., Hai, T., Feng, L., & Cabanillas, M. E. (2016). Role of CDKN2C copy number in sporadic medullary thyroid carcinoma. *Thyroid, 26*, 1553-1562.

Guilleret, I., Yan, P., Grange, F., Braunschweig, R., Bosman, F. T., & Benhattar, J. (2002). Hypermethylation of the human telomerase catalytic subunit (hTERT) gene correlates with telomerase activity. *International Journal of Cancer, 101*, 335-341.

Gundara, J. S., Zhao, J., Gill, A. J., Lee, J. C., Delbridge, L., Robinson, B. G., McLean, C., Serpell, J., & Sidhu, S. B. (2015). Noncoding RNA blockade of autophagy is therapeutic in medullary thyroid cancer. *Cancer medicine, 4*, 174-182.

Gundara, J. S., Zhao, J. T., Gill, A. J., Clifton-Bligh, R., Robinson, B. G., Delbridge, L., & Sidhu, S. B. (2014). Nodal metastasis microRNA expression correlates with the primary tumour in MTC. *ANZ journal of surgery, 84*, 235-239.

Guo, L.-C., Zhu, W.-D., Ma, X.-Y., Ni, H., Zhong, E.-J., Shao, Y. W., Yu, J., Gu, D.-M., Ji, S.-D., & Xu, H.-D. (2019). Mutations of genes including DNMT3A detected by next-generation sequencing in thyroid cancer. *Cancer biology & therapy, 20*, 240-246.

Hadoux, J., Pacini, F., Tuttle, R. M., & Schlumberger, M. (2016). Management of advanced medullary thyroid cancer. *The lancet Diabetes & endocrinology, 4*, 64-71.

Herfarth, K. K. F., Wick, M. R., Marshall, H. N., Gartner, E., Lum, S., & Moley, J. F. (1997). Absence of TP53 alterations in pheochromocytomas and medullary thyroid carcinomas. *Genes, Chromosomes and Cancer, 20*, 24-29.

Hofstra, R., Stelwagen, T., Stulp, R. P., de Jong, D., Hulsbeek, M., Kamsteeg, E. J., Van Den Berg, A., Landsvater, R., Vermey, A., & Molenaar, W. M. (1996). Extensive mutation scanning of RET in sporadic medullary thyroid carcinoma and of RET and VHL in sporadic pheochromocytoma reveals involvement of these genes in only a minority of cases. *The Journal of Clinical Endocrinology & Metabolism, 81*, 2881-2884.

Hofstra, R. M., Landsvater, R. M., Ceccherini, I., Stulp, R. P., Stelwagen, T., Luo, Y., Pasini, B., Hoppener, J. W., Van Amstel, H. K. P., & Romeo, G. (1994). A mutation in the RET proto-oncogene associated with multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma. *Nature, 367*, 375-376.

Huang, X., Yuan, T., Liang, M., Du, M., Xia, S., Dittmar, R., Wang, D., See, W., Costello, B. A., & Quevedo, F. (2015). Exosomal miR-1290 and miR-375 as prognostic markers in castration-resistant prostate cancer. *European urology, 67*, 33-41.

Hudson, J., Duncavage, E., Tamburrino, A., Salerno, P., Xi, L., Raffeld, M., Moley, J., & Chernock, R. D. (2013). Overexpression of miR-10a and miR-375 and downregulation of YAP1 in medullary thyroid carcinoma. *Experimental and molecular pathology, 95*, 62-67.

Ingenwerth, M., Goetz, M., Schmid, K. W., & Theurer, S. (2020). The mismatch repair system is not affected in medullary thyroid carcinoma independent of stromal desmoplasia or ret proto-oncogene mutation. *Annals of Diagnostic Pathology, 44*, 151445.

Iorio, M. V., & Croce, C. M. (2012). MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. *EMBO Molecular Medicine, 4*, 143-159.

Jiang, M., Shi, X., Zhu, H., Wei, W., & Li, J. (2019). Two GEO MicroRNA Expression Profile Based High-Throughput Screen to Identify MicroRNA-31-3p Regulating Growth of Medullary Thyroid Carcinoma Cell by Targeting RASA2. *Medical science monitor: international medical journal of experimental and clinical research, 25*, 5170.

Joo, L. J. S., Weiss, J., Gill, A. J., Clifton-Bligh, R., Brahmbhatt, H., MacDiarmid, J. A., Gild, M. L., Robinson, B. G., Zhao, J. T., & Sidhu, S. B. (2019). RET kinase-regulated microRNA-153-3p improves therapeutic efficacy in medullary thyroid carcinoma. *Thyroid, 29*, 830-844.

Karunamurthy, A., Panebianco, F., Hsiao, S. J., Vorhauer, J., Nikiforova, M. N., Chiosea, S., & Nikiforov, Y. E. (2016). Prevalence and phenotypic correlations of EIF1AX mutations in thyroid nodules. *Endocrine-related cancer, 23*, 295-301.

Kato, S., Subbiah, V., Marchlik, E., Elkin, S. K., Carter, J. L., & Kurzrock, R. (2017). RET aberrations in diverse cancers: next-generation sequencing of 4,871 patients. *Clinical Cancer Research, 23*, 1988-1997.

Ke, Z., Liu, Y., Zhang, Y., Li, J., Kuang, M., Peng, S., Liang, J., Yu, S., Su, L., & Chen, L. (2018). Diagnostic value and lymph node metastasis prediction of a custom-made panel (thyroline) in thyroid cancer. *Oncology reports, 40*, 659-668.

Kim, J., Gosnell, J. E., & Roman, S. A. (2019). Geographic influences in the global rise of thyroid cancer. *Nature Reviews Endocrinology*, 1-13.

Knudsen, E. S., & Wang, J. Y. (2010). Targeting the RB-pathway in cancer therapy. *Clinical Cancer Research, 16*, 1094-1099.

Komminoth, P., Kunz, E. K., Matias-Guiu, X., Hiort, O., Christiansen, G., Colomer, A., Roth, J., & Heitz, P. U. (1995). Analysis of RET protooncogene point mutations distinguishes heritable from nonheritable medullary thyroid carcinomas. *CANCER-PHILADELPHIA-, 76*, 479-479.

