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Indice

Abstract

Aim: To identify clinical and molecular prognostic factors in differentiated thyroid cancer (DTC)

Methods: A web based longitudinal database of newly diagnosed DTC was settled down. The risk of recurrence and the response to treatment were classified according to the American Thyroid Association (ATA) guidelines. Circulating miR analysis of sera collected before surgery and about 1 months and 1-2 years after surgery was performed with TaqMan MicroRNA Assay.

Results: 2730 patients had a follow-up ≥1 year. The ATA risk of recurrence was low in 1386 (50.8%), intermediate in 1168 (42.8%) and high in 176 (6.4%). The response to treatment was excellent in 1675 (61.3%), biochemical incomplete in 63 (2.3%), structural incomplete in 70 (2.6%), and indeterminate in 922 (33.8%). A significantly higher rate of structural disease was found in intermediate (2.7%, Odds ratio 4.85, 95% confidence interval 2.18 - 12.23, $p<0.01$) and high risk (17.1% Odds ratio 35.21, 95% confidence interval 15.41 - 90.66, $p<0.01$) patients compared with low risk patients (0.6%). Of the 829 patients that had a follow up of ≥3 years, only 3 (0.6%) intermediate risk patients experienced relapse.

Serum samples of 44 patients with papillary thyroid cancer (PTC) were available for miR profiling. After a screening analysis, miR-146a-5p and miR-221-3p were selected for validation because of superior accuracy in PTC identification from healthy controls and benign thyroid nodules. The trend over time of miR-146a-5p and miR-221-3p was decreasing in patients with disease remission and increasing in patients with structural disease. In 3 cases miR profile was more informative than the serum thyroglobulin.

Conclusion: The ATA risk stratification is an effective clinical prognostic tool for structural disease prediction in DTC. One third of the patients has an indeterminate response to treatment due to low detectable serum markers (thyroglobulin or anti thyroglobulin antibodies). miR profile may represents a promising alternative marker of disease status for these patients.

INTRODUCTION

Thyroid cancer

Thyroid cancer is the most frequent endocrine malignancy. It represents the 3% of all new cancer diagnoses, according to the data collected form the Surveillance, Epidemiology and End Results (SEER) program (https://seer.cancer.gov/statfacts/html/thyro.html last accessed 09 December 2018). The 96% of all thyroid cancers originates from follicular cells, and of these, 99% are differentiated thyroid cancer (DTC) and 1% are anaplastic thyroid cancers. DTCs are classified as papillary (PTC, 95%), follicular (FTC), Hürthle cell carcinoma (HCC) and poorly differentiated thyroid cancers (PDTCs) (5%) (Fagin and Wells 2016). Medullary thyroid carcinoma is rare, representing <3% of all thyroid cancers and originates from parafollicular cells (also known as C cells).

DTCs incidence has increased over the past three decades by >5% per year to reach 14 cases per 100,000 persons per year (Davies and Welch 2014). Overdiagnosis plays a relevant role in this incidence increase but is not possible to exclude the role of environmental risk factors, such as radiation exposure and obesity (Vaccarella, Dal Maso et al. 2015) (Grani, Lamartina et al. 2018).

Thyroid cancers have a global mortality at 5 years of less than 2% (SEER). Patients with advanced disease with increased risk of disease specific death are the minority (Milano 2018). The incidence-based mortality of PTCs increased by 1.1% per year during 1994–2013 (from 0.40 per 100,000 person-years in 1994–1997 to 0.46 per 100,000 person-years in 2010–2013). Over the same time period, the incidence-based mortality of PTCs with distant metastases increased by 2.9% per year (Lim, Devesa et al. 2017).

The one-size-fits-all treatment paradigm for DTCs used to consist the near-universal use of total thyroidectomy and radioactive iodine remnant ablation followed by TSH- suppressive therapy with levothyroxine (Cabanillas, McFadden et al. 2016). High risk DTCs are likely to benefit from such an aggressive approach. Nevertheless, the vast majority of DTCs in contemporary series are at

low risk of recurrence for whom a less aggressive and more individualized approach is appropriate. Management options now include active surveillance for select very low-risk tumors (Ito, Miyauchi et al. 2010, Tuttle, Fagin et al. 2017) and less extensive surgical treatment (Adam, Pura et al. 2015) (e.g. total thyroidectomy with no prophylactic lymph node dissection or thyroid lobectomy alone) for low and selected intermediate-risk tumors. Selective use of radioiodine remnant ablation is also advised (Lamartina, Durante et al. 2015, Lamartina, Durante et al. 2017). Also, the aims and drawbacks of levothyroxine treatment (notably the risks of long-term TSH suppression, which is now restricted to patients with persistent disease) are being redefined (Biondi and Cooper 2010, Lamartina, Montesano et al. 2018).

This paradigm shift was driven by the increased awareness on DTC patient's outcome and the availability of accurate diagnostic tools, less influenced by the presence of some amounts of normal thyroid tissue.

Most DTC patients have no evidence of disease after initial treatment, and recurrence, that is localized in the neck in the vast majority of cases, is highly unlikely in these cases (<1% in patients who are at low risk) (Durante, Attard et al. 2010, Schlumberger, Leboulleux et al. 2018). In terms of healthcare resources, the cost of low risk DTC follow up is 6–7 times higher than the cost of patients with an intermediate risk and high risk of recurrence (Wang, Roman et al. 2015). The proportion of patients who have a low risk of recurrence is high (about 2/3 of the patients) in contemporary prospective series (Lamartina, Durante et al. 2017). Appropriate risk stratification tools and predictive biomarkers are therefore crucial for the optimization of thyroid cancer follow up.

Tools for risk stratification

Risk of death from thyroid cancer

The American Joint Committee on Cancer and Union Internationale Contre le Cancer (AJCC/UICC) tumor node metastasis (TNM) staging system is the most diffused system for tumor death risk estimate used in clinical practice (Tuttle,

Haugen et al. 2017). The 8th edition of AJCC/UICC TNM system (published in 2017), introduced several changes aimed to improve the identification of the small subset of patients that have a substantial risk of thyroid cancer death. AJCC/UICC TNM 7th and 8th edition are summarized in Table 1.

Unlikely other tumors, age is a crucial risk factor of thyroid cancer death, the risk increases in a continuous fashion with increasing age but fixed cut off have been introduced to render the scores simpler in everyday clinical practice (Nixon, Wang et al. 2016, Tuttle, Haugen et al. 2017). In thyroid cancer staging the patients below the age cutoff are classified only according to the absence (stage I) or the presence of distant metastases (stage II). The first important change made in the 8th edition AJCC/UICC TNM staging system, was increasing the age threshold from 45 to 55 years. As a consequence, a larger proportion of patients will be classified as stage I or II based only on the absence or the presence of distant metastases, respectively (Lamartina, Grani et al. 2017). Another change in the 8th edition of the staging system is that tumors are no longer classified as T3 solely because they are associated with microscopic extrathyroidal extension to the perithyroidal soft tissues; such tumors are now classified only according to tumor size. Tumors with lymph node metastases in the central and lateral compartments have also been downgraded to stage II. The adoption of the 8th edition of the tumor node metastasis system is expected to result in the down-staging of approximately one-third of patients with DTC: most will be classified as stage I or II, while only a minority (5-10%) will fall in stage III and IV (Lamartina, Grani et al. 2017). A better prediction of disease-related mortality with AJCC/UICC TNM 8th edition compared with 7th edition has been confirmed in several retrospective series (Ito, Miyauchi et al. 2018), (Kim, Kim et al. 2017, Pontius, Oyekunle et al. 2017). We must stress, however, that the risk of thyroid cancer recurrence for any tumor node metastasis stage can be low, intermediate or high (Lamartina, Grani et al. 2017). Therefore, the tumor node metastasis stage, which was designed exquisitely for mortality prediction, should always be considered together with the risk of recurrence.

Table 1. AJCC/UICC TNM 7th and 8th edition

AJCC/UICC TNM 7th and 8th edition							
7th Edition	**Age <45 years**			**8th Edition**	**Age <55 years**		
I	Any T	Any N	M0	I	Any T	Any N	M0
II	Any T	Any N	M1	II	Any T	Any N	M1
7th Edition	**Age ≥45 years**			**8th Edition**	**Age ≥55 years**		
I	T1	N0/Nx	M0	I	T1/T2	N0/Nx	M0
II	T2	N0/Nx	M0	II	T1/T2/T3a T3b	N1 Any N	M0 M0
III	T1/T2 T3	N1a N0/Nx/N1a	M0 M0	III	T4a	Any N	M0
IVa	T1/T2/T3 T4a	N1b Any N	M0 M0	IVa	T4b	Any N	M0
IVb	T4b	Any N	M0	IVb	Any T	Any N	M1
IVc	Any T	Any N	M1	--	--	--	--

Risk of thyroid cancer recurrence

As discussed before, the risk of thyroid cancer death is low. Nonetheless, thyroid cancer patients can experience disease persistence or recurrence. Persistent disease refers to a patient that is not cured by the initial treatment and is found with evidence of disease on imaging studies at the first disease assessment (usually performed at about 1 year from primary treatment). Recurrent disease refers to patients that were free from disease at the first disease assessment and that are found with evidence of disease later during follow up. The risk estimate of persistent and recurrent disease for DTCs vary widely between cohorts: the risk of persistent DTC ranges from 7% to 28% (depending on the study), and the risk of recurrent DTC in patients who respond well to treatment is 0.2−1.4% (Durante, Montesano et al. 2013, Schlumberger, Leboulleux et al. 2018) (Tuttle, Tala et al. 2010). This data are based mainly on retrospective analyses or small longitudinal studies. The American Thyroid Association (ATA) risk stratification system classifies the likelihood of persistent and/or recurrent disease as low (<5%), intermediate (5–20%) or high (>20%) based on features documented at the time of diagnosis (Haugen, Alexander et al. 2016) (Table 2).

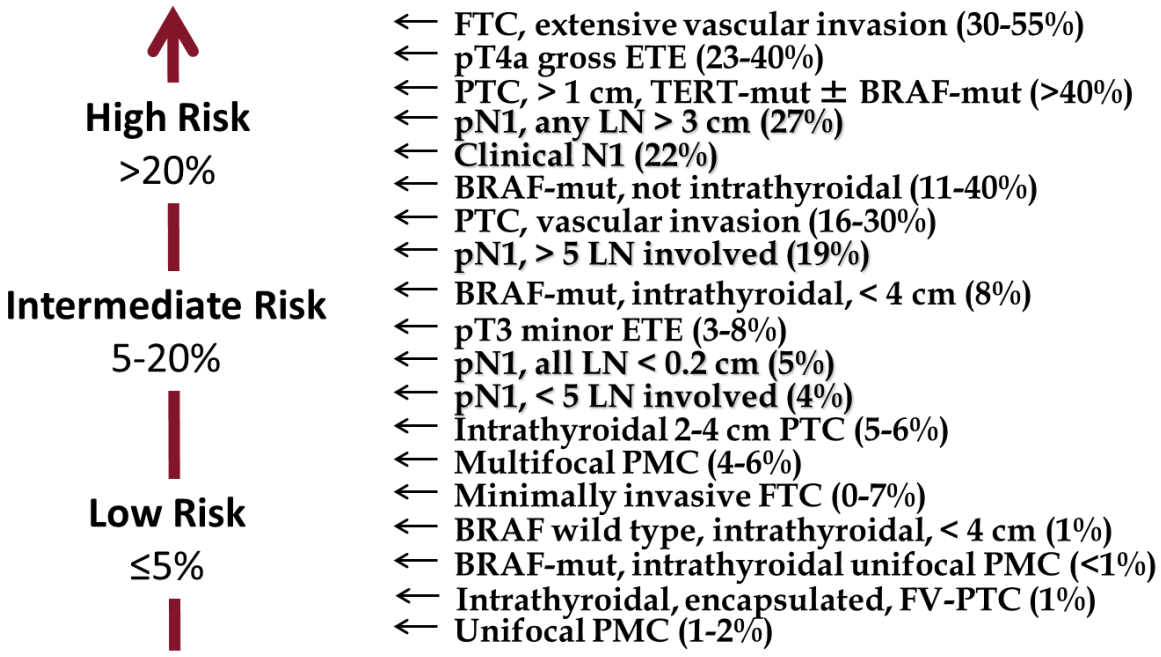
Table 2. American Thyroid Association risk of recurrence.