Kurzrock, R., Atkins, J., Wheler, J., Fu, S., Naing, A., Busaidy, N., Hong, D., & Sherman, S. (2013). Tumor marker and measurement fluctuations may not reflect treatment efficacy in patients with medullary thyroid carcinoma on long-term RET inhibitor therapy. *Annals of Oncology, 24*, 2256-2261.

Lairmore, T. C., Frisella, M. M., & Wells, S. A. (1996). Genetic testing and early thyroidectomy for inherited medullary thyroid carcinoma. *Annals of medicine, 28*, 401-406.

Landrum, M. J., Lee, J. M., Benson, M., Brown, G. R., Chao, C., Chitipiralla, S., Gu, B., Hart, J., Hoffman, D., & Jang, W. (2018). ClinVar: improving access to variant interpretations and supporting evidence. *Nucleic acids research, 46*, D1062-D1067.

Lassalle, S., Zangari, J., Popa, A., Ilie, M., Hofman, V., Long, E., Patey, M., Tissier, F., Belleannee, G., & Trouette, H. (2016). MicroRNA-375/SEC23A as biomarkers of the in vitro efficacy of vandetanib. *Oncotarget, 7*, 30461.

Lithwick-Yanai, G., Dromi, N., Shtabsky, A., Morgenstern, S., Strenov, Y., Feinmesser, M., Kravtsov, V., Leon, M. E., Hajdúch, M., & Ali, S. Z. (2017). Multicentre validation of a microRNA-based assay for diagnosing indeterminate thyroid nodules utilising fine needle aspirate smears. *Journal of clinical pathology, 70*, 500-507.

Liu, T., Wang, N., Cao, J., Sofiadis, A., Dinets, A., Zedenius, J., Larsson, C., & Xu, D. (2014). The age-and shorter telomere-dependent TERT promoter mutation in follicular thyroid cell-derived carcinomas. *Oncogene, 33*, 4978-4984.

Macià, A., Gallel, P., Vaquero, M., Gou-Fabregas, M., Santacana, M., Maliszewska, A., Robledo, M., Gardiner, J. R., Basson, M. A., & Matias Guiu, X. (2012). Sprouty1 is a candidate tumor-suppressor gene in medullary thyroid carcinoma. *Oncogene, 31*, 3961-3972.

Macías, M., Alegre, E., Díaz-Lagares, A., Patiño, A., Perez-Gracia, J. L., Sanmamed, M., López-López, R., Varo, N., & González, A. (2012). Liquid biopsy: from basic research to clinical practice. In *Advances in clinical chemistry* (Vol. 83, pp. 73-119): Elsevier.

Mancikova, V., Montero-Conde, C., Perales-Paton, J., Fernandez, A., Santacana, M., Jodkowska, K., Inglada-Pérez, L., Castelblanco, E., Borrego, S., & Encinas, M. (2017). Multilayer OMIC data in medullary thyroid carcinoma identifies the STAT3 pathway as a potential therapeutic target in RETM918T tumors. *Clinical Cancer Research, 23*, 1334-1345.

Mian, C., Pennelli, G., Barollo, S., Cavedon, E., Nacamulli, D., Vianello, F., Negro, I., Pozza, G., Boschin, I. M., & Pelizzo, M. R. (2011). Combined RET and Ki-67 assessment in sporadic medullary thyroid carcinoma: a useful tool for patient risk stratification. *European journal of endocrinology, 164*, 571.

Mian, C., Pennelli, G., Fassan, M., Balistreri, M., Barollo, S., Cavedon, E., Galuppini, F., Pizzi, M., Vianello, F., & Pelizzo, M. R. (2012). MicroRNA profiles in familial and sporadic medullary thyroid carcinoma: preliminary relationships with RET status and outcome. *Thyroid, 22*, 890-896.

Miele, E., Buttarelli, F., Arcella, A., Begalli, F., Garg, N., Silvano, M., Po, A., Baldi, C., Carissimo, G., Antonelli, M., & Spinelli, G. (2013). High-throughput microRNA profiling of pediatric high-grade gliomas. *Neuro-oncology*.

Mitchell, P. S., Parkin, R. K., Kroh, E. M., Fritz, B. R., Wyman, S. K., Pogosova-Agadjanyan, E. L., Peterson, A., Noteboom, J., O'Briant, K. C., & Allen, A. (2008). Circulating microRNAs as stable blood-based markers for cancer detection. *Proceedings of the National Academy of Sciences, 105*, 10513-10518.

Moarii, M., Boeva, V., Vert, J.-P., & Reyal, F. (2015). Changes in correlation between promoter methylation and gene expression in cancer. *Bmc Genomics, 16*, 873.

Mon, S. Y., & Hodak, S. P. (2014). Molecular diagnostics for thyroid nodules: the current state of affairs. *Endocrinology and Metabolism Clinics, 43*, 345-365.

Moura, M., Cavaco, B., Pinto, A., Domingues, R., Santos, J., Cid, M., Bugalho, M., & Leite, V. (2009). Correlation of RET somatic mutations with clinicopathological features in sporadic medullary thyroid carcinomas. *British journal of cancer, 100*, 1777-1783.

Moura, M. M., Cavaco, B. M., Pinto, A. E., & Leite, V. (2011). High prevalence of RAS mutations in RET-negative sporadic medullary thyroid carcinomas. *The Journal of Clinical Endocrinology & Metabolism*, *96*, E863-E868.

Mulligan, L. M., Eng, C., Healey, C. S., Clayton, D., Kwok, J. B., Gardner, E., Ponder, M. A., Frilling, A., Jackson, C. E., & Lehnert, H. (1994). Specific mutations of the RET proto-oncogene are related to disease phenotype in MEN 2A and FMTC. *Nature genetics*, *6*, 70-74.

Nikiforova, M. N., Mercurio, S., Wald, A. I., Barbi de Moura, M., Callenberg, K., Santana-Santos, L., Gooding, W. E., Yip, L., Ferris, R. L., & Nikiforov, Y. E. (2018). Analytical performance of the ThyroSeq v3 genomic classifier for cancer diagnosis in thyroid nodules. *Cancer*, *124*, 1682-1690.

Nikiforova, M. N., Wald, A. I., Roy, S., Durso, M. B., & Nikiforov, Y. E. (2013). Targeted next-generation sequencing panel (ThyroSeq) for detection of mutations in thyroid cancer. *The Journal of Clinical Endocrinology & Metabolism*, *98*, E1852-E1860.

Patocs, A., Klein, I., Szilvasi, A., Gergics, P., Toth, M., Valkusz, Z., Forizs, E., Igaz, P., Al-Farhat, Y., & Tordai, A. (2006). Genotype-phenotype correlations in Hungarian patients with hereditary medullary thyroid cancer. *Wiener Klinische Wochenschrift*, *118*, 417-421.

Pennelli, G., Galuppini, F., Barollo, S., Cavedon, E., Benuzzi, L., Fassan, M., Guzzardo, V., Pelizzo, M. R., Rugge, M., & Mian, C. (2015). The PDCD4/miR-21 pathway in medullary thyroid carcinoma. *Human pathology*, *46*, 50-57.

Pitt, S. C., Hernandez, R. A., Nehs, M. A., Gawande, A. A., Moore Jr, F. D., Ruan, D. T., & Cho, N. L. (2016). Identification of novel oncogenic mutations in thyroid cancer. *Journal of the American College of Surgeons*, *222*, 1036-1043. e1032.

Polivka Jr, J., Pesta, M., & Janku, F. (2015). Testing for oncogenic molecular aberrations in cell-free DNA-based liquid biopsies in the clinic: are we there yet? *Expert review of molecular diagnostics*, *15*, 1631-1644.

Priya, S., Dravid, C. S., Digumarti, R., & Dandekar, M. (2017). Targeted therapy for medullary thyroid cancer: a review. *Frontiers in oncology*, *7*, 238.

Qari, F. (2013). RET codon 618 mutations in Saudi families with multiple endocrine neoplasia Type 2A and familial medullary thyroid carcinoma. *Annals of Saudi medicine*, *33*, 155-158.

Qi, X.-P., Jin, B.-Y., Li, P.-F., Wang, S., Zhao, Y.-H., Cao, Z.-L., Yu, X.-H., Cheng, J., Fang, X.-D., & Zhao, J.-Q. (2015). RET S409Y Germline Mutation and Associated Medullary Thyroid Carcinoma. *Thyroid*, *29*, 1447-1456.

Roman, S., Lin, R., & Sosa, J. A. (2006). Prognosis of medullary thyroid carcinoma: demographic, clinical, and pathologic predictors of survival in 1252 cases. *Cancer: Interdisciplinary International Journal of the American Cancer Society*, *107*, 2134-2142.

Romei, C., Casella, F., Tacito, A., Bottici, V., Valerio, L., Viola, D., Cappagli, V., Matrone, A., Ciampi, R., & Piaggi, P. (2016). New insights in the molecular signature of advanced medullary thyroid cancer: evidence of a bad outcome of cases with double RET mutations. *Journal of medical genetics*, *53*, 729-734.

Romei, C., Elisei, R., Pinchera, A., Ceccherini, I., Molinaro, E., Mancusi, F., Martino, E., Romeo, G., & Pacini, F. (1996). Somatic mutations of the ret protooncogene in sporadic medullary thyroid carcinoma are not restricted to exon 16 and are associated with tumor recurrence. *The Journal of Clinical Endocrinology & Metabolism*, *81*, 1619-1622.

Romeo, P., Colombo, C., Granata, R., Calareso, G., Gualeni, A. V., Dugo, M., De Cecco, L., Rizzetti, M. G., Zanframundo, A., & Aiello, A. (2018). Circulating miR-375 as a novel prognostic marker for metastatic medullary thyroid cancer patients. *Endocrine-related cancer*, *25*, 217-231.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences*, *74*, 5463-5467.

Santarpia, L., Calin, G. A., Adam, L., Ye, L., Fusco, A., Giunti, S., Thaller, C., Paladini, L., Zhang, X., & Jimenez, C. (2013). A miRNA signature associated with human metastatic medullary thyroid carcinoma. *Endocr Relat Cancer, 20*, 809-823.

Schagdarsurengin, U., Gimm, O., Dralle, H., Hoang-Vu, C., & Dammann, R. (2006). CpG island methylation of tumor-related promoters occurs preferentially in undifferentiated carcinoma. *Thyroid, 16*, 633-642.

Schagdarsurengin, U., Gimm, O., Hoang-Vu, C., Dralle, H., Pfeifer, G. P., & Dammann, R. (2002). Frequent epigenetic silencing of the CpG island promoter of RASSF1A in thyroid carcinoma. *Cancer research, 62*, 3698-3701.

Schilling, T., Bürck, J., Sinn, H. P., Clemens, A., Otto, H. F., Höppner, W., Herfarth, C., Ziegler, R., Schwab, M., & Raue, F. (2001). Prognostic value of codon 918 (ATG→ ACG) RET proto-oncogene mutations in sporadic medullary thyroid carcinoma. *International Journal of Cancer, 95*, 62-66.

Schwarzenbach, H., Nishida, N., Calin, G. A., & Pantel, K. (2014). Clinical relevance of circulating cell-free microRNAs in cancer. *Nature Reviews Clinical Oncology, 11*, 145.

Shabani, N., Razaviyan, J., Paryan, M., Tavangar, S. M., Azizi, F., Mohammadi-Yeganeh, S., & Hedayati, M. (2018). Evaluation of miRNAs expression in medullary thyroid carcinoma tissue samples: miR-34a and miR-144 as promising overexpressed markers in MTC. *Human pathology, 79*, 212-221.

Shabani, N., Sheikholeslami, S., Paryan, M., Zarif Yeganeh, M., Tavangar, S. M., Azizi, F., Mohammadi-Yeganeh, S., & Hedayati, M. (2020). An investigation on the expression of miRNAs including miR-144 and miR-34a in plasma samples of RET-positive and RET-negative medullar thyroid carcinoma patients. *Journal of Cellular Physiology, 235*, 1366-1373.

Shakiba, E., Movahedi, M., Majd, A., & Hedayati, M. (2019). Investigating the expression and promoter methylation of RET gene in patients with medullary thyroid cancer with unmutated RET. *Journal of Cellular Physiology, 234*, 16304-16311.