Risk of recurrence class	Definition	Risk estimate
Low	Papillary thyroid cancer (all the following) - Absence of distant metastases - Intrathyroidal tumor, < 4cm; absence of macroscopic extra thyroid extension - Absence of clinically evident lymph node metastases - Five or less microscopic (< 2 mm) lymph node metastases - Absence of aggressive histology - Absence of vascular invasion - Complete macroscopic tumor resection - No uptake outside thyroid bed on post-therapeutic whole-body scan if radioiodine ablation is performed Follicular thyroid cancer (all the following) - Well differentiated - Capsular invasion only or less than 4 foci of vascular invasion	≤5%
Intermediate	Any of the following: Microscopic invasion of tumor into perithyroidal soft tissue Aggressive histology Papillary thyroid cancer with vascular invasion Presence of clinically evident lymph node metastases (<3 cm) or more than 5 microscopic (<2mm) lymph node metastases Multifocal papillary thyroid carcinoma with BRAFV600E mutation Uptake in the neck, outside thyroid bed, on post-treatment whole body scan	>5%-20%
High	Any of the following: Macroscopic invasion of perithyroidal tissue/structures Lymph node metastases of 3 cm or more or with extranodal extension Follicular thyroid cancer with extensive vascular invasion Incomplete tumor resection Distant metastases Serum thyroglobulin suggestive of distant metastases	>20%

Small (diameter of ≤10 mm), unifocal intrathyroidal PTCs with no aggressive histological variant have a very low risk of recurrence (<1%) (Durante, Attard et al. 2010, Haugen, Alexander et al. 2016). The presence of lymph node metastases and of minimal extrathyroidal extension (to perithyroidal soft tissue)

was considered a risk factor for recurrence, but this is now a subject of debate (Nixon, Ganly et al. 2011). The size of the metastatic lymph nodes (≤2 mm, >2 mm or ≥3 cm), their number (<5 or >5), the number of lymph node metastases with extracapsular invasion (Leboulleux, Rubino et al. 2005) and (probably) their location (central versus lateral compartments) should be considered in recurrence estimates (Randolph, Duh et al. 2012, Barbosa, Momesso et al. 2017). Several reports stated little influence of minimal extrathyroidal extension on disease recurrence when no other risk feature (such as lymph node metastasis) is present (Nixon, Ganly et al. 2011, Castagna, Forleo et al. 2018). It is though evident that in each risk class, there is a spectrum of characteristics associated to lower or higher risk: this has been proposed as the continuum of risk (Figure 1) by the 2015 ATA guidelines.

Figure 1. The continuum of risk (*Adapted from Haugen, Alexander et al 2016)*



Treatment options for differentiated thyroid cancer

Active surveillance

The mainstay of thyroid cancer treatment is surgery (Lamartina, Grani et al. 2018). As discussed before, an active surveillance approach is one of the options that can be taken into account for selected low risk patients (subcentimetric tumors, no evidence of extrathyroidal extension). Pioneering Japanese studies have demonstrated the safety and efficacy of this approach (Ito, Miyauchi et al. 2010, Oda, Miyauchi et al. 2016): after 10 years of sonographic surveillance only the 8% of the patients experienced tumor growth and the 4% had lymph node metastases development. Younger age (below 40 years) was found an independent risk factor for disease progression (either primary tumor growth or lymph node metastases development)(Ito, Miyauchi et al. 2014). Patients that had tumor progression were submitted to surgery with favorable outcomes (Ito, Miyauchi et al. 2014).

A US study included, in an active surveillance protocol, tumors up to 15 mm. Of the 291 patients the 2.5% experienced tumor growth after a median of two years and the 12% after 5 years (Tuttle, Fagin et al. 2017). This study confirmed younger age (under 50 years) as an independent predictor of tumor progression (Tuttle, Fagin et al. 2017). The percentage of tumors that displayed growth is slightly higher in the US study compared with the Japanese study: this may be due to differences in the populations studied but it may be explained also by the different method used to assess growth. Indeed, in the US study a more accurate method (i.e. tumor volume increase) was used to assess tumor growth. Other observational clinical trials to evaluate the "active surveillance" approach in PTCs of ≤10 mm are ongoing in Korea and Israel (NCT02952612, NCT02938702, and NCT02609685).

Surgery

Traditionally, total thyroidectomy was considered the standard of care for thyroid tumors. A retrospective analysis of 52,173 cases (data from the SEER

database), thyroid lobectomy for tumors with a maximum diameter of 1 cm or greater was associated with small but statistically significant increases in the risks for recurrence (9.8% versus 7.7%) and mortality (2.9% versus 1.6%) compared with total thyroidectomy (Bilimoria, Bentrem et al. 2007). A recent retrospective analysis of SEER data found no such difference in terms of overall survival after more extensive risk stratification (Adam, Pura et al. 2014). Nonetheless, another meta-analysis shows that the risk of recurrence after lobectomy was significantly higher than that after total thyroidectomy (8.3% versus 4.4%; $p<0.01$)(Macedo and Mittal 2015). Tumor recurrence in the contralateral lobe can occur in up to the 5% of patients treated with lobectomy (Matsuzu, Sugino et al. 2014) and that benign nodule relapse is experienced by 20–50% of the patients (Antunes and Taveira-Gomes 2013, Lytrivi, Kyrilli et al. 2016, Zatelli, Lamartina et al. 2018). Lobectomy offers several advantages over total thyroidectomy: surgical complications are reduced. Lobectomy eliminates the risk of permanent hypoparathyroidism and bilateral RLN palsy and reduces the rates of permanent unilateral RLN palsy (0.6% in lobectomy versus 1.3% in total thyroidectomy) (Rosato, Avenia et al. 2004). Moreover, a half to up to the 80% of the patients will not develop hypothyroidism and will not need levothyroxine replacement therapy. The rate of hypothyroidism after lobectomy varies from 23.6 to 47% (Balentine, Domingo et al. 2013, Lee, Seok et al. 2015).

Therefore, thyroid lobectomy is recommended by the ATA guidelines (Haugen, Alexander et al. 2016), for low-risk, intrathyroidal tumors up to 4 cm in size with no lesions in the contralateral lobe or other benign thyroidal conditions (e.g. contralateral benign nodules, diffuse autoimmune thyroid disease).

Patients with gross tumors (> 4cm), multifocal and bilateral tumors or in the presence of extrathyroidal extension or metastases total thyroidectomy is recommended (Haugen, Alexander et al. 2016). Lymph node surgery should always be performed in case of lymph node metastases found before or during surgery and should be a compartment-oriented neck dissection (Haugen, Alexander et al. 2016).

Prophylactic central neck dissection was advocated by some authors because of the high frequency of subclinical lymph node metastasis in DTC (Wada, Duh et al. 2003). It is worth to underline that microscopic lymph node metastases that are not clinically evident before surgery (not identified with neck ultrasound) have little influence in patient outcome (Randolph, Duh et al. 2012), but on the other hand, central compartment neck dissection carries an increased rate of surgical complications (namely hypoparathyroidism) (Lee, Oh et al. 2015, Viola, Materazzi et al. 2015).

A randomized prospective study compared the outcomes of the patients undergoing total thyroidectomy alone or total thyroidectomy and central compartment neck dissection. After five years of follow up no difference in the outcomes was found (Viola, Materazzi et al. 2015).

Radioiodine treatment

Routine use of radioiodine ablation (RRA) therapy used to be justified by the need to eliminate normal residual thyroid tissue in order to facilitate serum thyroglobulin (Tg) and 131I whole-body scan (WBS) interpretation. In addition, radioiodine treatment was performed with an adjuvant purpose (i.e. destroy any occult nests of neoplastic cells), thereby improving long-term outcomes. In recent years, due to the availability of accurate diagnostic tool and a better definition of the actual risk of DTC patients, these indications have been questioned (Lamartina, Durante et al. 2015). The preferred approach now days is to select patients for RRA based on the risk of recurrence estimate and eventually on the response to treatment (see below) (Lamartina, Grani et al. 2018). Only high risk patients have shown a benefit in terms of improved survival with radioiodine treatment (Jonklaas, Sarlis et al. 2006). For low and intermediate risk patients, that have a marginal thyroid cancer death risk as discussed before, no such benefit has been demonstrated. For low risk patients several reports have demonstrated no benefits in terms of reduced recurrence rates (Lamartina, Durante et al. 2015) and RRA is not recommended in these patients (Haugen, Alexander et al. 2016). In intermediate-risk patients, evidence

is more controversial, and the decision to perform RRA should be based on individual prognostic factors (Lamartina, Durante et al. 2015). An early disease assessment with neck ultrasound and serum Tg level may assist RRA decision. A low or undetectable serum Tg level and the absence of suspicious findings on neck ultrasound may support the decision to avoid RAI administration (Lamartina, Durante et al. 2015).

Two randomized European trials (HiLo and ESTIMABL) have demonstrated the non-inferiority of low (30 mCI) versus high (100 mCi) activities of radioiodine and preparation with either recombinant human TSH (rhTSH) or thyroid hormone withdrawal for remnant ablation (Mallick, Harmer et al. 2012, Schlumberger, Catargi et al. 2012). Therefore, low activities and rhTSH preparation should be preferred for RRA. Higher activities and thyroid hormone withdrawal are recommended when radioiodine is administered for a therapeutic purpose (i.e. treatment of iodine avid metastases).

Two randomized clinical trials in Europe are ongoing and aim to obtain reliable data on the indications for post-operative RAI administration. In the French ESTIMABL2 trial (NCT01837745), 750 patients with a T1bN0,Nx tumor will be randomly assigned to post-operative ablation with an activity of 30 mCi after rhTSH stimulation or simple follow-up. The IoN trial in the UK (NCT01398085) has a similar design. The primary outcomes in the two studies are disease-free survival rates evaluated at 3 and 5 years.

Response to treatment and dynamic risk assessment

The first disease assessment is usually performed 6–18 months after the initial treatment, and it should be based on serum thyroglobulin and anti-thyroglobulin antibody assays and imaging. The response to treatment is classified into four categories (Table 3). Two classes, excellent response (ER) and structural incomplete response (SIR) define the absence or the presence of disease respectively. The other two categories, indeterminate response (Ind) and biochemical incomplete response (BIR), are used when the patient cannot be

classified as free from disease for the presence of detectable serum markers of DTC (thyroglobulin or anti thyroglobulin antibodies) or in the presence non-specific imaging findings. BIR is usually associated to a higher risk of persistent/recurrent disease compared with Ind, but results in spontaneous remission in most of the cases (Vaisman, Momesso et al. 2012), (Lamartina, Montesano et al. 2016).

Table 3. Response to treatment

Response	Definition (2015 ATA guidelines)	Risk of recurrence
Excellent response	No abnormal finding on imaging AND basal Tg <0.2 ng/mL OR stimulated Tg <1 ng/mL AND No TgAb	<1-4%
Biochemical Incomplete Response	No abnormal finding on imaging AND basal Tg ≥1 ng/mL OR stimulated Tg ≥10 ng/mL OR Rising TgAb over time	20%
Indeterminate Response	Nonspecific findings on imaging AND / OR basal Tg ≥0.2 - <1 ng/mL OR stimulated Tg ≥1 - <10 ng/mL OR Stable or declining TgAb over time	15-20%
Structural Incomplete Response	Abnormal findings on imaging	100%

The initial risk estimate can be substantially refined by the response to treatment assessed during follow up (Tuttle, Tala et al. 2010) (Table 3). An ER in a low risk patient renders the risk of recurrence almost zero (Durante, Attard et al. 2010, Schlumberger, Leboulleux et al. 2018), and reduces the risk of intermediate risk patients to 1–2% (Vaisman, Momesso et al. 2012). Data on high risk patients are controversial: some reports found an important reduction of the recurrence rate in long term follow up equivalent to that observed for low and intermediate risk patients (Verburg, Stokkel et al. 2010). Other reports however found a non-negligible residual risk of recurrence in high risk patients with ER to treatment (up to 14%) (Tuttle, Tala et al. 2010). This risk increases even further (25%) following treatment of a recurrence (Lamartina, Borget et al. 2017).