Shan, N., Shen, L., Wang, J., He, D., & Duan, C. (2015). MiR-153 inhibits migration and invasion of human non-small-cell lung cancer by targeting ADAM19. *Biochemical and biophysical research communications, 456*, 385-391.

Shi, L., Zhao, S.-m., Luo, Y., Zhang, A.-w., Wei, L.-h., Xie, Z.-y., Li, Y.-y., & Ma, W. (2017). MiR-375: A prospective regulator in medullary thyroid cancer based on microarray data and bioinformatics analyses. *Pathology-Research and Practice, 213*, 1344-1354.

Simbolo, M., Mian, C., Barollo, S., Fassan, M., Mafficini, A., Neves, D., Scardoni, M., Pennelli, G., Rugge, M., & Pelizzo, M. R. (2014). High-throughput mutation profiling improves diagnostic stratification of sporadic medullary thyroid carcinomas. *Virchows Archiv, 465*, 73-78.

Skinner, H. D., Lee, J. H., Bhutani, M. S., Weston, B., Hofstetter, W., Komaki, R., Shiozaki, H., Wadhwa, R., Sudo, K., & Elimova, E. (2014). A validated miRNA profile predicts response to therapy in esophageal adenocarcinoma. *Cancer, 120*, 3635-3641.

Sozzi, G., Boeri, M., Rossi, M., Verri, C., Suatoni, P., Bravi, F., Roz, L., Conte, D., Grassi, M., & Sverzellati, N. (2014). Clinical utility of a plasma-based miRNA signature classifier within computed tomography lung cancer screening: a correlative MILD trial study. *Journal of Clinical Oncology, 32*, 768.

Spitschak, A., Meier, C., Kowtharapu, B., Engelmann, D., & Pützer, B. M. (2017). MiR-182 promotes cancer invasion by linking RET oncogene activated NF-κB to loss of the HES1/Notch1 regulatory circuit. *Molecular cancer, 16*, 24.

Tiedje, V., Ting, S., Walter, R. F., Herold, T., Worm, K., Badziong, J., Zwanziger, D., Schmid, K. W., & Fuhrer, D. (2016). Prognostic markers and response to vandetanib therapy in sporadic medullary thyroid cancer patients. *Eur J Endocrinol, 175*, 173-180.110.

Titov, S. E., Demenkov, P. S., Ivanov, M. K., Malakhina, E. S., Poloz, T. L., Tsivlikova, E. V., Ganzha, M. S., Shevchenko, S. P., Gulyaeva, L. F., & Kolesnikov, N. N. (2016). Selection and validation of miRNAs as normalizers for profiling expression of microRNAs isolated from thyroid fine needle aspiration smears. *Oncology reports, 36*, 2501-2510.

Titov, S. E., Ivanov, M. K., Demenkov, P. S., Katanyan, G. A., Kozorezova, E. S., Malek, A. V., Veryaskina, Y. A., & Zhimulev, I. F. (2019). Combined quantitation of HMGA2 mRNA, microRNAs, and mitochondrial-DNA content enables the identification and typing of thyroid tumors in fine-needle aspiration smears. *BMC cancer, 19*, 1010.

van Veelen, W., Klompmaker, R., Gloerich, M., van Gasteren, C. J., Kalkhoven, E., Berger, R., Lips, C. J., Medema, R. H., Höppener, J. W., & Acton, D. S. (2009). P18 is a tumor suppressor gene involved in human medullary thyroid carcinoma and pheochromocytoma development. *International Journal of Cancer, 124*, 339-345.

Vitale, G., Dicitore, A., Pepe, D., Gentilini, D., Grassi, E. S., Borghi, M. O., Gelmini, G., Cantone, M. C., Gaudenzi, G., & Misso, G. (2017). Synergistic activity of everolimus and 5-aza-2′-deoxycytidine in medullary thyroid carcinoma cell lines. *Molecular oncology, 11*, 1007-1022.

Wang, N., Kjellin, H., Sofiadis, A., Fotouhi, O., Juhlin, C. C., Bäckdahl, M., Zedenius, J., Xu, D., Lehtiö, J., & Larsson, C. (2016). Genetic and epigenetic background and protein expression profiles in relation to telomerase activation in medullary thyroid carcinoma. *Oncotarget, 7*, 21332.

Wang, Z., Hong, Z., Gao, F., & Feng, W. (2013). Upregulation of microRNA-375 is associated with poor prognosis in pediatric acute myeloid leukemia. *Molecular and cellular biochemistry, 383*, 59-65.

Wang, Z., & Liu, C. (2016). MiR-153 regulates metastases of gastric cancer through Snail. *Tumor Biology, 37*, 15509-15515.

Wasenius, V.-M., Hemmer, S., Karjalainen-Lindsberg, M.-L., Nupponen, N. N., Franssila, K., & Joensuu, H. (2005). MET receptor tyrosine kinase sequence alterations in differentiated thyroid carcinoma. *The American journal of surgical pathology, 29*, 544-549.

Wei, S., LiVolsi, V. A., Montone, K. T., Morrissette, J. J., & Baloch, Z. W. (2016). Detection of molecular alterations in medullary thyroid carcinoma using next-generation sequencing: an institutional experience. *Endocrine pathology, 27*, 359-362.

Wells Jr, S. A., Asa, S. L., Dralle, H., Elisei, R., Evans, D. B., Gagel, R. F., Lee, N., Machens, A., Moley, J. F., & Pacini, F. (2015). Revised American Thyroid Association guidelines for the management of medullary thyroid carcinoma: the American Thyroid Association Guidelines Task Force on medullary thyroid carcinoma. *Thyroid, 25*, 567-610.

Witte, T., Plass, C., & Gerhauser, C. (2014). Pan-cancer patterns of DNA methylation. *Genome medicine, 6*, 66.

Xia, W., Ma, X., Li, X., Dong, H., Yi, J., Zeng, W., & Yang, Z. (2015). miR-153 inhibits epithelial-to-mesenchymal transition in hepatocellular carcinoma by targeting Snail. *Oncology reports, 34*, 655-662.

Ye, X., & Chen, X. (2019). miR-149-5p inhibits cell proliferation and invasion through targeting GIT1 in medullary thyroid carcinoma. *Oncology letters, 17*, 372-378.

Yeganeh, M. Z., Sheikholeslami, S., Behbahani, G. D., Farashi, S., & Hedayati, M. (2015). Skewed mutational spectrum of RET proto-oncogene Exon10 in Iranian patients with medullary thyroid carcinoma. *Tumor Biology, 36*, 5225-5231.

Yuan, Y., Du, W., Wang, Y., Xu, C., Wang, J., Zhang, Y., Wang, H., Ju, J., Zhao, L., & Wang, Z. (2015). Suppression of AKT expression by mi R-153 produced anti-tumor activity in lung cancer. *International Journal of Cancer, 136*, 1333-1340.

Zane, M., Agostini, M., Enzo, M. V., Ide, E. C., Del Bianco, P., Torresan, F., Boschin, I. M., Pennelli, G., Saccani, A., & Rubello, D. (2013). Circulating cell-free DNA, SLC5A8 and

SLC26A4 hypermethylation, BRAFV600E: A non-invasive tool panel for early detection of thyroid cancer. *Biomedicine & Pharmacotherapy, 67*, 723-730.

Zawistowski, J. S., Nakamura, K., Parker, J. S., Granger, D. A., Golitz, B. T., & Johnson, G. L. (2013). MicroRNA 9-3p targets β1 integrin to sensitize claudin-low breast cancer cells to MEK inhibition. *Molecular and cellular biology, 33*, 2260-2274.

Zeng, J.-H., Liang, X.-Z., Lan, H.-H., Zhu, X., & Liang, X.-Y. (2018). The biological functions of target genes in pan-cancers and cell lines were predicted by miR-375 microarray data from GEO database and bioinformatics. *PLoS One, 13*.

Zhang, A., Wang, C., Lu, H., Chen, X., Ba, Y., Zhang, C., & Zhang, C.-Y. (2019). Altered Serum MicroRNA Profile May Serve as an Auxiliary Tool for Discriminating Aggressive Thyroid Carcinoma from Nonaggressive Thyroid Cancer and Benign Thyroid Nodules. *Disease markers, 2019*.

Zhang, L., Lu, D., Liu, M., Zhang, M., & Peng, Q. (2019). Identification and interaction analysis of key miRNAs in medullary thyroid carcinoma by bioinformatics analysis. *Molecular medicine reports, 20*, 2316-2324.

Zhang, X., Yan, Z., Zhang, J., Gong, L., Li, W., Cui, J., Liu, Y., Gao, Z., Li, J., & Shen, L. (2011). Combination of hsa-miR-375 and hsa-miR-142-5p as a predictor for recurrence risk in gastric cancer patients following surgical resection. *Annals of Oncology, 22*, 2257-2266.

Zhu, L., Lu, Z., & Zhao, H. (2015). Antitumor mechanisms when pRb and p53 are genetically inactivated. *Oncogene, 34*, 4547-4557.

Supplementary Section

We included a supplementary section on the different methods used for the extraction and evaluation of both microRNAs and DNA in order to provide an overview of the different processing steps and technologies that have been used.

DNA methylation in MTC cell models

The first study about methylation in MTC reported the characterization of a dense cluster of CpG islands at locus D10S84 that map to genes responsible for two dominantly inherited disorders, including MTC. *In vitro* data, using Lymphoblast cell line AC suggested that these CpG islands could represent the 5'ends of candidate genes for MTC development (Brooks-Wilson, Smailus, & Goodfellow, 1992).

Aberrant DNA methylation has been associated with the development of drug resistance through transcriptional suppression of genes implicated in drug metabolism, apoptosis, cell cycle control, and other biological processes. Combination analysis demonstrated that AZA (5-aza-20-deoxycytidine), a compound able to overcome resistance to chemotherapeutic and biological agents, combined with everolimus (mTOR inhibitor) was more effective in inhibiting cell proliferation than each agent alone in MTC cells. This cytotoxic activity was highly synergistic through a potent induction of apoptosis in MZ-CRC-1 cells, the most resistant cell line to everolimus alone. Gene expression analysis revealed potential molecular mechanisms implicated in the synergy of AZA and everolimus in MZ-CRC-1 cells: PI3K-Akt signaling, neurotrophin signaling pathway, extracellular matrix/receptor interaction, and focal adhesion. Interestingly, these pathways had a critical role in the regulation of both proliferation and migration/invasion of tumor cells. In this network, the "neurotrophin signaling pathway" appeared to exert a direct influence on the apoptotic machinery through the overexpression of NGFR (Nerve Growth Factor Receptor) and the activation of MAPK10-TP53-Bax (Mitogen-Activated Protein Kinase 10- tumor protein p53-BCL2 Associated X) pathway. A significant reduction in DNA methylation levels was observed after incubation with AZA alone, similar to the one observed after AZA plus everolimus compared to untreated control. A comparable trend was observed for NGFR and MAPK10, both methylated genes involved in the NGFR-MAPK10-TP53-Bax/Bcl-2 pathway. These results provided a new therapeutic scenario in MTC and probably other neuroendocrine tumors, where therapy with everolimus was approved (Vitale, et al., 2017).

Supplementary Methods

1. miRNA extraction, normalization and quantification

MiRNAs were extracted from tissue and fine needle aspiration (FNA) samples in 19 studies, plasma samples in 2 studies, serum samples in 1 study and cells in 5 studies.

1.2. miRNAs from tissue and FNA samples

MiRNAs were extracted from tissue samples using the following methods, miRNeasy FFPE Kit (Ehyaei, et al., 2017; Gundara, et al., 2014), miRNeasy mini Kit (Aubert, et al., 2018); MirVana miRNA Isolation kit (Duan, et al., 2014), RecoverAll Kit (Ambion) (Cavedon, et al., 2017; Hudson, et al., 2013; Pennelli, et al., 2015); miRCURY RNA isolation kit (Exiqon) (D. Abraham, et al., 2011) ; GeneAll Hybrid-R miRNa isolation kit (Biotechnology) (Shabani, et al., 2018) or TRizol reagent Lysis Buffer (Santarpia, et al., 2013). MiRNAs extraction from FNAs was performed by Guanidine Lysis Buffer or phenol (Lithwick-Yanai, et al., 2017; Titov, et al., 2016; Titov, et al., 2019).