Role of the histological type

Tumor histotype plays an important role in the risks of recurrence and DTC-related mortality. The world health organization (WHO) endocrine tumor classification system published in 2017 recognizes four histological classes of DTC:

- Papillary thyroid cancer,
- Follicular thyroid cancer,
- Hürthle cell thyroid cancer
- Poorly differentiated thyroid cancer (WHO 2017).

PTCs is the most common type of DTC (95% of the cases). Its metastatization pattern follows the lymphatic vessels. Neck lymph node metastases are frequent and involve the neck compartment in an ordered fashion (the central compartment first and then lateral compartments). Distant metastases from PTC are rare and seldom skip neck lymph nodes. Some histological variants (e.g. tall cell, columnar cell, hobnail cell, sclerosing and trabecular PTCs) are more aggressive than their classic counterparts. A new variant called non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) (Nikiforov, Seethala et al. 2016) was introduced with the 2017 WHO classification (WHO 2017). NIFTPs has indolent behavior with extremely low rates of local metastases (3–5%) and anecdotical reports of distant metastases (0.6–1%) (Cho, Mete et al. 2017, Parente, Kluijfhout et al. 2017). Given the recent introduction in DTC classification, data on NIFTPs are limited and, further evidence is needed to define their optimal management.

Compared with PTC, FTC — particularly the widely invasive form — carries a more severe prognosis (Grani, Lamartina et al. 2017). Metastatization occurs following the blood vessels, therefore spread to distant sites (mainly the lungs and the bones) occurs in up to 46% of FTC patients. On the other hand, metastases to neck lymph nodes are rare, occurring in only 2–8% of cases (Grani, Lamartina et al. 2017). The WHO recognizes 3 FTCs categories according to the presence and extent of vascular invasion (WHO 2017). Minimally invasive FTCs (limited neoplastic invasion of tumor capsule and no vascular invasion) have

generally a good prognosis (recurrence ≈0–5% and mortality <5%) and are considered low-risk tumors. FTCs with limited vascular invasion might be considered intermediate risk (5% recurrence and 3% mortality) (Ito, Hirokawa et al. 2013). Widely invasive FTCs are high risk tumors (recurrence ≈30–55% and disease-specific mortality ≈30%) (Haugen, Alexander et al. 2016).

HCC, traditionally considered as a subtype of FTC, is now recognized by the WHO as a separate entity (WHO 2017). It spreads frequently to distant sites as widely invasive FTC but also to neck lymph nodes in up to the 30% of the patients and should be considered a high-risk DTC. HCC molecular profile is different from that of other DTCs (Ganly, Ricarte Filho et al. 2013).

PDTC is another high risk thyroid cancer. The diagnosis is based on precise histopathological criteria: the Turin criteria (Volante, Landolfi et al. 2004). These tumors have a poor prognosis and the 10-year survival is only 46% (Haugen, Alexander et al. 2016).

Molecular profile

Molecular profiling of the tumor provides prognostic and therapeutic information and helps to identify high-risk tumors. The Cancer Genome Atlas initiative has identified the 97% of the driver mutations of PTCs (Cancer Genome Atlas Research Network 2014). In most cases these driver mutations are mutually exclusive (Cancer Genome Atlas Research Network 2014). Two subgroups can be identified based on the genetic phenotype of PTCs: RAS-like tumors and BRAF-like tumors. BRAF-like tumors are less differentiated and has a more aggressive behavior (Elisei, Viola et al. 2012, Xing, Alzahrani et al. 2013, Tallini, de Biase et al. 2015, Xing, Alzahrani et al. 2015, Huang, Qu et al. 2017). BRAFV600E is the most common driver mutation, present in 40–60% of PTCs, namely in classical PTCs and the tall cell PTCs (Cancer Genome Atlas Research Network 2014). This mutation is associated with several pathological features (such as multifocality, extrathyroidal extension and lymph node metastases) and, in some series, with an increased risk of persistent or recurrent disease, and

in older patients (>45 years), this mutation has been associated with an increased risk of mortality (Shen, Zhu et al. 2018). Given the heterogeneity of the phenotype associated with the presence of the BRAFV600E mutation, confirmation of its presence should be considered together with the other risk features, and this evaluation is not routinely recommended for risk stratification. Profiling microRNAs seems a promising tool for identifying high-risk BRAF-like PTC (Rosignolo, Memeo et al. 2017). The BRAFV600E mutation can impair iodine metabolism (Durante, Puxeddu et al. 2007, Rosignolo, Maggisano et al. 2015), possibly reducing the effectiveness of radioiodine. RAS mutations occur in 10–30% of PTCs, notably in the follicular variant and in NIFTP (Nikiforov, Seethala et al. 2016) (Cancer Genome Atlas Research Network 2014).

Gene fusions were found in 15% of PTCs, with the RET–PTC translocation being the most frequent (6%) (Cancer Genome Atlas Research Network 2014). Several other gene fusions have been identified (NRTK1, NRTK3, BRAF, ALK, FGFR2, THADA, MET, LTK and ROS) (Cancer Genome Atlas Research Network 2014). Gene fusions are more common in children than in adults and occur frequently in radiation-induced PTCs. The coexistence of BRAFV600E and TERT promoter mutations has been associated with poor clinical outcomes in terms of both persistence and recurrence of the disease as well as survival (Xing, Liu et al. 2014). The majority of these oncogenic gene fusions can be targeted with specific drugs. In FTC, RAS mutations are found in 40% of tumors, the most common being the codon 61 N-RAS mutation (Fukahori, Yoshida et al. 2012, Jang, Song et al. 2014). RAS mutations are also found in 20% of follicular adenomas. PAX8 and PPARG are observed in both follicular adenomas (14%) and FTCs (10–60%) (Lacroix, Lazar et al. 2005). PTEN somatic mutations are observed in 10% of FTCs. Cowden syndrome (an inheritable autosomal dominant disorder) is driven by a PTEN germline mutation and is associated with an increased risk of FTC. Other genetic abnormalities found in FTCs are PI3KCA-activating mutations (10%)(Liu, Hou et al. 2008). Finally, TERT promoter mutations have been observed in FTC and, as is the case with the mutations found in PTC, were associated with a more aggressive phenotype (Liu, Wang et al. 2014). In HCC, two molecular pathways (PI3K–AKT– mTOR and WNT–CTNNB1) are typically

activated (Ganly, Ricarte Filho et al. 2013). In PDTC, several mutations were found (BRAF, RAS, EIF1AX, TP53 and TERT promoter), the most frequent being the RAS mutation (Volante, Rapa et al. 2009). The accumulation of genetic abnormalities in a given tumor might represent the pathogenetic mechanism of transition from well-differentiated tumor towards PDTC (Landa, Ibrahimpasic et al. 2016, Ibrahimpasic, Xu et al. 2017).

Tools used for follow-up

Serum thyroglobulin

The serum level of thyroglobulin is a highly sensitive marker for the presence of thyroid tissue (anywhere in the body). Thyroglobulin is produced by both normal and neoplastic thyrocytes. Thyroglobulin should always be measured using the same method (due to lack of standardization between assays) and can be assessed with assays with a functional sensitivity ≈1 ng/ml or with assays with a functional sensitivity ≈0.1–0.2 ng/ml (the so-called ultrasensitive assays). The sensitivity of serum thyroglobulin can be improved with TSH increase that can be obtained with levothyroxine withdrawal (that renders the patient hypothyroid) or with injections of rhTSH (Pacini, Molinaro et al. 2001). The negative predictive value of undetectable TSH-stimulated thyroglobulin level is close to 100%. Therefore, if the initial assay reveals undetectable levels, further monitoring of stimulated thyroglobulin offers no benefit (Castagna, Brilli et al. 2008). Ultrasensitive thyroglobulin assays seem to remove the need for routine TSH-stimulated thyroglobulin determinations (Kloos and Mazzaferri 2005, Castagna, Brilli et al. 2008). TSH stimulation produces a fivefold to tenfold increase over basal values in serum levels of thyroglobulin, and, using a sensitive assay, TSH-stimulated serum levels of thyroglobulin can be predicted with the basal serum thyroglobulin determination(Spencer, Fatemi et al. 2010, Brassard, Borget et al. 2011). The cut-off, using the best accuracy for basal sensitive thyroglobulin, was reported as 0.2 ng/ml (Schlumberger, Hitzel et al. 2007, Malandrino, Latina et al. 2011).

Patients treated with simple thyroid lobectomy or total thyroidectomy not followed by RRA have some amounts of residual thyroid tissue. In these patients a single detectable thyroglobulin value is not a reliable indicator for the presence of residual thyroid tumor (Haugen, Alexander et al. 2016). Some reports have demonstrated that the trend of thyroglobulin over time is a reliable predictor of the disease status of the patients (Durante, Montesano et al. 2012, Angell, Spencer et al. 2014): a stable or declining trend is consistent with disease remission while a rising trend is highly suspicious for disease relapse.

Anti-thyroglobulin antibodies

Thyroglobulin antibodies should always be measured at each thyroglobulin assessment because they can interfere with thyroglobulin assays leading false negative or more rarely false positive results (Haugen, Alexander et al. 2016). Mass spectrometry has demonstrated to overcome this problem in experimental settings (Netzel, Grebe et al. 2015, Netzel, Grant et al. 2016), but this approach seems to be less effective in clinical practice than it is in the research setting (Azmat, Porter et al. 2017). Thyroglobulin antibody titres over time (using the same assay) can provide surrogate information on disease recurrence, and declining titres are usually associated with remission (Durante, Tognini et al. 2014, Rosario, Carvalho et al. 2016, Matrone, Latrofa et al. 2018).

Neck ultrasonography and cytology

Most recurrences of PTCs are localized in the neck. The ultrasonographic characteristics use for the identification of metastatic neck lymph nodes and thyroid bed relapses has variable sensitivity and specificity (Leboulleux, Girard et al. 2007, Shin, Han et al. 2007, Leenhardt, Erdogan et al. 2013, Lamartina, Deandreis et al. 2016). Of note, up to 18% of histologically benign lymph nodes display suspicious features on ultrasonography (Leenhardt, Erdogan et al. 2013). The European Thyroid Association guidelines identify groups of characteristics

that can be used to distinguish indeterminate from suspicious findings (Leenhardt, Erdogan et al. 2013).

Roughly two-thirds of neck lymph nodes classified as indeterminate will spontaneously disappear (Lamartina, Grani et al. 2016), and watchful waiting is thus appropriate for small indeterminate nodes. Overall, only a small proportion of lesions will grow over time, and complications due to size increases have not been reported to date (Rondeau, Fish et al. 2011, Robenshtok, Fish et al. 2012) (Lamartina, Grani et al. 2016). Recurrences in the surgical bed usually appear hypoechoic — i.e. tissue darker than normal thyroid tissue — with microcalcifications, cystic component, irregular margins and increased vascularity, but this appearance can be found also in scar tissue or granulomas leading to false-positive ultrasonographic findings in 26–35% of the cases (Shin, Han et al. 2007, Kamaya, Gross et al. 2011, Leenhardt, Erdogan et al. 2013). Fine-needle aspiration cytology of thyroid bed nodules or neck lymph nodes can be employed in those cases that will be referred to surgery in case of findings consistent with malignancy (i.e. lesions of at least 8 mm in small diameter or lesions that represent a potential threat for their location) (Choudhary, Wartofsky et al. 2015, Haugen, Alexander et al. 2016). The sensitivity of fine-needle aspiration of lymph nodes can be increased by means of thyroglobulin measurement in the washout fluid and molecular testing (Arturi, Russo et al. 1997, Grani and Fumarola 2014). Smaller lesions and lesions not located in threatening sites can be managed with active surveillance (Rondeau, Fish et al. 2011, Robenshtok, Fish et al. 2012) (Lamartina, Grani et al. 2016). The documented increased use of neck ultrasonography in patients with DTC observed between 1998 and 2011 was associated with increased rates of treatment (surgery or radioiodine) but not with improved survival (Banerjee, Wiebel et al. 2016).