1.3. miRNAs from liquid biopsy

MiRNAs were extracted from serum samples using Trizol Reagent (A. Zhang, et al., 2019) and from plasma using miRNeasy serum/plasma kit (Qiagen) (Romeo, et al., 2018; Shabani, et al., 2020).

1.4. miRNAs from cells

RNA extraction was performed with Trizol reagent (Lassalle, et al., 2016; Ye & Chen, 2019), NucleoSpi RNA Kit (Macherey-Nagel) (Spitschak, et al., 2017) or miRNeasy mini Total RNA Isolation Kit (Qiagen) (Gundara, et al., 2015).

1.5. miRNAs normalization and quantification

In the studies using qRT-PCR quantification, a single endogenous reference molecule (miR-16, RNU6-6P, RNAU6B (Aubert, et al., 2018; Galuppini, et al., 2017; Santarpia, et al., 2013), U6 (Cavedon, et al., 2017; Y. Chen, et al., 2017; Duan, et al., 2014; Ehyaei, et al., 2017; Jiang, et al., 2019), RNU48 (D. Abraham, et al., 2011; Gundara, et al., 2014), RN5-8S6, snord47 or 18SrRNA (Chu, et al., 2017) was used. In the qRT-PCR based studies for the analysis of multiple miRNAs using TaqMan Low Density Arrays (TLDA) the combination of let-7d/g/I was used as endogenous control for serum miRNAs (A. Zhang, et al., 2019).

In the study using NanoString the results were normalized using the 100 most abundant miRNAs, the "Top 100 method" one of the recommended methods by Nanostring (Titov, et al., 2016).

Studies with single assays RT-qPCR analysis (D. Abraham, et al., 2011; Aubert, et al., 2018; Cavedon, et al., 2017; Y. Chen, et al., 2017; Chu, et al., 2017; Duan, et al., 2014; Ehyaei, et al., 2017; Galuppini, et al., 2017; Gundara, et al., 2015; Gundara, et al., 2014; Hudson, et al., 2013; Jiang, et al., 2019; Lassalle, et al., 2016; Mancikova, et al., 2017; Mian, et al., 2012; Pennelli, et al., 2015; Santarpia, et al., 2013; Shabani, et al., 2018; Ye & Chen, 2019) or Microarray (D. Abraham, et al., 2011; Hudson, et al., 2013; Lassalle, et al., 2016; Romeo, et al., 2018; Spitschak, et al., 2017) and one study based on serum samples using TaqMan Low Density Arrays (TLDA) (A. Zhang, et al., 2019) reported miRNAs quantification relative to the respective control group.

Absolute miRNA quantification was reported in the study using NanoString (Titov, et al., 2016).

Expression of specific miRNAs was evaluated in a few studies by in situ Hybridation (ISH) on tissue section using miRCURY LNA detection probes (Exiqon) (Chu, et al., 2017; Mian, et al., 2012; Santarpia, et al., 2013).

2. DNA extraction and analysis

DNA mutations were evaluated in 43 studies, 3 of which concerned cfDNA. DNA methylation profile analysis was investigated in the other 9 studies (Table 7).

2.1. DNA from tissues and FNA

DNA was extracted from FFPE with QIAamp DNA FFPE Tissue Kit (Qiagen) (Cho, et al., 2014; Grubbs, et al., 2016; Guo, et al., 2019; Simbolo, et al., 2014), MagNA Pure instrument (Roche) (Nikiforova, et al., 2018), Maxwell® 16 FFPE Plus LEV DNA Purification kit (Ciampi, et al., 2019) or Qiamp DNA mini kit (Qiagen) (D. T. Abraham, et al., 2011).

DNA was isolated from FNA samples with PicoPure DNA Extraction Kit (ThermoFisher Scientific) (H. Chen, et al., 2018).

From frozen tissues, DNA was extracted using Maxwell® 16 FFPE Plus LEV Blood DNA Purification kit (Ciampi, et al., 2019), Qiamp DNA mini kit (Qiagen) (D. T. Abraham, et al., 2011), Qiagen DNeasy tissue KIT (Qiagen) (Ameur, et al., 2009), by phenol/chloroform extraction and ethanol precipitation (Elisei, et al., 2008; Herfarth, et al., 1997; Romei, et al., 2016) or DNeasy blood and tissue handbook (Qiagen) (Mian, et al., 2011).

2.2. DNA from peripheral blood leukocytes

DNA was isolated from peripheral blood leukocytes by MagNA Pure LC 2.0 with automation (Roche Applied Science) (Aydoğan, et al., 2016) using the QIAMP blood kit (QIAGEN) (Romei, et al., 2016; Schilling, et al., 2001) or using the standard salting out/proteinase K method, dissolved in TE buffer (Yeganeh, et al., 2015).

2.3. Cell-free DNA (cfDNA) from liquid biopsy samples

The range volume of plasma samples for cfDNA isolation was from 700ul to 4mL. The following methods were used: QIAsymphony DSP Circu-lating DNA Kit (Qiagen) with 4 ml of plasma (Allin, et al., 2018), QIAmp UltraSens Virus Kit (Qiagen) with 700 uL of plasma (Zane, et al., 2013) and QIAamp Circulating Nucleic Acid Kit (Qiagen) with 3 ml of plasma (Cote, et al., 2017). cfDNA was measured by quantitative real-time PCR (Zane, et al., 2013) or via droplet polymerase chain reaction (Allin, et al., 2018; Cote, et al., 2017).

2.4. DNA mutation analysis in tissue samples

DNA mutations were analyzed using polymerase chain reaction (PCR)-based single strand conformation polymorphism analysis (PCR-SSCP) (Komminoth, et al., 1995), PCR (Lairmore, et al., 1996) or/and restriction enzyme digestion, and direct sequencing of the PCR-amplified DNA for mutation not detectable with restriction enzyme digestion (Patocs, et al., 2006; Qari, 2013). Wasenius et al. investigated DNA sequence alterations using DHPLC (Denaturing High-Performance Liquid Chromatography) and direct sequencing of the PCR products (Wasenius, et al., 2005). In most studies, DNA alterations analyses were performed through direct sequencing (Ciampi, et al., 2019; Elisei, et al., 2008; Komminoth, et al., 1995; Lairmore, et al., 1996; M. Moura, et al., 2009; M. M. Moura, et al., 2011; Patocs, et al., 2006; Qari, 2013; Romei, et al., 2016).

Three studies performed Sanger sequencing (Aydoğan, et al., 2016; Cho, et al., 2014; Simbolo, et al., 2014).

2.5. DNA extraction for Next Generation Sequencing studies

Next generation sequencing studies (NGS) were performed from tissue (FFPE and frozen), and FNA samples. DNA extraction was performed from FFPE with QIAamp DNA FFPE Tissue Kit (Qiagen) (Guo, et al., 2019; Ke, et al., 2018; Simbolo, et al., 2014; Tiedje, et al., 2016; Wei, et al., 2016), with the QIAamp DNA Micro kit (QIAGEN) (Chang, et al., 2018), MagNA Pure instrument (Roche) (Nikiforova, et al., 2018) or Maxwell® 16 FFPE Plus LEV DNA Purification kit (Ciampi, et al., 2019). FFPE samples were used for DNA extraction and purification utilizing the PicoPure DNA Extraction Kit (ThermoFisher Scientific) and Agencourt AMPureXP Kit (H. Chen, et al., 2018). DNA was isolated from FNA samples with MagNA Pure instrument (Roche) (Nikiforova, et al., 2018) or with the DNeasy blood and tissue kit on the automated QIAcube (QIAGEN) (Nikiforova, et al., 2013). DNA was extracted from fresh frozen tissue using Maxwell® 16 FFPE Plus LEV Blood DNA Purification kit (Ciampi, et al., 2019) or QIAmp DNA Mini kit (Qiagen) (Nikiforova, et al., 2013).

Targeted NGS analysis was performed using panels of tumor-related genes (Borre, et al., 2017; Guo, et al., 2019; Ingenwerth, et al., 2020; Kato, et al., 2017; Pitt, et al., 2016), or thyroid/MTC cancer-related genes (Ciampi, et al., 2019; Tiedje, et al., 2016; Wei, et al., 2016). The gene panels were: AmpliSeq Hot Spot Cancer Panel v2 (Life Technologies) that explored selected regions of 50 cancer-associated genes (Simbolo, et al., 2014); ThyroSeq v3 that analyzed 112 genes for a variety of genetic alterations including single nucleotide variations (SNVs), insertions/deletions (indels) gene fusion (GF) types, abnormal gene expression alterations (GEAs) and copy number alterations (CNAs) (Nikiforova, et al., 2018); OncoMap-4 or OncoPanel high-throughput genotyping platforms that detected up to 275 cancer genes and 91 introns for DNA rearrangement (Pitt, et al., 2016); Thyroline was designed to detect 15 target gene mutations and 2 fusions type (fusion type) (Ke, et al., 2018). ThyroSeq (Nikiforova, et al., 2013) and ThyroSeq v2 panel designed for the analysis of point mutations in the hotspots of thyroid cancer-related genes and gene fusions (Karunamurthy, et al., 2016); point mutations and small indels in cancer related genes were examined using Ampliseq Cancer Hotspot v1 panel and v2 panel (H. Chen, et al., 2018).

2.6. DNA extraction for methylation profiling

DNA from frozen tissue was extracted using a phenol/chloroform procedure (Schagdarsurengin, et al., 2006; Schagdarsurengin, et al., 2002) or DNeasy Blood and Tissue Kit (Qiagen) (Mancikova, et al., 2017; N. Wang, et al., 2016). DNA from FFPE samples was extracted using DNeasy Blood and Tissue Kit (Qiagen) (Mancikova, et al., 2017; N. Wang, et al., 2016) or QIAamp DNA FFPE protocol (Qiagen) (Shakiba, et al., 2019). DNA from cell lines was extracted using the DNeasy Blood and Tissue Kit (Qiagen) (Mancikova, et al., 2017; Vitale, et al., 2017; N. Wang, et al., 2016).

DNA from MTC samples was initially modified with sodium bisulfite. For assessing DNA methylation status, methylation-specific PCR (MSP) was exploited (Macià, et al., 2012; Schagdarsurengin, et al., 2006; Schagdarsurengin, et al., 2002; Shakiba, et al., 2019). Genome-wide promoter DNA methylation was determined using the Illumina Infinium Human Methylation 27K Platform (Illumina) (Mancikova, et al., 2017), The Imprint Methylated DNA Quantification Kit

(MDQ1; Sigma Aldrich) (Ceolin, et al., 2018), Pyrosequencing (N. Wang, et al., 2016) or Infinium HumanMethylation 450K BeadChip (Illumina) (Vitale, et al., 2017).