Whole-body radioactive iodine scan

Before the advent of neck ultrasonography, diagnostic whole-body scan with radioactive iodine was the cornerstone of DTC follow-up. Given the superior

diagnostic accuracy of other imaging techniques, in patients with a post-therapeutic whole-body scan that is considered normal (defined as the absence of radioactive iodine uptake outside of the thyroid bed), there is no longer any need for diagnostic whole-body scans (Cailleux, Baudin et al. 2000, Pacini, Capezzone et al. 2002). Several retrospective studies have shown that diagnostic whole-body scans do not improve the detection of neck recurrences during follow-up over the detection that can be achieved with serum thyroglobulin assays and neck ultrasonography (Pacini, Agate et al. 2001, Torlontano, Crocetti et al. 2006, Gonzalez Carvalho, Gorlich et al. 2017).

Cross-sectional imaging

Given the rarity of distant metastases in patients with DTC, second-line tools, such as cross-sectional imaging (CT scan or MRI) and 18F-fluorodeoxyglucose (FDG)– PET, are not routinely used in patients with DTC but can provide valuable information in certain cases. Cross-sectional imaging is the method of choice for identifying distant metastases owing to its high anatomical definition. The RECIST criteria can be applied to CT and MRI images to identify morphological disease progression or to evaluate responses to treatment. A CT scan without contrast enhancement is an effective tool for lung imaging, and CT with contrast medium can be used for the assessment of lesions in the neck and mediastinum (Lamartina, Deandreis et al. 2016). MRI provides complementary results for neck imaging and is the best tool for detecting respiratory and digestive tract spread. MRI is also the method of choice for imaging bone lesions88. 18F-FDG–PET–CT is effective to screen lesions, including in bones, in a single procedure and provides prognostic information (Leboulleux, Schroeder et al. 2007).

Novel biomarkers for thyroid cancer: role of miRNA

As discussed in the previous sections, thyroglobulin, the serum biomarker used together with imaging to assess disease status of DTC patients, has several limitations (notably in the presence of residual thyroid tissue or in the presence

of anti-thyroglobulin antibodies that can interfere with thyroglobulin assays). The research for alternative biomarkers is therefore an active field of research. miRNAs are endogenous, noncoding RNAs. Their length ranges from 19 to 25 nucleotides. miRNA have an important role in the posttranscriptional regulation of gene expression (Bartel 2009, Shukla, Singh et al. 2011). miRNAs can reduce the stability of a target gene transcript or inhibit its translation resulting in the downmodulation of the target gene expression (Bartel 2009, Shukla, Singh et al. 2011). This regulatory role on gene expression is important for several physiologic process, including those involved in embryogenesis and postnatal development. miRNA dysregulation is associated to several pathological conditions, including malignant tumors (Lagos-Quintana, Rauhut et al. 2001, Iorio and Croce 2017, Oliveto, Mancino et al. 2017). Over 2000 annotated human miRNAs are reported in the miRBase database (Kozomara and Griffiths-Jones 2014). Thyroid tumors (like other cancers) display alterations involving miRNA biogenesis at different levels. Malignant thyroid tissues and cell lines have downregulated transcription of DICER as compared with normal thyroid tissues and benign thyroid neoplasms (Penha, Sepe et al. 2017). This alteration was associated with “aggressive” tumor features (e.g. extrathyroidal extension, lymph node and distant metastases) and recurrence (Erler, Keutgen et al. 2014). DICER downregulation was also associated with the presence of the BRAFV600E mutation (Erler, Keutgen et al. 2014). Specific patterns of miRNA expression have been described in thyroid carcinomas (Nikiforova, Tseng et al. 2008, Dettmer, Vogetseder et al. 2013), and several miRNAs were found overexpressed or downregulated in major types of thyroid tumors (Nikiforova, Chiosea et al. 2009, Fuziwara and Kimura 2017). The overexpression of miRNA-146b, miRNA-221, miRNA-222, and miRNA-181b in PTCs as compared with expression levels in normal thyroid tissues was described in several reports. This upregulation is found associated with a more aggressive behavior of the tumors (Chou, Chen et al. 2010, Zhou, Liu et al. 2012, Wang, Zhang et al. 2013, Rosignolo, Memeo et al. 2017) [33–37]. miRNA-146b, miRNA-221, and miRNA-222, are also upregulated in FTC, HCC, and anaplastic thyroid carcinoma (Fuziwara and Kimura 2014, Wojtas, Ferraz et al. 2014, Petric, Gazic et al. 2016). In contrast, miRNA-197 and

miRNA-346 are upregulated specifically in FTC (Weber, Teresi et al. 2006). The miRNA-17-92 cluster is highly expressed in ATC (Fuziwara and Kimura 2014)[39].

Some of miRNA molecular targets are genes with a regulatory role in cell proliferation, migration invasion and survival. Some examples are: proto-oncogene receptor tyrosine kinase (KIT) (target of miRNA-146b, miRNA-221, miRNA-222), C-X-C motif chemokine ligand 12 (CXCL12) (target of miRNA-137), connective tissue growth factor (CTGF) (target of miRNA-199a-5p), NF-kB (target of miRNA-146a) (He, Jazdzewski et al. 2005, Pacifico, Crescenzi et al. 2010, Dong, Jin et al. 2016, Sun, Han et al. 2016).

miRNA Detection in Biological Samples

Several pathologic conditions including several malignant tumors are associated with significant changes in the miRNA profile (Di Leva, Garofalo et al. 2014). miRNAs can be found in cell lines, fresh-frozen and formalin-fixed tissues, fine needle aspiration biopsy samples, blood plasma and serum, and urine and ill all this specimens demonstrate a good stability (Makarova, Shkurnikov et al. 2016). Therefore, several approaches have been developed to isolate miRNA and study their expression, with the aim to identify profiles or single miRNAs associated with specific pathological condition.

The first step of the process is the isolation of total RNA. The extraction protocols used are designed to enrich the fraction containing miRNAs and other small RNA. Chemical miRNA extraction methods and column-based miRNA extraction methods are the most used. Chemical methods are suitable for all biological specimens and provide high amounts of RNA but may be prone to chemicals contamination. Column based methods provide more pure and high quality RNA but specific kit for each kind of biological tissue studied is needed (Celano, Rosignolo et al. 2017). The isolated miRNA is then quantified and subjected to quality assessment. The quantity of the RNA obtained depends on the specimen, while the quality depends on the extraction method adopted. The methods currently used to analyze miRNA expression are: microarrays, quantitative

reverse-transcription PCR (qRT-PCR), high-throughput sequencing (RNA-seq), and digital PCR (dPCR). Microarray analysis allows rapid parallel analysis of large numbers of miRNAs. The miRNAs in a biological sample are labeled using fluorescent, chemical, or enzymatic techniques and then hybridized to DNA-based probes on the array. Microarray-based profiling cost is relatively inexpensive, but its sensitivity and specificity are lower compared with other miRNA profiling methods. QRT-PCR entails reverse transcription of miRNA to cDNA, followed by real-time monitoring of the accumulation of polymerase reaction products. Platforms with preplated PCR primers distributed across microfluidic cards containing nanoliter-scale wells allow qRT-PCR detection of hundreds of miRNAs. This approach is more specific and sensitive than microarray profiling. An internal control is needed for relative quantification of the expression; for absolute quantification, a standard curve can be employed. RNA-seq provides quantification data as well as sequence data and can therefore be used to identify novel miRNAs or sequence variations. A cDNA library of small RNAs is prepared from the samples of interest. The second step adaptor ligation and immobilization of the cDNA on a support that can be either solid (for solid-phase PCR) or bead-based (for emulsion PCR). Massive parallel sequencing of millions of cDNA molecules from the library is then performed allowing simultaneous analysis of the expression patterns of a huge number of targets. This technique of miRNA profiling is the most informative but also the most expensive. Digital PCR allows quantitative analysis of miRNA expression. It is the most sensitive technique, internal controls are not needed for quantification, and is the only technique capable of direct miRNA absolute copy number quantification. A cDNA sample is partitioned into multiple parallel PCR reactions performed with nanofluidics partitioning or emulsion chemistry. Digital PCR is superior to previously described methods in terms of sensitivity and precision, and it is the most suitable technique to study miRNA expression in plasma or serum samples, where stable endogenous controls are laking (Moldovan, Batte et al. 2014, Hunt, Broyles et al. 2015, Celano, Rosignolo et al. 2017).

miRNAs as Diagnostic Markers in Thyroid Cancer

Molecular testing is a useful tool for the identification of malignant or benign nodules when fine needle cytology yields indeterminate findings (Durante, Grani et al. 2018). miRNAs are promising molecular marker in this setting (Paskas, Jankovic et al. 2015, Boufraqech, Klubo-Gwiezdzinska et al. 2016, Stokowy, Wojtas et al. 2016, Wei, Shen et al. 2016).

Another field of active research are the so called "liquid biopsies". The liquid biopsies aim to identify molecules in peripheric blood as markers for tumor diagnosis and potentially provide a picture of both primary and metastatic tumor lesions (Ilie and Hofman 2016, Larrea, Sole et al. 2016). Some reports on the role of miRNA in PTC show variable results. Differences in the populations studied and in methodology (i.e., sample type, storage conditions, and/or sample processing, RNA extraction protocol, quantification methods) probably contribute to this variability. The miRNA studied as circulating markers in DTCs are reported in Table 4.

Table 4. Circulating miRNA in PTC and FTC *(adapted form Celano et al. 2017)*

Histotype	Sample analyzed	miRNA	Regulation
PTC	Serum	let-7e, 151-5p, 222, hsa-let7b-5p, hsa-miR-10a-5p, hsa-miR-93-5p, hsa-miR-191, 24-3p, 28-3p, 103a-3p, 146a-5p, 146b-5p, 191-5p, 221-3p, 222-3p	Up
	Serum	95, 21	Down
	Plasma	146b, 222, 190, let-7i, 25-3p, 140-3p, 451a, 146b, 155, 31-5p, 126-3p, 145-5p, 181a	Up
	Plasma	hsa-miR-146a-5p, hsa-miR-150-5p, hsa-miR-199b-3p, has-miR-342-3p	Down
	Plasma-derived exosomes	31-5p, 126-3p, 145-5p, 181a	Up
FTC	Plasma-derived exosomes	21	Up

Circulating levels of miRNA-146b5p, miRNA-221-3p, and miRNA-222-3p can discriminate PTC patients form healthy controls (Yu, Liu et al. 2012, Lee, Zhao et al. 2013), while miRNA-222 and miRNA-146b levels discriminate between PTCs and benign nodules (Yu, Liu et al. 2012, Lee, Lim et al. 2015). Higher levels miRNA-21 are found in FTC patients compared with patients with benign nodules or PTC (Samsonov, Burdakov et al. 2016) (Samsonov, Burdakov et al. 2016). A higher expression of miRNA-181a was found in PTC patients compared with FTC patients (Samsonov, Burdakov et al. 2016). Circulating levels of miRNA-146a-5p, miRNA-146b-5p, miRNA-221-3p, and miRNA-222-3p decline after thyroid surgery in PTC patients (Yu, Liu et al. 2012, Lee, Zhao et al. 2013) (Yoruker, Terzioglu et al. 2016).

miRNAs as Prognostic Markers in Thyroid Cancer

Some evidence is suggesting that prognostic information can be provided by miRNA profiling in PTCs. The expression levels of some miRNAs in thyroid tumor tissues are associated with: tumor size (miRNA-221, miRNA-222, miRNA-135b, miRNA-181b, miRNA-146a, and miRNA-146b), multifocality (miRNA-146a, and miRNA-146b), capsular or vascular invasion (miRNA-146b, miRNA-221, and miRNA-222), extrathyroidal extension (miRNA-221, miRNA-222, miRNA-146a, miRNA146b, miRNA-199b-5p, and miRNA-135b), and both lymph node (miRNA-221, miRNA-222, miRNA-21- 3p, miRNA-146a, miRNA-146b, and miRNA-199b-5p), distant metastases (miRNA-146b, miRNA-221, and miRNA-222), and advanced TNM stage (miRNA-146a, miRNA146b, miRNA-221, and miRNA-222). (Huang, Liao et al. 2013, Sun, Yu et al. 2013, Wang, Zhang et al. 2013, Acibucu, Dokmetas et al. 2014, Peng, Li et al. 2014, Sun, Fang et al. 2015). Higher risk of recurrence class, defined according to the ATA guidelines, has been positively associated with higher expression of miRNA-146b-5p, miRNA-146b-3p, miRNA-21-5p, miRNA-221, miRNA222-3p, miRNA-31-5p, miRNA-199a-3p/miRNA-199b-3p, miRNA-125b, and miRNA-203 and lower expression levels of miRNA-1179, miRNA-7-2-3p, miRNA-204-5p, miRNA138, miRNA-30a, and let-7c (Geraldo and Kimura 2015, Rosignolo, Memeo et al. 2017). One report studied the prognostic

role of miRNAs in follicular thyroid cancer. The expression in tumor tissue of miRNA-10b was found an independent predictor of worse prognosis in FTCs as it was associated with the presence of distant metastases in minimally invasive follicular thyroid cancer and to the aggressive widely invasive histotype (Jikuzono, Kawamoto et al. 2013). Finally, serum levels of miRNA-221-3p and miRNA-146a-5p appear to predict disease response to treatment in PTC: their levels were found increased in patients with structural evidence of disease (Rosignolo, Sponziello et al. 2017).