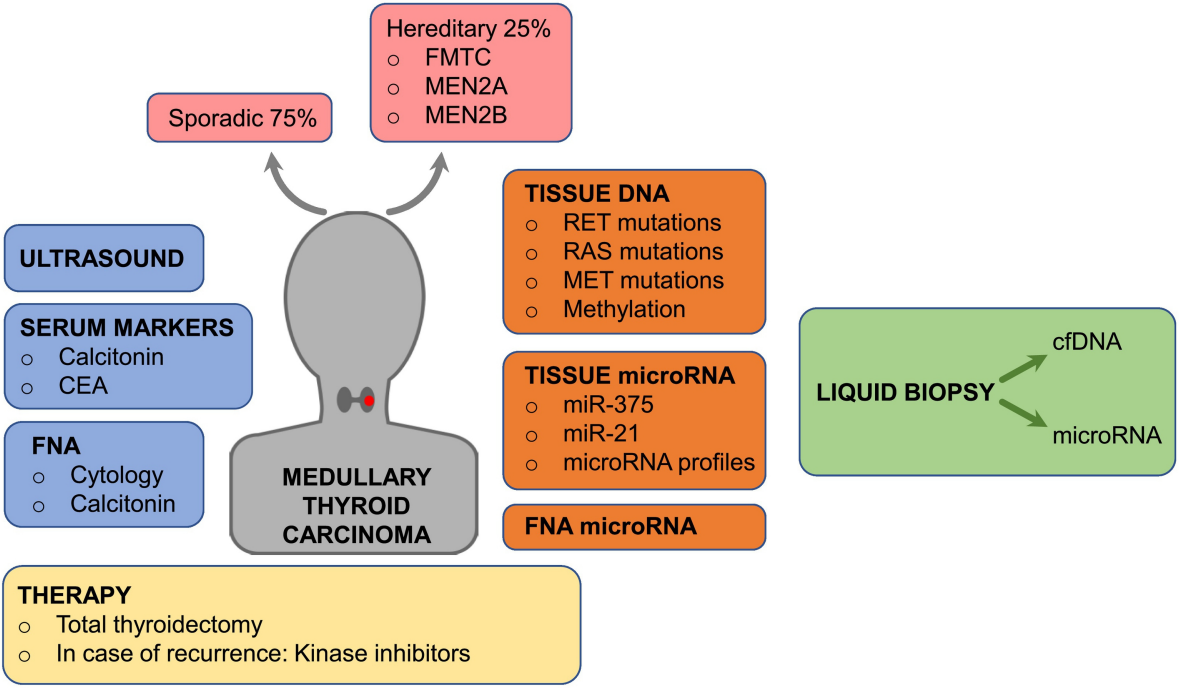
Hereditary 25%
FMTC
MEN2A
MEN2B
Sporadic 75%
ULTRASOUND
SERUM MARKERS
Calcitonin
CEA
FNA
Cytology
Calcitonin
MEDULLARY THYROID CARCINOMA
TISSUE DNA
RET mutations
RAS mutations
MET mutations
Methylation
TISSUE microRNA
miR-375
miR-21
microRNA profiles
FNA microRNA
LIQUID BIOPSY
cfDNA
microRNA
THERAPY
Total thyroidectomy
In case of recurrence: Kinase inhibitors

Figure 1

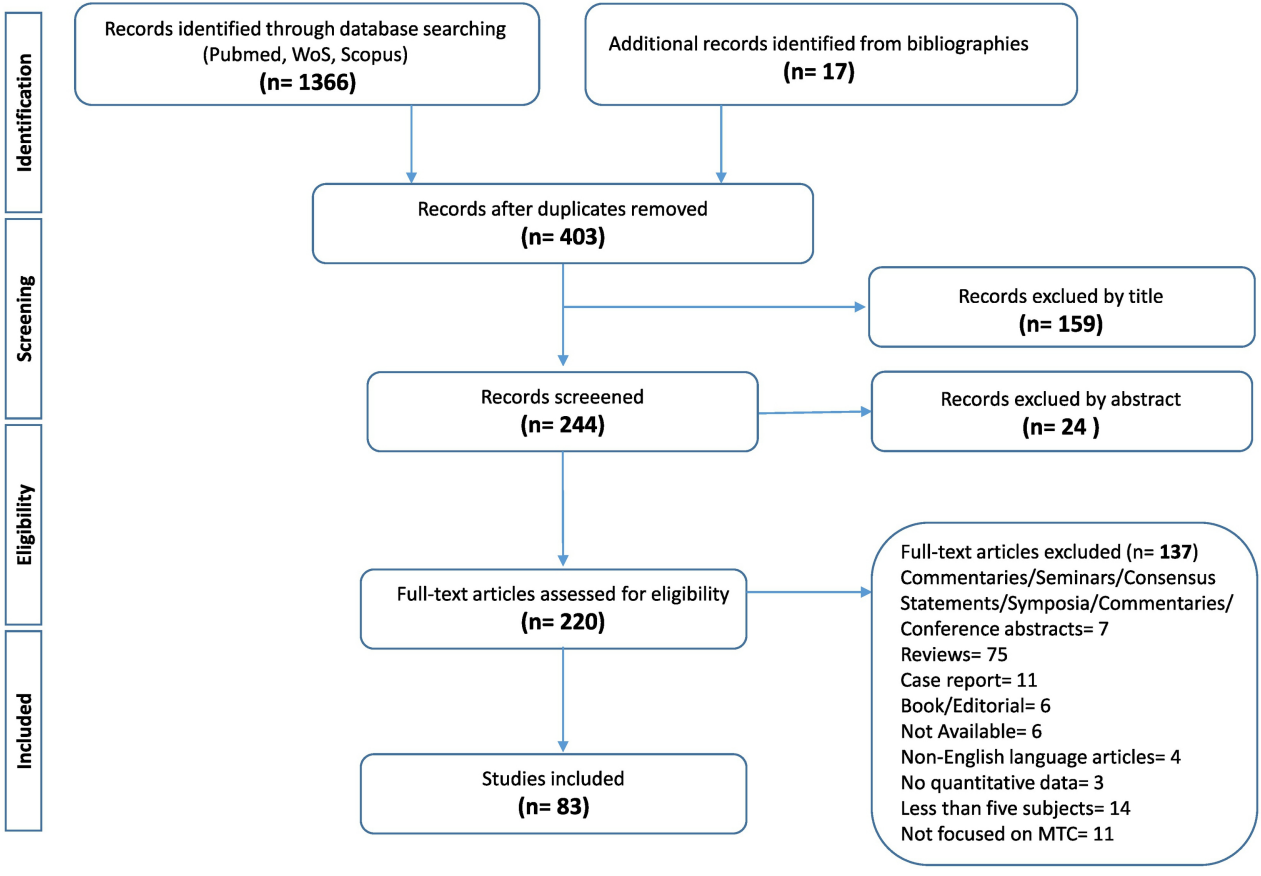
Identification
Screening
Eligibility
Included
Records identified through database searching (Pubmed, WoS, Scopus) (n= 1366)
Additional records identified from bibliographies (n= 17)
Records after duplicates removed (n= 403)
Records exclued by title (n= 159)
Records screeened (n= 244)
Records exclued by abstract (n= 24)
Full-text articles assessed for eligibility (n= 220)
Full-text articles excluded (n= 137)
Commentaries/Seminars/Consensus Statements/Symposia/Commentaries/ Conference abstracts= 7
Reviews= 75
Case report= 11
Book/Editorial= 6
Not Available= 6
Non-English language articles= 4
No quantitative data= 3
Less than five subjects= 14
Not focused on MTC= 11
Studies included (n= 83)

Figure 2