The Italian Thyroid Cancer Repository

Italian Thyroid Cancer Observatory (ITCO) foundation involves physicians, scientists, and patients with interests in thyroid cancer (www.itcofoundation.org). The principal aims of the foundation are: a) promote thyroid cancer basic, clinical and translational research; b) create a multidisciplinary network of experts willing to share knowledge and tools for thyroid cancer patient care; and c) to provide scientific support for healthcare policy makers (Lamartina, Durante et al. 2017). The ITCO network currently includes 46 centers involved in the care of thyroid cancer patients, spread throughout the Italian territory. They include tertiary referral centers operating at the national level, teaching-hospitals and academic institutions as well as local or regional hospital-based units.

AIM OF THE STUDY

The aims of this study were to:

- describe the response to treatment evaluated after 1 and 3 years from surgery;
- validate the 2015 ATA risk of recurrence classification;
- study the role of miRNA profiles in the assessment of the response to treatment of DTCs.

MATERIALS AND METHODS

Study population

A web-based thyroid cancer database was set up in the setting of the ITCO network. The Web-based database started the enrollment of patients with newly diagnosed and histologically confirmed thyroid cancer in 2013 at the Thyroid Cancer Center of the University of Rome Sapienza (the ITCO network's coordinating center). It was then expanded to include data from centers that joined the ITCO. It now includes prospectively collected data on 6000 Italian patients with histologically confirmed diagnoses of thyroid cancer of either follicular of C-cell origin. Cases are considered eligible if contact with the reporting center began within 12 months from primary treatment. Each case record includes information on patient demographics and biometrics, circumstances of the diagnosis, tumor pathology, surgical and radioactive iodine treatments, and the results of periodic follow-up examinations. Sensitive data are encrypted, and the database is managed in an anonymous fashion.

For the purpose of this study DTC patients were included in the analysis. Cases with medullary and anaplastic thyroid cancer were excluded as well as those with tumors of uncertain malignancy potential. Cases with incomplete information on the initial treatment, tumor pathology or 1-year follow up assessment were excluded.

For each case, the following information was recorded:

A) Initial treatment: Thyroid surgery procedure (total thyroidectomy, thyroid lobectomy, or lobectomy followed by completion thyroidectomy); cervical lymph node dissection (none, central compartment dissection, lateral compartment dissection, central and lateral compartment dissection). For patients who had undergone total thyroidectomy (in one or two procedures), the use of RRA (performed or omitted) was also recorded. The date of last surgical procedure was considered as the date of primary treatment.

In patients submitted to RRA the administered activity, the method of preparation (thyroid hormone withdrawal or recombinant human TSH stimulation) and the results of iodine post-therapeutic whole-body scan (RxWBS) were recorded.

B) The risk of recurrence category was assigned in accordance with the 2015 ATA guidelines as follow:

- Low risk: intrathyroidal tumors of any size, uni- or multifocal, without lymph node or distant metastases, no aggressive histotype, no foci of vascular invasion

- Intermediate risk: either presence of lymph node metastases (except for bulky metastases of >30 mm), minimal extrathyroidal extension, aggressive histotype (i.e. solid, insular, tall-cell, columnar-cell, hobnail-cell, sclerosing, poorly differentiated, trabecular), or foci of vascular invasion

- High risk: either presence of gross extrathyroidal extension, bulky lymph node metastases of >30 mm, grossly incomplete tumor resection (R2) or distant metastases.

When the information on the size of lymph node metastasis was not available and no other high-risk features were present patients where included in the intermediate risk category.

FTCs and HCCs were also analyzed separately from other DTCs. FTCs were classified as widely invasive (presence of extensive vascular or capsular invasion) or minimally invasive (absence of such extensive invasion).

The response to treatment was evaluated at about 1 year after surgery (range 6-18 months) and at about 3 years from surgery (range 30 – 42 months) and was categorized according to 2015 ATA guidelines as:

- ER: no evidence of disease on imaging, undetectable serum thyroglobulin (Tg) (≤0.2 ng/mL on levothyroixine treatment or <1 after TSH stimulation), absence of anti-thyroglobulin antibodies (TgAbs)
- BIR: no evidence of disease on imaging, detectable serum Tg ≥1 ng/mL on levothyroxine treatment or ≥10 ng/mL after TSH stimulation or TgAbs positivization
- SIR: evidence of disease on imaging
- Ind: aspecific findings on imaging or low detectable serum Tg >0.2 and <1 ng/mL on levothyroxine treatment or ≥1 and <10 ng/mL after TSH stimulation or presence of TgAbs.

The response to treatment was also categorized in two classes as absence of SIR (absence of disease on imaging, i.e. ER+BIR+Ind) and SIR (evidence of disease on imaging).

Presence of SIR after 1 year from primary treatment was considered synonym of persistent disease. Disease relapse was defined as SIR found at the 3 years evaluation in a patient classified ER at 1 year. At the 3 years evaluation a composite outcome of persistent and recurrent diseases was also evaluated.

Statistical analysis

In descriptive analysis, continuous variables were expressed as medians with interquartile ranges (IQR), and nominal variables as numbers and percentages. Differences between categoric variables were calculated with the chi square test; differences between continuous variables were assessed with the Mann–Whitney test. All statistical analyses were performed with SAS software (SAS Institute, Inc.).

Methods: miRNA profile analysis

Patients and sample collection

Consecutive PTC patients followed at the thyroid cancer unit of the university of Rome Sapienza and referred to total thyroidectomy that provided consent to participate to the study were enrolled. Serum samples were collected before surgery (just after anesthesia induction) and 30 days after surgery. For HCs a single sample was obtained at the moment of study enrollment.

Age- and sex-matched controls and healthy controls (HC) were enrolled in the study. Controls were patients who underwent total thyroidectomy for nodules with indeterminate cytology (Bethesda Class IV) that were subsequently diagnosed as benign on the basis of surgical histology. HC were patients seen in the hospital's outpatient clinics for health problems unrelated to the thyroid. All the HCs had no evidence of thyroid-disease based on the results of a screening examination performed by the study team (complete patient and family histories, neck ultrasound findings, and results of thyroid hormone and thyroid antibody assays).

Blood samples were collected and centrifuged at 3000 rpm for 10 minutes. The serum obtained was stored at –80°C.

RNA Isolation from Serum Samples

Total RNA (including small RNAs) was extracted from 200 mL of serum using the miRNeasy Serum/Plasma kit and cel-miR-39 as the spiked-in control (Qiagen, Hilden, Germany) following the manufacturer's instructions. To perform miRNA profiling without the preamplification step, we used a multiple extraction method: isolation of total RNA was made from up to 5 200-mL aliquots of each serum sample. This solution yeld over 1000 ng of total RNA from each sample. NanoDrop Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA) was used to verify the quality and quantity of RNA samples.

Retrotranscription and real time PCR

The reaction of retrotranscription was prepared in a volume of 15 µl, containing 5 µl of total RNA (5 ng), 3 µl of RT Primers (5X), 0.15 µl of dNTPs with dTTP (100 mM), 1 µl of MultiScribe Reverse Transcriptase (50 U/µL), 1.5 µl of RT Buffer (10X), 0.19 µl of RNase Inhibitor (20 U/µL) and 4.16 µl of Nuclease-free H2O.

For the first cDNA strand synsthesis the retrotranscription mix was incubated at 16°C for 30 min, at 42°C for 30 min, at 85°C for 5 min and finally stored at 4°C.

Then, 1.33 µl of cDNA was amplified by Real-Time PCR using 10 µl of TaqMan Universal PCR Master Mix, No AmpErase UNG (2X), 1µl of microRNA TaqMan Assay and 7.67 µl of nuclease-free H2O in a final volume of 20 µl. The amplification reaction was undertaken with Real-Time PCR 7900HT (Thermo Fisher Scientific) using the following schedule: 10 min at 95°C, followed by 40 cycles of 15s at 95°C and 1 min at 60°C.

Screening cohort

A first analysis of miR profiles was performed in the samples obtained from a cohort of PTC patients. In this cohort (screening cohort) miRNA profiling was performed with TaqMan Array Human MicroRNA A+B Cards, version 3.0 (Thermo Fisher Scientific, Inc.), constituted by a set of two 384-well microfluidic cards for the quantitative expression of 754 miRNAs. Reverse transcription and real-time polymerase chain reaction reactions were performed without a preamplification step (as for manufacturer's instructions) which requires preamplification only if the total amount of RNA is less than 350 ng. Expression Suite software, version 1.0.3 (Thermo Fisher Scientific, Inc.), was used to calculate cycle threshold (Ct) values (cutoff: 35) and for the analysis of the data. U6 demonstrated the lowest between-sample variance and was used as the endogenous control. The relative miRNA expression levels were calculated using the comparative 2-ΔΔCt method. Differences in preoperative and postoperative miRNA expression levels were assumed significant if the fold change was >2 or

<0.5 and $p < 0.05$. Based on these results a subset of miRNAs was selected for validation analysis.

Validation cohort

The validation cohort included PTC patients and age- and sex-matched controls. The miRNAs analyzed were selected on the basis of the results obtained from the analysis of the screening cohort and data in the literature. Expression levels were quantified in duplicate using specific TaqMan MicroRNA Assays (Thermo Fisher Scientific, Inc.) as indicated by manufacturer's instructions. The abundance of circulating miRNA was quantified with standard curves constructed with synthetic RNA oligonucleotides corresponding to mature miRNA sequences (miScript miRNA mimics, Qiagen, miRBase Release, version 21). The mimics were reverse transcribed with specific primers (5x) together with the cDNA of the samples diluted serially 10-fold and then used to generate standard curves for each miRNA TaqMan assay. The absolute number of miRNA copies was determined from each sample Ct using the following equation: y=kx+ m, where y is the Ct value, k is the slope, x is the logarithm of the number of miRNA copies, and m is the y intercept. A normalization factor (NF) was calculated for each sample using the following formula: NF = 1/[2^(median spike in Ct value) – (spike in average Ct value of the given sample)]. A normalized copy number value was obtained for each sample by multiplying the number of copies of the given miRNA by the NF. Assuming that in each PCR reaction 0.4 ng of cDNS were added, the normalized copy number was converted to normalized copies of cDNA per nanogram and then to normalized copies of miRNA per milliliter of serum (considering the total amount of RNA isolated from 200 mL of serum).

Statistical Analysis

The Mann-Whitney test was used to assess differences in miRNA expression levels between preoperative and postoperative serum samples. The Kruskal Wallis test followed by Dunn's multiple comparisons test was used when more

than 2 groups were compared. P values < 0.05 were considered statistically significant. All these analyses were performed with GraphPad Prism software, version 5.0 (GraphPad Software Inc., San Diego,CA). Receiver-operating characteristic (ROC) curves and areas under the ROC curve were analyzed with the p-ROC package in R software, version 3.1.1, using the Youden Index and the DeLong method.

RESULTS

Study population with at least 1 year of follow up

After the exclusion of patients that didn't reach 1 year of follow up and those with incomplete data on treatment, pathology or for treatment response classification (i.e. serum markers and imaging), 2730 subjects were enrolled in the study.

The characteristics of these subjects are summarized on Table 5.

Table 5. Characteristics of the study population

No. patients	**2730**
Gender - N (%)	
Female	2019 (74%)
Male	711 (26%)
Age - median (range)	49 (10 - 89)
Histology - N (%)	
- Papillary	2532 (92.7%)
- Follicular	138 (5%)
- Hürthle	49 (2%)
- Other (mixed tumors)	11 (0.3%)
Tumor size – median (range)	12 (2 - 162)
Risk of recurrence ATA - N (%)	
- Low	1386 (50.8%)
- Intermediate	1168 (42.8%)
- High	176 (6.4%)
Surgery - N (%)	
- Total/near total thyroidectomy	2619 (95.9%)
- Lobectomy+completion thyroidectomy	51 (1.9%)
- Lobectomy	60 (2.2%)
Lymph node dissection - N (%)	
- No	1634 (60%)
- Central compartment	786 (29%)
- Central and lateral compartments	261 (10%)
- Lateral compartments	49 (1%)
Radioiodine remnant ablation/treatment - N (%)	
- Performed	1793 (65.7%)
- Not performed	937 (34.3%)
Radioiodine preparation - N (%)	
- rhTSH	795 (44.3%)
- thyroid hormone withdrawal	938 (52.3%)
- unknown	60 (3.4%)
Radioiodine activity (mCi) - median (range)	99 (30 - 200)

The 74% of the cohort was female. The median age at diagnosis was 49 years old. The most common histotype was PTC (about 93%). Median tumor size was 12 mm (range 2 – 162 mm). The most used surgical approach was total or neat total thyroidectomy (in one or two procedures) while only the 2.2% of the patients underwent a simple thyroid lobectomy. Lymph node dissection was performed in the 40% of the patients. Radioiodine remnant ablation (RRA) was performed in 2/3 of the cases, with a median activity of radioiodine of about 100 mCi. Preparation to RRA was thyroid hormone withdrawal in about a half of the cases, 44% had recombinant human TSH stimulation (the information was missing in 60 subjects).

The risk of recurrence was low in a half of the patients (50.6%), intermediate in the 43% and high in 6.4%.

Treatment according to ATA risk

Thyroid lobectomy was the ultimate surgical procedure in 46 (3.3%) low risk patients and in 14 (1.2%) intermediate risk patients. All the 6 high risk patients initially treated with thyroid lobectomy underwent completion thyroidectomy (Table 6).

Table 6. Surgical approach according to risk of recurrence

	Surgical approach			
ATA risk of recurrence	**Bilateral procedure**	**Lobectomy**	***p***	**OR [95% CI]**
Low	1340 (96.7%)	46 (3.3%)	ref	ref
Intermediate	1154 (98.8%)	14 (1.2%)	<0.01	2.8285 [1.5184 - 5.6008]
High	176 (100%)	-	<0.01	INF [1.5544 - INF]
Total	**2670 (97.8%)**	**60 (2.2%)**		

Fisher exact test

Of the 2670 patients treated with bilateral thyroid tissue removal (i.e. total/near total thyroidectomy or thyroid lobectomy followed by completion thyroidectomy) RRA was performed in 1793 (67.2%) and omitted in 877 (32.8%). RRA was omitted more frequently in low risk patients (722/1340 - 54%) than

intermediate risk patients (150/1155 – 13%) and seldom in high risk patients (5/176 – 2.8%). The odds ratio of receiving RRA increased with increasing risk of recurrence (Table 7).

Table 7. Radioiodine remnant ablation according to risk of recurrence

ATA risk of recurrence	RRA Omitted	RRA Performed	*p*	OR [95% CI]
Low	722 (54%)	618 (46%)	ref	ref
Intermediate	150 (13%)	1004 (87%)	<0.01	7.8128 [6.3609 – 9.637]
High	5 (2.8%)	171 (97.2%)	<0.01	39.8965 [16.607 – 125.4165]
Total	**877 (32.8%)**	**1793 (67.2%)**		

Fisher exact test

Follow up tools

All patients underwent serum thyroglobulin and anti-thyroglobulin antibodies. Most patients had neck ultrasound (2675 – 98%); 55 patients were evaluated with RAI scan (N=16), 18 FDG PET (N=30), cross sectional imaging (CT, MRI, N=9).

Response to treatment evaluated at 1 year from primary surgery

1. Whole cohort

The response to treatment after 1 year was ER in 1675 (61.4%), BIR 63 (2.3%), SIR in 70 (2.6%) and Ind 922 (33.8%). The outcome at 1 year form primary surgery according to ATA risk is represented in Table 8. The probability of SIR increased with increasing risk while the probability of ER decreased with increasing risk. Taking into account the outcome in two classes (presence or absence of SIR), the OR for SIR was significantly higher in intermediate (4.85) and high risk (35.21) compared with low risk patients (Table 9).

Table 8. Response to treatment (four classes) according to ATA risk

	Outcome				
ATA risk of recurrence	**ER**	**BIR**	**SIR**	**Ind**	**Total**
Low	900 (64.9%)	42 (3%)	8 (0.6%)	436 (31.5%)	1386
Intermediate	713 (61.1%)	19 (1.6%)	32 (2.7%)	404 (34.6%)	1168
High	62 (35.2%)	2 (1.1%)	30 (17.1%)	82 (46.6%)	176
Total	**1675 (61.3%)**	**63 (2.3%)**	**70 (2.6%)**	**922 (33.8%)**	**2730**

Table 9. Response to treatment (two classes) according to ATA risk

	Outcome				
ATA risk of recurrence	**Absence of SIR**	**SIR**	**Total**	***p***	**OR [95%CI]**
Low	1378 (99.4%)	8 (0.6%)	1386	ref	ref
Intermediate	1136 (97.3%)	32 (2.7%)	1168	<0.01	4.85 [2.1776 - 12.23]
High	146 (82.9%)	30 (17.1%)	176	<0.01	35.2163 [15.4088 - 90.6602]
Total	**2660 (97.4%)**	**70 (2.6%)**	**2730**		

Fisher exact test

The outcome in two classes according to surgical approach in shown in Table 10. None of the patients treated with lobectomy alone had persistent disease after 1 year from surgery.

Table 10. Outcome in two classes according to surgical approach

Surgical approach	**Absence of SIR**	**SIR**	**Total**
Total thyroidectomy	2550 (97.4%)	69 (2.6%)	2619
Lobectomy	60 (100%)	-	60
Lobectomy+ completion thyroidectomy	50 (98%)	1 (2%)	51
Total	**2660 (97.4%)**	**70 (2.6%)**	**2730**

2. Papillary thyroid cancer

The outcome evaluated at 1 year from primary surgery in PTC patients treated with bilateral thyroid tissue removal according to ATA risk class and RRA treatment is shown in Table 11 (RRA omitted) and Table 12 (RRA performed). The odds ratio for SIR increases along with recurrence risk class in both RRA treated and not treated groups. The increased frequency of SIR in intermediate- and high-risk groups compared with low-risk group reaches statistical significance only in the RRA treated group.

Table 11. Outcome of PTC patients treated with bilateral surgery and not submitted to RRA

	Outcome				
ATA risk of recurrence	**Absence of SIR**	**SIR**	**Total**	***p***	**OR [95%CI]**
Low	700 (99.3%)	5 (0.7%)	705	ref	ref
Intermediate	132 (98.5)	2 (1.5%)	134	0.31	2.1188 [0.1998 - 13.1098]
High	3 (75%)	1 (25%)	4	0.03	44.6496 [0.742 - 700.1687]
Total	835 (99.1%)	8 (0.9%)	843		

Fisher exact test

Table 12. Outcome of PTC patients treated with bilateral surgery and RRA

	Outcome				
ATA risk of recurrence	**Absence of SIR**	**SIR**	**Total**	***p***	**OR [95%CI]**
Low	583 (99.5%)	3 (0.5%)	586	ref	ref
Intermediate	873 (96.8%)	29 (3.2%)	902	<0.01	6.4491 [1.9852 - 33.252]
High	130 (83.3%)	26 (16.7%)	156	<0.01	38.6272 [11.5769 - 202.1478]
Total	**1586 (96.5%)**	**58 (3.5%)**	**1644**	-	-

Fisher exact test

3. Follicular thyroid cancer and Hürthle-cell thyroid cancer

Follicular thyroid cancer presented with SIR after 1 year form primary surgery in the 2.2% of the cases: none of the FTC without a precise histopathologic classification had SIR, one patients with minimally invasive FTC (FTC MI) and 2 patients with widely invasive FTC (FTC WI). A tendency to a more frequent SIR in

FTC WI compared with FTC MI was observed (OR of 7.6) even though the difference was not statistically significant (Table 13).

Table 13. Outcome of FTC patients

	Outcome				
Histology subtypes	**Absence of SIR**	**SIR**	**Total**	***p***	**OR [95%CI]**
FTC NOS	3 (100%)	-	3	-	-
FTC MI	105 (99%)	1 (1%)	106	ref	ref
FTC WI	27 (93.1%)	2 (6.9%)	29	0.11	7.6176 [0.3834 - 461.5782]
Total	**135 (97.8%)**	**3 (2.2%)**	**138**		

Fisher exact test

Only one over 49 (2%) HCCs had SIR after 1 year from primary surgery.

Response to treatment evaluated at 3 years

The 30% (829 patients) of the cohort had a 3-year disease evaluation available. The characteristics of these patients are consistent with those of the original cohort and are listed in Table 14.

Table 14. Characteristics of the study population that reached 3 years of follow up

No. patients	**829**
Gender - N (%)	
Male	215 (26%)
Female	711 (74%)
Age - median (range)	48 (12 - 84)
Histology - N (%)	
- Papillary	771 (93%)
- Follicular	44 (5.3%)
- Hürthle	13 (1.6%)
- Other (mixed tumors)	1 (0.1%)
Tumor size – median (range)	11 (1 - 100)
Risk of recurrence ATA - N (%)	
- Low	445 (53.7%)
- Intermediate	346 (41.7%)
- High	38 (4.6%)
Surgery - N (%)	
- Total/near total thyroidectomy	821 (99%)
- Lobectomy+completion thyroidectomy	3 (0.4%)
- Lobectomy	5 (0.6%)
Lymph node dissection - N (%)	
- No	476 (57.4%)
- Central compartment	272 (32.8%)
- Central and lateral compartments	67 (8.1%)
- Lateral compartments	14 (1.7%)
Radioiodine remnant ablation/treatment - N (%)	
- Performed	335 (65.7%)
- Not performed	494 (34.3%)
Radioiodine preparation - N (%)	
- rhTSH	145 (44.3%)
- thyroid hormone withdrawal	338 (52.3%)
- unknown	11 (3.4%)
Radioiodine activity (mCi) - median (range)	100 (30 - 200)

The outcome at 3 years from primary surgery was ER in 537 (64.8%), BIR in 57 (6.9%), SIR in 39 (4.7%) and Ind in 196 cases (23.6%). The rate of SIR at 3 years, considered as composite outcome of persistence or recurrence, increased significantly along with the initial risk estimate as observed with persistent disease at 1 year (Table 15).

Table 15. Outcome at 3 years

	Outcome				
ATA risk of recurrence	**Absence of SIR**	**SIR**	**Total**	***p***	**OR [95%CI]**
Low	440 (98.9%)	5 (1.1%)	445	ref	ref
Intermediate	319 (92.2%)	27 (7.8%)	346	<0.01	7.4304 [2.7802 – 24.9835]
High	31 (81.6%)	7 (18.4%)	38	<0.01	19.5482 [5.0189 - 82.7853]
Total	**790 (95.3%)**	**39 (4.7%)**	**829**		

Fisher exact test

The outcome in 4 classes at 3 years according to initial response to treatment classification is represented in Table 16. ER status at 1 year was confirmed after 3 years in 410 (82.8%) over 495 patients. One third of the patients initially classified as BIR changed their status as ER and 1/3 as Ind while only the 16.7% was confirmed as BIR. Patients with SIR changed their status without any treatment in 5 cases (suspicious imaging findings at 1 year not confirmed on further assessments), while further treatments (radioiodine, surgery or both) were performed in 6 cases. Ind patients remained Ind in 44% of the cases, spontaneously achieved ER status in the 38% of the cases, while in about 9% of the cases were upstaged to BIR.

Table 16. Outcome in 4 classes at 3 years according to initial response to treatment

	Outcome at 3 years				
Outcome at 1 year	**ER**	**BIR**	**SIR**	**Ind**	**Total**
ER	410 (82.8%)	26 (5.3%)	3 (0.6%)	56 (11.3%)	495
BIR	2 (33.3%)	1 (16.7%)	1 (16.7%)	2 (33.3%)	6
SIR	7 (36.8%)	3 (15.8%)	8 (42.1%)	1 (5.3%)	19
Ind	118 (38.2%)	27 (8.7%)	27 (8.7%)	137 (44.3%)	309
Total	**537 (64.8%)**	**57 (6.9%)**	**39 (4.7%)**	**196 (23.6%)**	**829**

The rate of SIR found at 3 years increased form the 0.6% in patients initially classified as ER (true disease relapse), to 8.7% in patients initially classified as

Ind and 16.7% in patients initially classified as BIR. The 42% of patients who had persistent disease at 1 year had SIR in 42% of the cases (Table 17).

Table 17. Outcome in 4 classes at 3 years according to initial response to treatment

	Outcome at 3 years				
Outcome at 1 year	**Absence of SIR**	**SIR**	**Total**	***p***	**OR [95%CI]**
ER	492 (99.4%)	3 (0.6%)	495	ref	ref
Ind	282 (91.3%)	27 (8.7%)	309	<0.01	15.6566 [4.7537 - 81.327]
BIR	5 (83.3%)	1 (16.7%)	6	0.05	31.44 [0.5215 - 482.5554]
SIR	11 (57.9%)	8 (42.1%)	19	<0.01	112.9494 [23.4439 - 733.984]
Total	**790 (95.3%)**	**39 (4.7%)**	**829**		

Fisher exact test

The outcome according to initial response to treatment classification stratified by initial risk estimate is reported in Table 18 panels a, b, c. Overall, the probability of spontaneous improvement of the response class was higher for low risk patients compared with intermediate and high risk. Both low risk patients initially classified as SIR changed their status to ER without any treatment, half of BIR and about 40% of Ind were finally classified as ER. On the other hand, all true relapses were observed in patients initially classified as intermediate risk while none of the low risk patients had disease relapse.

Table 18. Outcome at 3 years according to initial response to treatment divided for ATA risk of recurrence estimate

Panel a. Low risk patients					
	3 years Outcome				
1 year Outcome	**ER**	**BIR**	**SIR**	**Ind**	**Total**
ER	231 (84.3%)	6 (2.3%)	-	37 (13.5%)	274
BIR	1 (50%)	-	-	1 (50%)	2
SIR	2 (100%)	-	-	-	2
Ind	66 (39.5%)	18 (10.8%)	5 (3%)	78 (46.7%)	167
Total	**300 (67.4%)**	**24 (5.4%)**	**5 (1.1%)**	**116 (26.1%)**	**445**
Panel b. Intermediate risk patients					
	3 years Outcome				
1 year Outcome	**ER**	**BIR**	**SIR**	**Ind**	**Total**
ER	168 (81.2%)	18 (8.7%)	3 (1.4%)	18 (8.7%)	207
BIR	1 (25%)	1 (25%)	1 (25%)	1 (25%)	4
SIR	4 (40%)	1 (10%)	4 (40%)	1 (10%)	10
Ind	48 (38.4%)	6 (4.8%)	19 (15.2%)	52 (41.6%)	125
Total	**221 (63.9%)**	**26 (7.5%)**	**27 (7.8%)**	**72 (20.8%)**	**346**
Panel c. High risk patients					
	3 years Outcome				
1 year Outcome	**ER**	**BIR**	**SIR**	**Ind**	**Total**
ER	11 (78.6%)	2 (14.3%)	-	1 (7.1%)	14
BIR	-	-	-	-	-
SIR	1 (14.3%)	2 (28.6%)	4 (57.1%)	-	7
Ind	4 (23.5%)	3 (17.6%)	3 (17.6%)	7 (41.2%)	17
Total	**16 (42.1%)**	**7 (18.4%)**	**7 (18.4%)**	**8 (21.1%)**	**38**

miRNA profile analysis: miRNA population

A subset of 55 PTC patients followed at the Thyroid cancer clinic of University of Rome Sapienza and referred to total thyroidectomy was enrolled for the miRNA analysis. Sera of eleven patients were used for the screening analysis (screening cohort) and 44 for validation analysis (validation cohort) together with 39 age and sex matched controls (19 subjects with benign thyroid nodules and 20 healthy controls).

Screening Analysis

Preoperative and postoperative sera (collected 30 days after surgery) of the patients of the screening cohort were analyzed and the quantification of 754 miRNAs was performed. Eleven miRNAs had a significantly lower level in the postoperative samples than in the preoperative samples (Table 19). None of the 754 miRNAs analyzed demonstrated a significantly increased expression comparing pre and post-operative samples.

Table 19. miRNAs that decreased after surgery in the screening group

miRNA ID	Pre-operative samples	Post-operative samples	*P-value**
hsa-miR-103a-3p	1(0.473-1.682)	0.121(0.025-0.286)	0.0061
hsa-miR-222-3p	1(0.310-2.424)	0.390(0.190-0.877)	0.0071
hsa-miR-24-3p	1(0.282-2.285)	0.379(0.097-0.973)	0.0079
hsa-miR-28-3p	1(0.057-2.190)	0.253(0.109-0.412)	0.0091
hsa-miR-191-5p	1(0.125-1.869)	0.350(0.155-0.832)	0.0117
hsa-miR-146a-5p	1(0.104-2.026)	0.340(0.028-0.711)	0.0129
hsa-miR-454-3p	1(0.101-2.986)	0.105(0.002-0.265)	0.0205
hsa-miR-186-5p	1(0.205-2.428)	0.425(0.157-0.908)	0.0288
hsa-miR-489-3p	1(0.097-2.709)	0.126(0.092-0.194)	0.0333
hsa-miR-126-3p	1(0.176-2.946)	0.424(0.080-1.086)	0.0356
hsa-miR-320a	1(0.159-2.004)	0.326(0.074-0.752)	0.0398

Mann-Whitney test. Results are expressed as RQ (RQ min- RQ max). Post-operative levels are normalized to levels found in pre-operative serum samples (equal to 1).

Validation Analysis

Ten miRNA were selected for validation analysis according to the following criteria:

- 6 miRNA (miRNA-146a-5p, miRNA-28-3p, miRNA-103a-3p, miRNA-222-3p, miRNA-191-5p, miRNA-24-3p) that disclosed a marked decline in the expression comparing pre and post-operative samples (cut-off of <0.02 vs mean preoperative levels)

- 4 miRNA (miRNA-146b-5p, miRNA-2213p, miRNA-95-3p, miRNA-190a-5p) that were found associated with PTC in the literature with high quality evidence(Lee,

Zhao et al. 2013, Cancer Genome Atlas Research Network 2014, Graham, Hart et al. 2015, Hu, Wang et al. 2016).

The levels of these 10 miRNAs were analyzed in the sera of 44 PTC (collected pre and post-operatively) and those of 39 controls (preoperatively in the 19 subjects with benign thyroid nodules and at study enrollment for the 20 healthy controls).

The level of two miRNAs (miRNA-95-3p and miRNA-190a-5p) was below the detection limits of our assay (Ct values higher than 35) and so they were excluded from further analysis.

All the remaining 8 miRNAs levels were less abundant in the postoperative samples compared with pre-operative sera (Figure 2). The miRNAs that disclosed the most significant decrease were: miRNA-146a-5p (*p* 0.0007), miRNA-221-3p (*p* <0.0001), and miRNA-222-3p (*p* <0.0001).

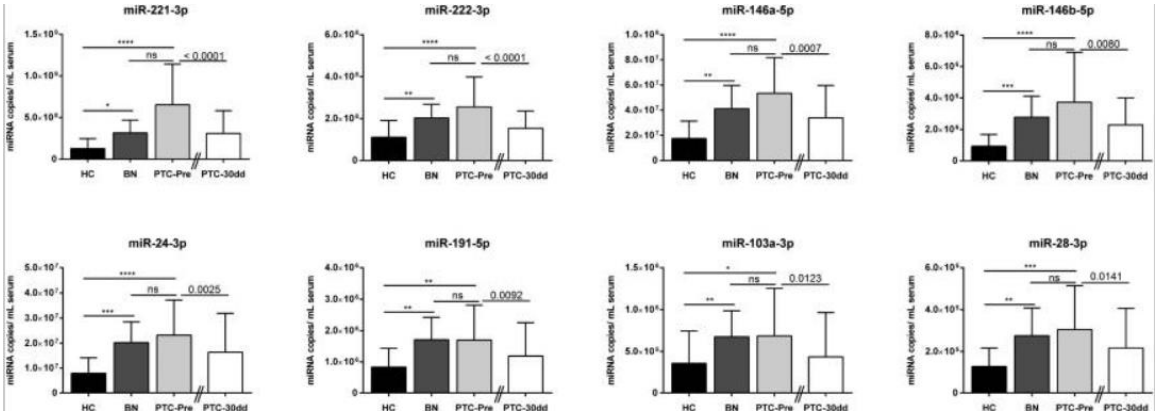


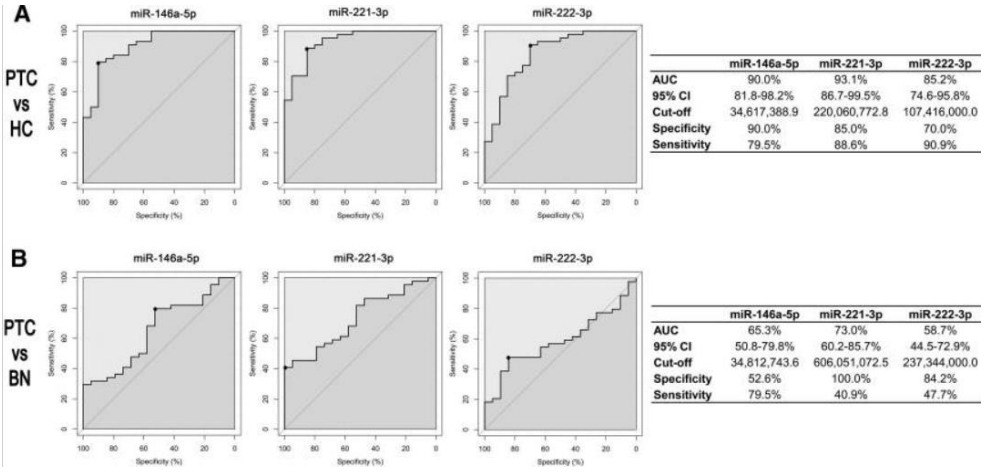
Figure 2. Validation of 8 miRNA serum levels in 44 patients with PTC (preoperative and postoperative samples), 19 controls with benign nodules (preoperative sample), and 20 healthy controls (enrollment sample). Data are reported as mean copies/mL ± standard deviation; *P <0.05, **P <0.01, and ***P <0.001. (Kruskal-Wallis test followed by Dunn's multiple comparison test for intergroup differences; Mann-Whitney test for intraindividual differences.) ns, not significant.

Compared with healthy controls, all the 8 miRNAs were significantly more abundant in the preoperative sera from the patients with PTC. The preoperative expression level of the 8 miRNAs in the PTC patients was not significantly

different form that of the patients with benign nodules, although the mean serum level of miRNA-221-3p in preoperative samples of PTC patients appreciably exceeded that found in samples from patients with benign nodules (653,242,460 ± 490,942,066 copies/mL versus 316,148,953 ± 153,115,869 - p = 0.076).

Three miRNAs (miRNA-146a-5p, miRNA-221-3p, and miRNA-222-3p) that disclosed the most marked decrease in postoperative samples in PTC patients were selected for ROC curve analysis.

The 3 miRNAs demonstrated a good accuracy in discriminating between PTC patients and healthy controls (Figure 3, panel A) but a lower performance was found in the differentiation of PTC and benign nodules (Figure 2, panel B). Both miRNA-146a-5p and miRNA-221-3p had better accuracy in the discrimination of PTC than miRNA-222-3p.



	miR-146a-5p	miR-221-3p	miR-222-3p
AUC	90.0%	93.1%	85.2%
95% CI	81.8-98.2%	86.7-99.5%	74.6-95.8%
Cut-off	34,617,388.9	220,060,772.8	107,416,000.0
Specificity	90.0%	85.0%	70.0%
Sensitivity	79.5%	88.6%	90.9%

	miR-146a-5p	miR-221-3p	miR-222-3p
AUC	65.3%	73.0%	58.7%
95% CI	50.8-79.8%	60.2-85.7%	44.5-72.9%
Cut-off	34,812,743.6	606,051,072.5	237,344,000.0
Specificity	52.6%	100.0%	84.2%
Sensitivity	79.5%	40.9%	47.7%

Figure 3. ROC curve analyses to explore the diagnostic value of miRNA-146a5p, miRNA-221-3p, and miRNA-222-3p levels for discriminating (A) between PTC patients and healthy controls and (B) between PTC patients and patients with benign nodules. Tables on the right show areas under the curve (AUC) with 95% confidence intervals (95% CI) and cutoffs (indicated on curves as solid black circles and reported as copies per milliliter) with associated sensitivity and specificity. BN: benign nodules; HC: healthy controls.

Follow up analysis

The levels of miRNA-146a-5p and miRNA-221-3p were analyzed in additional serum samples collected at the moment of first follow up disease assessment (performed 12-24 months from primary surgery i.e. follow up sample) from 20 of the 44 PTC patients (from whom samples were available) (Figure 4). The response to treatment was ER or Ind in 15 (75%) of the patients, BIR in one patient (5%) and SIR in 4 (20%). The expression levels of miRNA-146a-5p and miRNA-221-3p decreased in the postoperative samples and remained low in the follow up sample in the 15 ER patients and in the BIR patient. On the other hand, in the 4 SIR patients the levels of expression of miRNA-146a-5p and miRNA-221-3p increased in the follow up sample to levels comparable or higher to the preoperative ones.

Serum Tg levels evaluated in these 20 PTC patients at 1 months and 12-24 months after primary surgery were consistent with clinical findings and with circulating levels of the 2 miRNAs in 17 cases.

Three patients, initially treated with total thyroidectomy not followed by RRA, were better defined by miRNA profile than conventional thyroid cancer markers. In one patient (case 1) a rising trend of Tg (from 0.17 ng/mL to 0.95 ng/mL) and a declining trend of TgAbs (from 61.4 IU/mL to 14.3 IU/mL) with stable TSH levels was observed, neck ultrasound revealed no evidence of structural disease; the trend of the two miRNAs analyzed was declining. Two cases had evidence of structural disease on neck US (suspicious lymph nodes) and declining trend of serum Tg (from 0.7 and 0.36 ng/mL to 0.11 and 0.15 ng/mL respectively) and of TgAbs (case 2 and 3). In both cases the levels of miRNA-146a-5p and miRNA-221-3p were found more abundant in the follow up sample.

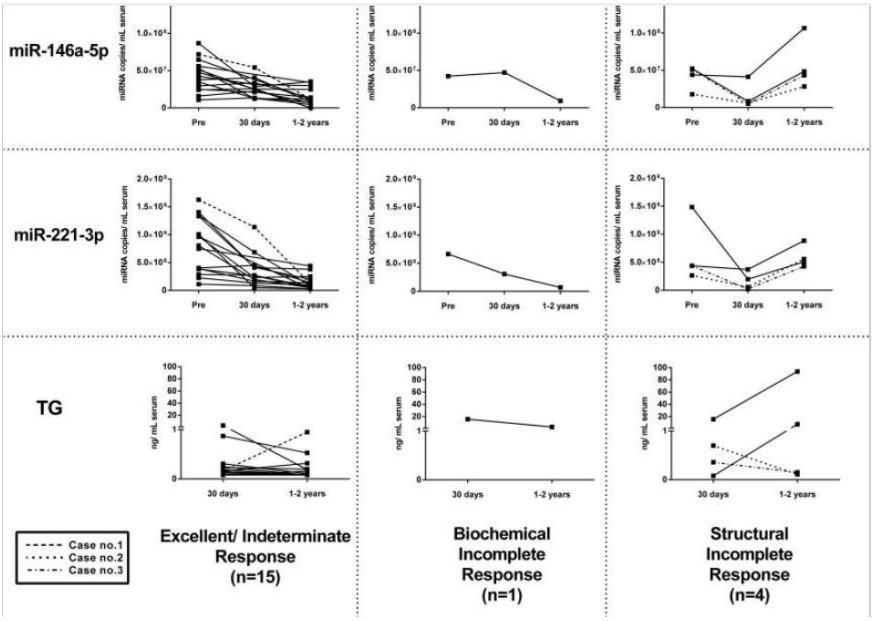


Figure 4. Levels of miRNA-146a, miRNA-221-3p, and Tg in preoperative and postoperative (1 month and 12-24 months) serum samples of PTC patients classified according to response to treatment classes. Case 1: Patient with an indeterminate response and increasing serum Tg levels. Cases 2 and 3: Patients with structural incomplete responses and decreasing serum Tg levels (less than 1 ng/mL).

Discussion

Our data demonstrates that the risk stratification system proposed by the ATA is effective in predicting the risk of persistent disease evaluated at about 1 year from primary surgery. It has also shown a good prediction of the composite outcome (persistent/recurrent disease) at about 3 years from primary surgery. The miRNA profiling analysis demonstrated that the trend of miRNA-146a-5p and miRNA-221-3p is a promising tool for disease assessment of PTC patients.

This study has several limitations. Complete information on pathology was used as an inclusion criterion but some details on lymph node pathologic features (e.g. metastasis size, extra-nodal invasion) that may have an important prognostic role in risk stratification (Randolph, Duh et al. 2012) were not available for all patients. Also, data on molecular characterization of the tumors are lacking. Even if relevant prognostic information is provided by molecular characterization of thyroid tumors (Cancer Genome Atlas Research Network

2014, Fagin and Wells 2016), this kind of analysis is not offered by the public health system in Italy and is performed with research purpose in some referral centers. Like other multicentric studies, heterogenous disease assessment methods were used in each ITCO center. Serum biomarkers were not centralized in the same laboratory and several assays with different functional sensitivities were employed with potential bias in response to treatment result assessment. For this reason, the outcome was also evaluated as presence and absence of SIR that is defined only by imaging studies results, avoiding the influence serum biomarkers variability. Neck ultrasound was the imaging tool employed in almost all patients, but it is an operator dependent technique with high interobserver variability (Grani, Lamartina et al. 2018). This limit may be partly overcome by the use of scoring systems (Grani, Lamartina et al. 2018) as the one proposed by the European Thyroid Association for the classification of neck lymph nodes and thyroid bed nodules (Leenhardt, Erdogan et al. 2013, Lamartina, Grani et al. 2016) that was adopted in the ITCO database to standardize ultrasound reports. From another point of view, these limitations reflect the clinical clues faced by clinicians in the real-life setting.

Some subgroups, namely FTC and HCC, were too small and the number of events too low for robust statistical analysis. This histotypes represent only the 6% of all DTCs in contemporary series (Lamartina, Durante et al. 2017). Most of the evidence available on these tumors relies on small series or larger retrospective series of cohorts diagnosed during a very long time period (up to the 50s-70s). During the same period, the pathologic criteria for FTCs and HCCs have changed several times (Tallini, Tuttle et al. 2017). The information available on these histotypes remains limited (Grani, Lamartina et al. 2017).

As for PTCs the number of subjects enrolled was not negligible. Nevertheless, non-RRA treated PTC patients (representing about 1/3 of the population) only a tendency to significant prediction of SIR by the ATA risk stratification system was observed. It is worth note that patients who were not submitted to RRA were more likely to be low risk. Therefore, a larger population is probably needed to provide more statistically robust results in this subgroup.

The observational design precludes to draw conclusions on the influence of the kind of treatment performed and patient outcome. On the other hand, a picture of clinical choices made in thyroid cancer treatment in the ITCO Italian centers is provided. Bilateral thyroid surgery is performed in the vast majority (97.8%) of patients often together with lymph node surgery (40% of the patients). More limited surgery is performed only in a limited number of low and intermediate risk patients. This attitude might reflect some inertia in the adoption of most recent guideline recommendations (Lamartina, Durante et al. 2017) but also the high prevalence of bilateral nodular thyroid disease may have played a role (Durante, Costante et al. 2015). The use of RRA is more selective and appears guided by the risk of recurrence evaluated at the moment of diagnosis (Lamartina, Durante et al. 2017). Still, a high proportion of low risk patients (46%) is treated regardless their excellent prognosis: only the 0.6% had persistent disease at the 1-year assessment and none experienced disease recurrence.

Only 3 patients experienced recurrent disease at 3 years form primary surgery. A short follow up period was considered, and some disease recurrences may become evident later during follow up. Thyroid cancer relapses have been described to occur up to 20-40 years form diagnosis (Mazzaferri and Jhiang 1994). This study reflects an era where therapeutic and diagnostic tools where much different from those available now days (Lamartina, Deandreis et al. 2016, Lamartina, Grani et al. 2018). More recent series of PTC patients, followed with neck ultrasound and sensitive thyroglobulin assays, have shown that true relapses are rare (1.4%) and occur early after diagnosis (Durante, Montesano et al. 2013). More than a half of these recurrences are observed within 3 years from diagnosis (Durante, Montesano et al. 2013).

Overall the rates of persistent disease observed in this large and prospective cohort are low (2.6%) and the most frequent response category at 1 year was excellent response (about two thirds of the population). The rate of true recurrences was only 0.6% and none of the low risk patients experienced recurrence. Aggressive treatment and follow-up protocols are probably not

justified in these patients that represent the vast majority of DTC patients. Roughly one third of the patients had an indeterminate response at the 1-year assessment. As described in other studies (Lamartina, Montesano et al. 2016), a good number of the patients (38%) with indeterminate response will spontaneously achieve excellent response later during follow up. Less than 10% will develop SIR, the likelihood of SIR in patients initially classified as indeterminate response grew with recurrence risk class: 3% in low risk patients, 15.2% in intermediate risk patients and 17.6% in high risk patients. Still the need for biomarkers alternative to thyroglobulin, such as miRNAs, is therefore of great interest.

The miRNA profile analysis was performed in a small subgroup of patients and further validation on larger cohorts is needed. Nonetheless, a rigorous methodology of analysis was employed on a well characterized longitudinal population (Rosignolo, Sponziello et al. 2017). The kits used RNA isolation (miRNeasy Serum/Plasma kit, Qiagen), allowed the detection of low-abundance miRNAs and the isolation of more concentrated RNA samples than other kits of this type (Farina, Wood et al. 2014). The multiple-extraction approach allowed us to obtain over 1,000 ng total RNA from each serum sample, thereby eliminating the need for cDNA preamplification (that is a potential source of technical bias). Reliable endogenous controls for circulating miRNA analysis are lacking (Farina, Wood et al. 2014), to overcome this problem absolute quantitative polymerase chain reaction was used for all the analyses performed in the validation cohort. miRNA levels were consistent with serum thyroglobulin in all but three cases. In these cases, miRNA profile reflected the actual patient status better than serum thyroglobulin.

In conclusion the analysis of miRNA profiles could represent a valuable biomarker for early definition of the disease status namely in those cases in which traditional markers are less informative.

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